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Original article

Antibacterial and antibiofilm potentials of *Rumex dentatus* root extract characterized by HPLC-ESI-Q-TOF-MS

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ABSTRACT

The control of infections is one of the key strategies to treat cuts, wounds, lung, and skin infections. In this study the folkloric use of *Rumex dentatus* (*R. dentatus*) roots in the mentioned conditions was scientifically investigated. The methanolic (MeOH) crude extract of *R. dentatus* root was fractionated (n-hexane, ethyl acetate and water) via bioassay-guided method, and its antibacterial activity was evaluated using the agar well diffusion and Minimum inhibitory concentration (MIC) assays against clinical isolate of *Pseudomonas aeruginosa* (*P. aeruginosa*). The antibiofilm activity was measured using the crystal violet staining method. The crude extract, fractions and sub-fractions tested showed the MICs values ranging from 200 to 1000 μg/mL respectively. Among the fractions, notably, the water fraction exhibited the highest activity against *P. aeruginosa*. The water fraction was then subjected to thin layer chromatography (TLC). Following spectrometric analysis using HPLC-ESI-Q-TOF-MS, gallic acid and emodin were identified as the primary components within the same fraction, responsible for eliciting antibacterial and antibiofilm effects. The in-silico studies conducted with AutoDock Vina on the LasR protein, using both isolated gallic acid and emodin, confirm the binding affinity of these molecules to the active sites of the LasR protein that has regulatory role in building of biofilm formation and its pathogenicity. By scientifically validating the infection-controlling properties of *R. dentatus*, this research provides compelling evidence that supports its traditional use as reported in folklore. Moreover, this study contributes to our understanding of the plant's potential in managing infections, thereby substantiating its traditional therapeutic application in a scientific context.

1. Introduction

P. aeruginosa is frequently observed in chronic wounds and the respiratory systems of individuals with cystic fibrosis. The formation and persistence of biofilms further enhance its ability to trigger opportunistic infections (Bjarnsholt et al., 2008). Biofilms constitute clusters of microorganisms that thrive within a self-generated matrix of biopolymers. These includes DNA, proteins, and polysaccharides. Their physiological characteristics differ from those of free-living planktonic cells. The intricate structure of biofilm-organized microorganisms leads to altered responses to antibiotics (Mah et al., 2003, Parsek and Singh, 2003, Khan et al., 2010). This dilemma is further complicated by a growing reservoir of clinically and veterinary relevant bacterial strains that are resistant to antibiotics (Chen et al., 2009). Hence, novel strategies and compounds that can effectively tackle these challenges are imperative.

Throughout history, plants have served as sources of healing and

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vitality. The use of plant-based remedies can be traced back to ancient civilizations, dating as far back as 4000–5000 BC. Even in the new era, plants continue to play a crucial role in healthcare, about 80 % of developing world is still relying on it. It's noteworthy that over 50 % of the drugs currently employed in clinical practice have their roots in plant sources. Considering the availability of plant based medicine and the rich traditional wisdom regarding wound treatment, communities in developing regions assimilate natural medicine into the management of diabetic wounds and complications (Oguntibeju, 2019).

R. dentatus L. belongs to family Polygonaceae is known for its diverse chemical constituents that are usually biologically active compounds. It has been reported for its anti-inflammatory (Süleyman et al., 1999), antitumor, astringent, and anti-dermatitis properties (Litvinenko and MuzychKina, 2003), as well as for its diuretic, cholagogue, tonic, and laxative effects (Demirezer, 1993). Some studies have also indicated the bactericidal potential of *R. dentatus* extract (Yıldırım et al., 2001). Nevertheless, limited information is available on the specific active components associated with its antibiofilm activity.

In today's pharmaceutical landscape, the quest for ideal drug candidates extends beyond exceptional pharmacological properties and encompasses considerations of accessibility and cost-effectiveness. The choice of *R. dentatus* as the subject of our current antibiofilm investigation was motivated by several factors. Firstly, it holds a significant place in traditional medicine for treating chronic wounds, particularly among the local populations in remote areas of Pakistan. Secondly, it is readily available, making it accessible to economically disadvantaged individuals. We assessed the crude methanolic root extract, fractions, and isolated compounds, namely emodin and gallic acid, using HPLC and LC-MS, from *R. dentatus* for their antibacterial and antibiofilm potential against clinical isolate of *P. aeruginosa*. Additionally, we conducted in silico evaluations for emodin and gallic acid to gain further insights into their activity.

