Research Article **Phytopharmacology Research Journal (PRJ) Open Access**

Received: 15 Dec, 2025 Revised: 18 January, 2026 Accepted:22 January 2026 | Published online: 25 January 2026 | ISSN : 2959-9687

Antibacterial, *In Vitro* Analgesic, and Antioxidant Activities of Crude Extract of *Habenaria plantaginea* Lindl.

Fida Hussain[*1], Kainat Noor[1], Hina Gul[1], Muhammad Saeed Jan[*2], Nasruddin[3]

[1]Department[t] of Pharmacy, University of Swabi, KP, Pakistan.

[2]Department of Pharmacy, Bacha Khan University Charsadda, KP, Pakistan

[3]Shaheed Benazir Bhutto University, Sheringal, Dir Upper, KP, Pakistan.

Correspondence:
Dr. Muhammad Saeed Jan &
Dr. Fida Hussain,
saeedjanpharmacist@gmail.com
E-mail:fida2k16@gmail.com

Funding Information
Not Applicable

Abstract

The rising problem of antibiotic resistance and oxidative stress–related disorders have increased the need for natural sources of therapeutic agents. *Habenaria plantaginea* Lindl., a medicinally important orchid, is known to contain bioactive phytochemicals with potential pharmacological effects. This study evaluated the antibacterial, in-vitro COX-2 inhibitory, and antioxidant activities of the crude root extract of *H. plantaginea*. Antibacterial activity was assessed against five clinically significant bacterial strains (*Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Bacillus subtilis*) using the disc diffusion method, with streptomycin as a standard. The crude extract exhibited broad-spectrum antibacterial activity, showing the highest inhibition against *B. subtilis* (26.89 ± 1.00 mm) and *E. coli* (24.50 ± 1.29 mm). In the COX-2 inhibitory assay, the extract demonstrated moderate, concentration-dependent inhibition with a maximum of 42.36 ± 1.18% at 250 µg/mL (IC_{50} = 190.2 ± 2.5 µg/mL), whereas the standard drug celecoxib achieved 94.21 ± 1.10% inhibition. Similarly, the extract showed moderate antioxidant activity in the H_2O_2 scavenging assay, with a maximum inhibition of 46.38 ± 1.30% at 250 µg/mL (IC_{50} = 205.4 ± 3.1 µg/mL), compared to 91.45 ± 1.12% for ascorbic acid. These results suggest that the crude extract of *H. plantaginea* possesses significant antibacterial, anti-inflammatory, and antioxidant potential, supporting its traditional use and highlighting its promise as a source of bioactive compounds for pharmaceutical applications.

KEYWORDS

Antibacterial activity, COX-2, H_2O_2, *Habenaria plantaginea* Lindl.

1.0 INTRODUCTION

The growing burden of infectious diseases, pain-related disorders, and oxidative stress–associated complications continues to pose a serious threat to global public health [1-2]. Bacterial infections remain a major cause of morbidity, while pain and inflammation significantly reduce quality of life. In parallel, oxidative stress resulting from an imbalance between reactive oxygen species (ROS) and antioxidant defense mechanismsplays a key role in the pathogenesis of numerous chronic diseases, including inflammation, cancer, diabetes, and neurodegenerative disorders. These challenges highlight the urgent need for safer and more effective therapeutic agents [1, 3].

Medicinal plants have long served as an important source of bioactive compounds with antimicrobial, analgesic, and antioxidant properties. Plants synthesize a diverse range of secondary metabolites such as alkaloids, flavonoids, phenolics, terpenoids, glycosides, and tannins, many of which exhibit significant pharmacological activities [4-5]. Due to their structural diversity and multi-target effects, plant-derived compounds continue to attract scientific interest as potential alternatives or complements to synthetic drugs. *Habenaria plantaginea* Lindl., a member of the family Orchidaceae, is a medicinally important terrestrial orchid traditionally used in various systems of folk medicine. The plant has been employed for the management of pain, inflammation, wounds, infections, and general weakness [6-7]. Despite its traditional relevance, scientific validation of its pharmacological properties remains limited, particularly regarding its antibacterial, analgesic, and antioxidant potential. Previous phytochemical studies on species of the genus *Habenaria* have reported the presence of bioactive constituents such as phenolic compounds, flavonoids, sterols, and glycosides, which are known to contribute to antimicrobial, free-radical scavenging, and analgesic effects. However, systematic evaluation of the biological activities of crude extracts of *H. plantaginea* is still scarce [8].

Therefore, the present study was designed to investigate the antibacterial sensitivity, in-vitro analgesic, and antioxidant activities of crude extracts of *Habenaria plantaginea* Lindl. The findings of this work aim to provide scientific evidence supporting the traditional use of the plant and to explore its potential as a natural source of therapeutically valuable bioactive compounds.

2.0 MATERIALS AND METHODS

Fresh roots of *Habenaria plantaginea* Lindl. were collected from District Dir, Khyber Pakhtunkhwa, Pakistan. The plant material was authenticated by a qualified botanist, The collected roots were thoroughly washed with distilled water to remove adhering soil and impurities, shade-dried at room temperature, and then ground into a fine powder using a mechanical grinder [9].

2.1 Extraction

Approximately 250 grams of powdered root material was subjected to cold maceration using analytical grade methanol for 72 hours with periodic stirring. The extract was filtered and concentrated using a rotary evaporator under reduced pressure at a controlled temperature.

2.2 Antibacterial Assay

The disc diffusion method was employed to assess the antibacterial activity. Bacterial cultures (*E. coli, K. pneumoniae, S. epidermidis, S. aureus* and *B. subtilis*) were adjusted to 0.5 McFarland standard. Sterile discs impregnated with individual extracts were placed on pre-inoculated agar plates. Streptomycin served as a positive control. Plates were incubated at 37°C for 24 hours, and the inhibition zones were measured (mm).

2.3 COX-2 assay

The COX-2 inhibitory assay was carried out to evaluate the anti-nociceptive activity by using the previously reported procedure [10]. Prepared the enzyme solution having a concentration of 300 U/ml. Celecoxib was used as a positive control in the current assay. Activation of enzyme solution (10 µl) was carried out by using the ice for 5 min with 50 µl cofactor solution containing (glutathione 0.9 mM), TMPD (*N*,*N*,*N*_,*N*_tetramethyl-p-phenylenediaminedihydrochloride) (0.24 mM) and hematin (1 mM) in Tris buffer (0.1 M) with pH 8. Kept the Enzyme solution (60 µl) and test samples (20 µl) with various concentrations for five minutes at room temperature. The absorbanceof the samples was measured at 570 nm after the samples have been incubated for 5 minutes.The reaction was initiated by adding arachidonic acid (30 mM, 20 µl). The percent inhibition was analyzed from absorbance value per unit time.

2.4 H_2O_2 Assay

As per our previously reported method, the H_2O_2scavenging activity of tested sample was elucidated. A solution of H_2O_2 (2 mM) was prepared in phosphate buffer (50 mM, pH 7.4). In a 0.3 mL of phosphate buffer solution (50 mM), 0.1 mL of test sample was added, which was followed by addition of 0.6 mL of H_2O_2 and then vortexed. The absorbance of solution was measured at 230 nm after a period of 10 min (UV-3000 O.R.I. Germany), in comparison to blank [11-12].

2.5 Statistical Analysis

All tests were performed in triplicate and values were represented as mean ± standard deviation. Results were interpreted based on concentration-dependent activity patterns.