2. Experimental

2.1. Chemicals and reagents

Muller-Hinton (MH) broth, Tryptic Soy Agar (Becton, Dickinson and Company, USA), MH agar plates, methanol, ethyl acetate, n-hexane, formic acid, acetic acid and other chemical reagents (Sigma Chemical Co. St. Louis, USA) were used. The chemicals and solvents used were of analytical reagent grade.

2.2. Bacterial culture

A clinical isolate of *P. aeruginosa*, isolated from an infected wound from a tertiary care hospital named Pakistan Institute of Medical Sciences was used in the present study. The isolate was identified using the Gram-stain assay, catalase test, oxidase test, its ability to grow on selective medium (*Pseudomonas* cetrimide agar) and finally using API-20NE system (Merieux, France).

2.3. Plant material

In April-May, when the plant was in full bloom, the roots of *R. dentatus* were collected from fields in Mansehra, Pakistan. A voucher specimen (No. 5228) has been duly archived in the Herbarium of the Botany Department within the Faculty of Science at Hazara University, Mansehra, Pakistan.

2.4. Extraction and isolation of constituents

The dried roots of *R. dentatus* (1 kg), previously kept in the shade (15 days), were finely ground into powder using grinder. The powdered material then underwent extraction using methanol (MeOH; 5 L × 4) via maceration at room temperature for a period of four-days. Following the extraction process, the resulting extract was filtered, and the solvent was removed using rotary evaporation, with temperature control kept below 45 °C. This procedure resulted in a crude extract (RDI) weighing 203 g, which was subsequently stored at 4 °C for future analysis. A portion of the crude extract (183 g) was dissolved in a methanol–water solution and subjected to liquid–liquid fractionation using n-hexane (Hex), ethyl acetate (EtOAc), and water (H2O), respectively. This process produced three distinct fractions, as illustrated in Fig. 1. The crude methanol extract (Crd-MeOH) and its subsequent fractions (Hex, EtOAc, and aqueous fractions) were employed in microbial bioassays. The RDIc, exhibiting the most promising antibacterial activity, was deemed worthy of further investigation.

Fraction RDIc (35 g) underwent column chromatography (CC) using silica gel (Merck 60 F254) and was eluted with a gradient of n-Hex-EtOAc and EtOAc-MeOH solvent mixtures. Based on the results of thin-layer chromatography (TLC), we collected four distinct fractions, which were labeled as RDIc1 (5.8 g), RDIc2 (10.7 g), RDIc3 (15.8 g), and RDIc4 (2 g) as shown in Fig. 1. RDIc1 (5.6 g) was then further subjected to CC, with elution utilizing EtOAc-MeOH mixtures of increasing polarity, resulting in the collection of six new sub-fractions labeled as RDIc11, RDIc12, RDIc13, RDIc14, RDIc15, and RDIc16. Due to limited antibacterial activity RDIc2 was not subjected to further analysis. RDIc3 (15.5 g) was processed through CC over silica gel, with elution involving EtOAc-MeOH-water mixtures of increasing polarity, leading to the collection of five sub-fractions named RDIc31, RDIc32, RDIc33, RDIc34, and RDIc35. Fraction RDIc4 (1.8 g) was passed through a silica column with elution employing mixtures of acetic acid, water, and butanol of increasing polarity, yielding RDIc41, RDIc42, and RDIc43. Fractions were identified using TLC. Notably, among all the sub-fractions, RDIc13 exhibited the most potent antimicrobial activity, that were further analyzed through LC-MS to identify the active compounds (Santos et al., 2009).