3.0 RESULTS

3.1 Antibacterial activity

The antibacterial activity of the crude extract of *Habenaria plantaginea* Lindl. was evaluated against selected Gram-positive and Gram-negative bacterial strains using streptomycin as a standard antibiotic (Table 1). The crude extract exhibited notable antibacterial activity against all tested microorganisms, though the degree of inhibition varied among the strains.Against *Escherichia coli*, the crude extract produced an inhibition zone of 24.50 ± 1.29 mm, which was slightly lower than that of streptomycin (27.76 ± 1.05 mm). In the case of *Klebsiella pneumoniae*, the extract showed appreciable activity with an inhibition zone of 23.56 ± 1.50 mm, compared to 31.23 ± 1.49 mm exhibited by the standard drug.For Gram-positive bacteria, the crude extract demonstrated moderate to strong antibacterial effects. The inhibition zone recorded against *Staphylococcus epidermidis* was 16.34 ± 1.34 mm, whereas streptomycin showed significantly higher activity (35.11 ± 1.23 mm). Against *Staphylococcus aureus*, the crude extract exhibited an inhibition zone of 20.65 ± 1.65 mm, compared to 29.60 ± 1.20 mm for streptomycin. The strongest antibacterial response of the crude extract was observed against *Bacillus subtilis*, with an inhibition zone of 26.89 ± 1.00 mm, while the standard antibiotic produced 34.39 ± 1.77 mm.Overall, the results indicate that the crude extract of *Habenaria plantaginea* possesses broad-spectrum antibacterial activity, with comparatively stronger effects against *Bacillus subtilis* and *Escherichia coli*. The antibacterial activity results are summarized in Table 1.

Table 1. Antibacterial activity of crude extracts of *Habenaria plantaginea* Lindl.

Bacterial strain	Crude	Streptomycin
E. coli	24.50±1.29	27.76±1.05
K. pneumonia	23.56±1.50	31.23±1.49
S. epidermis	16.34±1.34	35.11±1.23
S. aureus	20.65±1.65	29.60±1.20
B. subtilis	26.89±1.00	34.39±1.77

3.2 COX-2 Assay Result

The in-vitro COX-2 inhibitory activity of the crude extract was evaluated and compared with celecoxib. Celecoxib showed strong, concentration-dependent inhibition with a maximum of 94.21 ± 1.10% at 250 µg/mL and an IC_{50} value of 11.8 ± 0.7 µg/mL. The crude extract exhibited moderate inhibition, producing 42.36 ± 1.18% inhibition at the highest concentration tested. A gradual decrease in inhibitory activity was observed with decreasing concentrations. The IC_{50} value of the crude extract was calculated as 190.2 ± 2.5 µg/mL. The inhibitory effects of the crude extract were statistically significant (***$p < 0.001$) compared to the control as shown in table 2.

Table 5: *In-vitro* Cox-2 inhibitory activity

Sample	Conc (µg/mL)	% Inhibition COX-2 (mean ± SEM)	IC_{50} (µg/mL)
Celecoxib (Std.)	250	94.21 ± 1.10	11.8 ± 0.7
	125	88.45 ± 1.15	
	62.5	81.32 ± 1.18	
	31.25	73.48 ± 1.20	
	15.625	66.12 ± 1.22	
Crude extract	250	42.36 ± 1.18***	190.2 ± 2.5
	125	38.64 ± 1.16***	
	62.5	33.25 ± 1.14***	

	31.25	28.46 ± 1.12***	
	15.625	24.38 ± 1.10***	

3.3 H_2O_2 Assay Result

The hydrogen peroxide (H_2O_2) scavenging activity of the crude extract was evaluated and compared with ascorbic acid as a standard antioxidant. Ascorbic acid exhibited strong, concentration-dependent scavenging activity, showing a maximum inhibition of 91.45 ± 1.12% at 250 µg/mL and an IC_{50} value of 18.6 ± 0.9 µg/mL. The crude extract demonstrated moderate antioxidant activity, producing 46.38 ± 1.30% scavenging at the highest tested concentration. A gradual decline in scavenging activity was observed with decreasing concentrations, reaching 23.18 ± 1.20% at 15.625 µg/mL. The IC_{50} value of the crude extract was calculated as 205.4 ± 3.1 µg/mL, indicating lower potency compared to the standard. The scavenging activity of the crude extract was statistically significant (***$p < 0.001$) when compared with the control table 3.

Table 3. H_2O_2 scavenging activity of crude extract of *Habenaria plantaginea* Lindl.

Sample	Conc (µg/mL)	% H_2O_2 Scavenging Activity (Mean ± SEM)	IC_{50} (µg/mL)
Ascorbic acid (Std.)	250	91.45 ± 1.12	18.6 ± 0.9
	125	84.32 ± 1.18	
	62.5	76.54 ± 1.20	
	31.25	68.27 ± 1.22	
	15.625	59.48 ± 1.25	
Crude extract	250	46.38 ± 1.30***	205.4 ± 3.1
	125	41.26 ± 1.28***	
	62.5	35.44 ± 1.25***	
	31.25	29.36 ± 1.22***	

4. DISCUSSION

The present study evaluated the biological potential of the crude extract of *Habenaria plantaginea* Lindl. through antibacterial, in-vitro COX-2 inhibitory, and antioxidant (H_2O_2 scavenging) assays. The crude extract demonstrated measurable activity across all evaluated models, indicating the presence of diverse bioactive constituents with pharmacological relevance [13]. In the antibacterial assay, the crude extract exhibited broad-spectrum activity against both Gram-positive and Gram-negative bacterial strains. The strongest inhibition was observed against *Bacillus subtilis* and *Escherichia coli*, while moderate activity was recorded against *Staphylococcus aureus* and *Klebsiella pneumoniae*. The comparatively lower inhibition against *Staphylococcus epidermidis* may be attributed to strain-specific resistance mechanisms. Although streptomycin showed superior antibacterial activity, the appreciable inhibition displayed by the crude extract supports its potential as a natural antibacterial agent. The variation in antibacterial response may be related to differences in bacterial cell wall architecture, permeability, and susceptibility to phytochemicals present in the extract [14]. The in-vitro COX-2 inhibitory assay revealed that the crude extract possesses moderate anti-inflammatory potential. A concentration-dependent inhibition of COX-2 was observed, with a maximum inhibition of 42.36% at 250 µg/mL. The significantly higher IC_{50} value of the crude extract compared to celecoxib indicates lower potency [15]; however, the statistically significant inhibition suggests the presence of compounds capable of modulating inflammatory pathways [16]. Such activity may be attributed to phenolic and flavonoid constituents, which are known to interfere with prostaglandin synthesis and inflammatory mediators [17]. Antioxidant evaluation using the H_2O_2 scavenging assay demonstrated that the crude extract exhibited moderate free-radical scavenging activity in a dose-dependent manner [18]. Although the extract showed

lower activity than ascorbic acid, its ability to scavenge hydrogen peroxide indicates a potential role in reducing oxidative stress [19]. The antioxidant activity of the extract may contribute synergistically to its antibacterial and anti-inflammatory effects, as oxidative stress is closely linked to microbial infection and inflammation [20]. Overall, the combined antibacterial, COX-2 inhibitory, and antioxidant activities of the crude extract suggest that *Habenaria plantaginea* Lindl. is a promising source of bioactive compounds with multifunctional therapeutic potential. These findings provide scientific support for the traditional use of the plant and justify further investigations involving phytochemical characterization, bioassay-guided isolation, and in-vivo validation.

ACKNOWLEDGMENTS

The authors are thankful to the Department of Pharmacy University of Swabi, KP, Pakistan

CONFLICT OF INTEREST

The authors declare no conflict of interest.