2.5. HPLC analysis of RDIc13

HPLC-ESI-MS was used to know about the chemical nature of the RDIc13 sub-fraction. This involved employing an Agilent 1290 Infinity LC System equipped with an online degasser, binary pump, autosampler, and diode array detector, as detailed by Siu et al. in 2015. The mass spectrometry analysis was conducted using the Agilent 6530 Accurate-Mass Q-TOF LC-MS System (Siu et al., 2015). The sample solution was introduced into an Agilent ZORBAX Eclipse Plus C18 column (1.8 μm, 3 mm x 100 mm). Before usage, all solvents were passed through a 0.22 μm filter disk and degassed. We employed a gradient elution method using the following solvent systems: mobile phase A, consisting of 0.1 % formic acid in water, and mobile phase B, composed of 0.1 % methanol. The gradient profile was as follows: 0 to 10 min, a linear change from 2 % to 100 % B; 10 to 20 min, holding at 100 % B; 20 to 21 min, maintaining 100 % B. The flow rate was set at 0.5 mL/min, and the injection volume was 2 μL. MS analysis was carried out using the ESI interface with negative ionization mode, with a full-scan range of 100–2000 *m/z*.

2.6. Agar well diffusion assay

Different concentrations of crude extract, fractions and sub-fractions were examined to evaluate their antibacterial potential using the agar diffusion assay, following the established protocols with minor adaptations (Clinical and Institute, 2012). Petri dishes were prepared with Tryptic Soy Agar, a non-selective culture medium, and were uniformly coated with 100 μL of a bacterial strain suspension at a concentration of 106 CFU/mL. Subsequently, a sterile cork borer was utilized to create a well with a 6 mm diameter in the agar. Then, 100 μL of the antimicrobial agent was introduced into the well. As a positive control, discs containing 5 μg of ciprofloxacin were included. After 24 h of incubation at 37 °C, any clear zone where bacterial growth was inhibited was measured in mm to assess the antibacterial efficacy (Klančnik et al.,

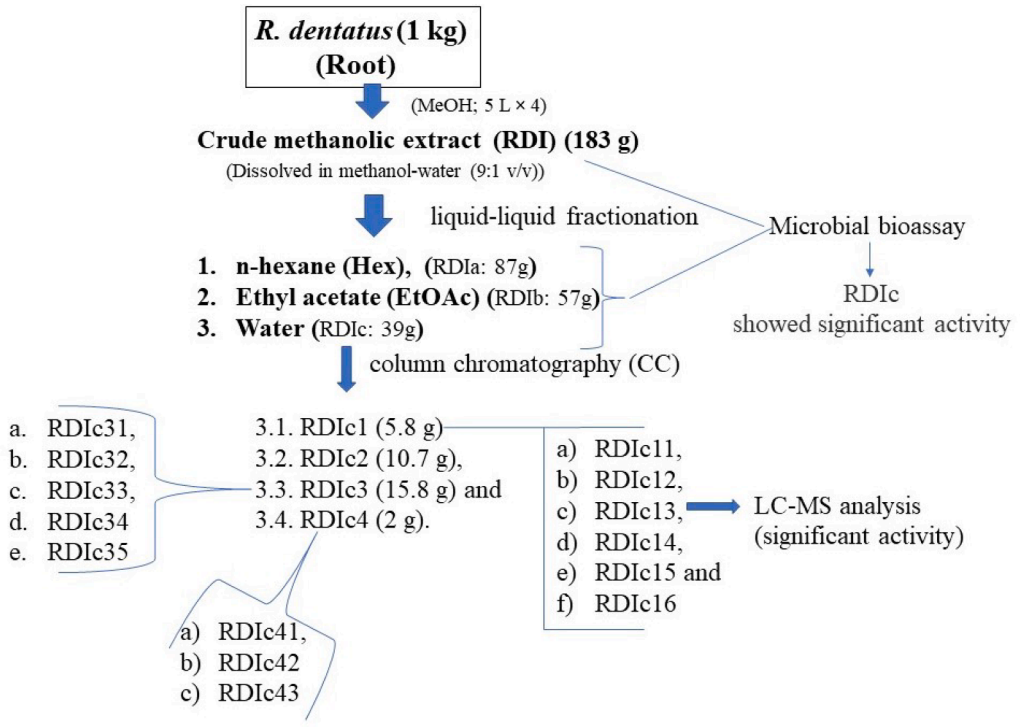

Fig. 1. Fractionation and characterization sketch.

2010).

2.7. Determination of MIC

Antibacterial susceptibility assessments were conducted using a modified broth microdilution method, following a protocol outlined in previous research. Bacterial cells were introduced into Muller-Hinton (MH) broth at a concentration of 10^6 CFU/ml, with each well of a 96-well microtiter plate containing 200 μL of this bacterial suspension. Minimum Inhibitory Concentrations (MICs) were determined by performing sequential 2-fold dilutions of the test compound in MH broth. The bacterial culture was then incubated for 18 h at 37 °C, and visual assessment was made to detect inhibited bacterial growth. To quantify bacterial growth in each well, the absorbance at 620 nm was measured using a spectrophotometer. The percentage of growth in each well was calculated using the already reported method (Chan et al., 2013).

2.8. Anti-biofilm activity

The ability of *R. dentatus* root to prevent biofilm formation was evaluated employing the crystal violet staining method. Biofilms cultured in 96-well flat bottom polystyrene plates were exposed to varying concentrations of extracts or compounds and incubated at 37^OC for 24 hrs. Following incubation, each plate was washed using distilled water to discard any planktonic cells and left for some time until dry. After drying, 125 μL crystal violet dye (0.1 %) was used as staining adherent. The adherent biofilm was re-solubilizes with the addition of 130 μL of 30 % acetic acid and each plate was analyzed using Microplate absorbance reader at 550 nm for biofilm inhibition. The process was carried out three times, and the averages were computed (Khan et al., 2010, Sandasi et al., 2010).

2.9. Molecular docking

Molecular simulation studies investigated the binding efficiency of emodin and gallic acid [ligand, Pubchem Id: **3220** & **370**, into the active sites of proteins LasR from LBD-QslA (4NG2) https://doi.org/10.2210/pdb4NG2/pdb of *P. aeruginosa.* Autodock 1.5.7 software was used for docking studies, the 3d structures of the ligands (emodin and gallic acid were downloaded from PubChem website and save as sdf file format which was converted to PDB format using Discovery studio 2021, the proteins/receptors were downloaded from PDB website, the proteins were prepared using Autodock 1.5.7, water molecules were removed, polar hydrogens were added, followed by adding Kollman charges, than using Lamarckian algorithm and saved the file as pdbqt format. The Grid command were using the fix the grid preferably at default status and the configuration file was prepared for docking via Autodock Vina using command prompt.

2.10. Statistics

Statistical analyses were performed using the Student's *t*-test, were carried out using Prism software version 5.0. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Structural determination

For identification of the presence of the active ingredients LC-MS analysis was performed. However, through the Agilent MS database of Traditional Chinese Medicine (TCM) only two compounds could be identified i.e., gallic acid and emodin (Table 1). Figs. 2a, b shows the HPLC profile of standards of the bioactive markers gallic acid and emodin, respectively. The total ion chromatogram of RDIc13 fraction in

Table 1
Information related to Gallic acid and Emodin identification.

5	Rt (mint	UV (nm)	$[M - Z]^-$	Mw	Formula	Identification
1	3.62	254, 280, 330	169	170.12	$C_7H_6O_5$	Gallic acid
2	18.13	254, 280, 330	269	270.24	$C_{15}H_{10}O_5$	Emodin