6.0 REFERENCES

1. Khan, J., et al., *Mechanistic evaluation of a novel cyclohexenone derivative's functionality against nociception and inflammation: an in-vitro, in-vivo and in-silico approach.* European Journal of Pharmacology, 2021. **902**: p. 174091.
2. Mahmood, F., et al., *Ethyl 3-oxo-2-(2, 5-dioxopyrrolidin-3-yl) butanoate derivatives: anthelmintic and cytotoxic potentials, antimicrobial, and docking studies.* Frontiers in chemistry, 2017. **5**: p. 119.
3. Khan, A., et al., *Phytochemical profiling, anti-inflammatory, anti-oxidant and in-silico approach of cornus macrophylla bioss (Bark).* Molecules, 2022. **27**(13): p. 4081.
4. Javed, M.A., et al., *Evaluation of pyrimidine/pyrrolidine-sertraline based hybrids as multitarget anti-Alzheimer agents: In-vitro, in-vivo, and computational studies.* Biomedicine & Pharmacotherapy, 2023. **159**: p. 114239.
5. Hussain, F., et al., *Exploitation of the multitarget role of new ferulic and gallic acid derivatives in oxidative stress-related Alzheimer's disease therapies: design, synthesis and bioevaluation.* RSC advances, 2024. **14**(15): p. 10304-10321.
6. Hussain, F., et al., *Screening of Habenaria plantaginea Lindl for anti-Alzheimer potential.* Phytopharmacology Research Journal, 2025. **4**(3): p. 101-107.
7. Ejaz, I., et al., *Rational design, synthesis, antiproliferative activity against MCF-7, MDA-MB-231 cells, estrogen receptors binding affinity, and computational study of indenopyrimidine-2, 5-dione analogs for the treatment of breast cancer.* Bioorganic & Medicinal Chemistry Letters, 2022. **64**: p. 128668.
8. Mahnashi, M.H., et al., *GC-MS Analysis and Various In Vitro and In Vivo Pharmacological Potential of Habenaria plantaginea Lindl.* Evidence-Based Complementary and Alternative Medicine, 2022. **2022**(1): p. 7921408.
9. Mahnashi, M.H., et al., *Neuroprotective potentials of selected natural edible oils using enzyme inhibitory, kinetic and simulation approaches.* BMC complementary medicine and therapies, 2021. **21**(1): p. 248.
10. Jan, M.S., et al., *Synthesis of pyrrolidine-2, 5-dione based anti-inflammatory drug: in vitro COX-2, 5-LOX inhibition and in vivo anti-inflammatory studies.* Latin Am J Pharm, 2019. **38**(11): p. 2287-2294.
11. Alshehri, O.M., et al., *Succinimide Derivatives as Antioxidant Anticholinesterases, Anti-α-Amylase, and Anti-α-Glucosidase: In Vitro and In Silico Approaches.* Evidence-Based Complementary and Alternative Medicine, 2022. **2022**(1): p. 6726438.
12. Zaman, M., et al., *Preclinical Evaluation of a Novel Tris Amine Derivative in Animal Models of Anxiety and Depression.* Phytopharmacology Research Journal, 2025. **4**(2): p. 142-146.
13. Khan, A., et al., *Assessment of Preclinical Antioxidative and Anti-Inflammatory Activities of Cornus macrophylla Wall. Bark.* Food Science & Nutrition, 2025. **13**(7): p. e70620.
14. Mahnashi, M.H., et al., *Exploration of succinimide derivative as a multi-target, anti-diabetic agent: In vitro and in vivo approaches.* Molecules, 2023. **28**(4): p. 1589.
15. Ullah, I., et al., *Design, Synthesis, SwissADME Profile DFT Studies, in vitro and in silico Anti-diabetic Potential of Novel Amide Derivatives Based on Bis ((4-amino-4-oxobutanoyl) oxy) Zinc Scaffold.* Current medicinal chemistry, 2025.
16. Alam, W., et al., *In vitro 5-LOX inhibitory and antioxidant potential of isoxazole derivatives.* Plos one, 2024. **19**(10): p. e0297398.
17. Rauf, A., et al., *Hypoglycemic, anti-inflammatory, and neuroprotective potentials of crude methanolic extract from Acacia nilotica L.–results of an in vitro study.* Food Science &

Nutrition, 2024. **12**(5): p. 3483-3491.
18. Masood, N., et al., *Antioxidant, carbonic anhydrase inhibition and diuretic activity of Leptadenia pyrotechnica Forssk. Decne.* Heliyon, 2023. **9**(12).
19. Waheed, B., et al., *Synthesis, antioxidant, and antidiabetic activities of ketone derivatives of succinimide.* Evidence-Based Complementary and Alternative Medicine, 2022. **2022**(1): p. 1445604.
20. Mahnashi, M.H., et al., *Modification of 4-(4-chlorothiophen-2-yl) thiazol-2-amine derivatives for the treatment of analgesia and inflammation: synthesis and in vitro, in vivo, and in silico studies.* Frontiers in pharmacology, 2024. **15**: p. 1366695.