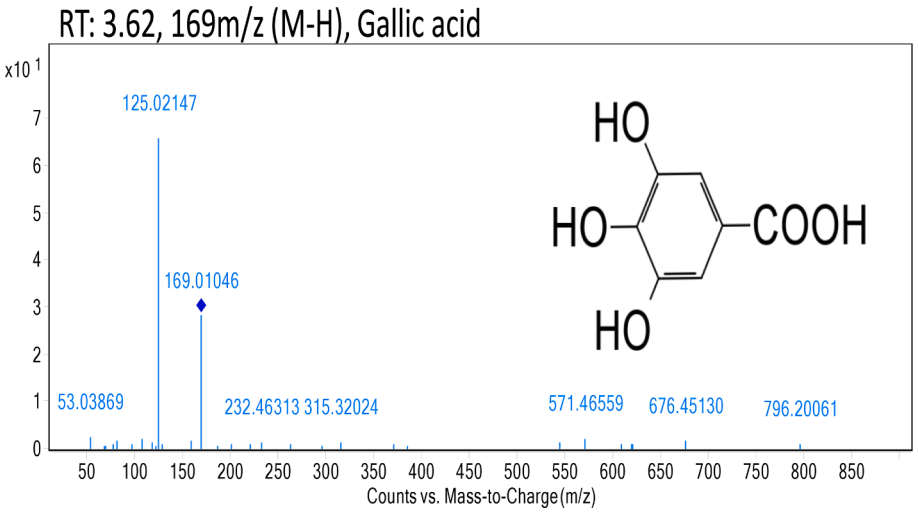

Fig. 2a. The HPLC-MS profile of the bioactive markers gallic acid.

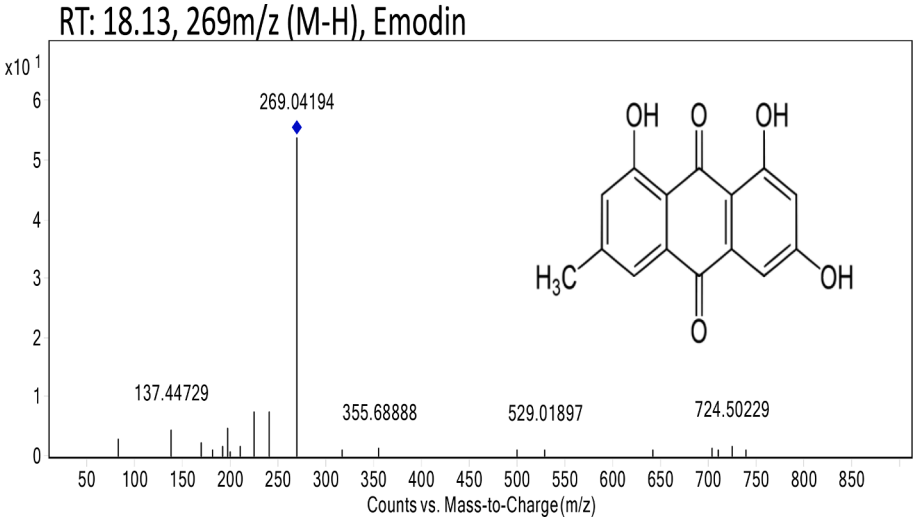

Fig. 2b. Shows the HPLC-MS profile of the bioactive markers emodin.

a negative ion mode is shown in Fig. 2c.

3.2. Antibacterial and antibiofilm activity

The antibacterial activity for crude methanolic extract (15 mg/ml), fractions and sub-fractions (250 to 500 µg/ml) were determined against *P. aeruginosa* using by agar well diffusion method using ciprofloxacin as standard. The zones of inhibition recorded in mm are given in Table 2. The MICs against *P. aeruginosa* of the plant extracts extracted in Hex, EtOAc and water are shown in Table 2. The MIC values recorded for each

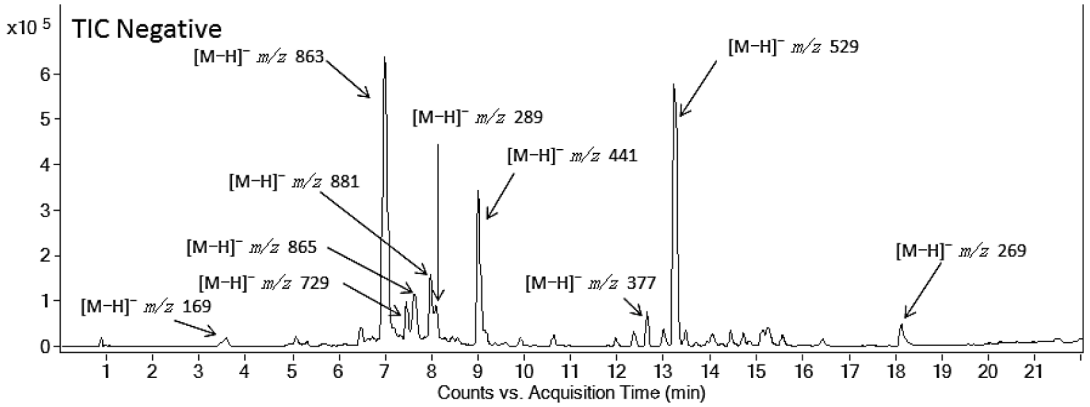

Fig. 2c. The total ion chromatogram of RDIc13 fraction in a negative ion mode.

Table 2
Antibacterial activity of *R. dentatus* extracts against *P. aeruginosa.*

Sample/ extract	Concentration	Zone of inhibition (mm)	MIC (µg/ml)	% Inhibition
RDI	15 mg/ml	20	> 250	98
RDIa	15 mg/ml	8	> 1000	70
RDIb	15 mg/ml	10	> 1000	85
RDIc	15 mg/ml	20	> 250	98
RDIc1	500 µg/ml	18	> 250	94
RDIc2	500 µg/ml	ND	ND	ND
RDIc3	500 µg/ml	10	> 500	87
RDIc4	500 µg/ml	8	> 500	84
RDIc11	250 µg/ml	8	> 250	85
RDIc12	250 µg/ml	8	> 250	84
RDIc13	250 µg/ml	18	> 125	93
RDIc14	250 µg/ml	10	> 250	85
RDIc15	250 µg/ml	8	> 250	84
Ciprofloxacin	5 µg/ml	21	> 7	100
DMSO	100 %	ND	ND	ND

Values are mean ± SD of three parallel measurements, ND = Not detected; RDI = crude methanol extract; RDIabc = n-hexane, ethyl acetate & water fractions.

extract ranged from 250 to 1000 µg/mL. In the antibiofilm activity, a concentration dependent activity was obtained for both fractions and crude extracts as shown in Fig. 3.

The antibacterial and antibiofilm potential of the biomarker emodin and gallic acid was determined using MH-broth dilution and crystal violet staining assays and the results of inhibition are given Fig. 4 respectively. These results underscore the potential antimicrobial potency of *R. dentatus* extracts against *P. aeruginosa.*

3.3. Molecular docking

The antibacterial potential of gallic acid and emodin was determined using 100 to 200 µg/ml concentration and up to 61 % inhibition of bacterial growth was recorded against *P. aeruginosa.* The antibacterial effects of these constituents may be attributed to various molecular mechanisms but we targeted LasR which is a transcription factor of *P. aeruginosa,* a component of quorum sensing system, ligands binds to LasR play role in regulating genes associated with pathogenicity and virulence (Rutherford and Bassler, 2012). After docking analysis, we have found the binding energy for emodin −7 kcal/mol (Table 3) and −6.8 kcal/mol for gallic acid. The active sites of LasR with both ligands have been shown in Figs. 5-7. Thus, experimental results are in line with the molecular docking results however, antibacterial or antibiofilm results may not be linked to only single ligand or one receptor, as there is always multichannel involvement.

4. Discussion

Antibacterial agents serve the crucial purpose of either eliminating bacteria or impeding their growth to curb rapid reproduction. The escalating global issue of antibiotic resistance has taken center stage in contemporary scientific discourse. The gradual development of new antibiotics is outpaced by diminishing bacterial susceptibility, leading to a worrying scarcity of effective treatments against common infections. Urgent preventive measures are essential to address this challenge. At present, the global scientific community is dedicated to refining the precision of infection targeting through the advancement of innovative tools. This pursuit has propelled the exploration of plant-derived medicinal options, as a response to the time-intensive nature of synthetic drug production (Khaliq et al., 2023). Prior studies have reported the diverse medicinal uses of *R. dentatus* in traditional folk medicine, encompassing treatments for ailments such as acariasis, eczema, diarrhea, constipation, astringent conditions, dermatitis, diuretic effects, cholagogue properties, and tonic applications (Abou Elfotoh et al., 2013, Humeera et al., 2013). This historical knowledge provides a foundational context for the present investigation into the antibacterial and antibiofilm activities of *R. dentatus* root extract.

The RDIc13 fraction exhibited notable antibacterial activity, prompting further spectrometric analysis. Subsequent examination of the total ion chromatogram of the RDIc13 fraction in negative ion mode

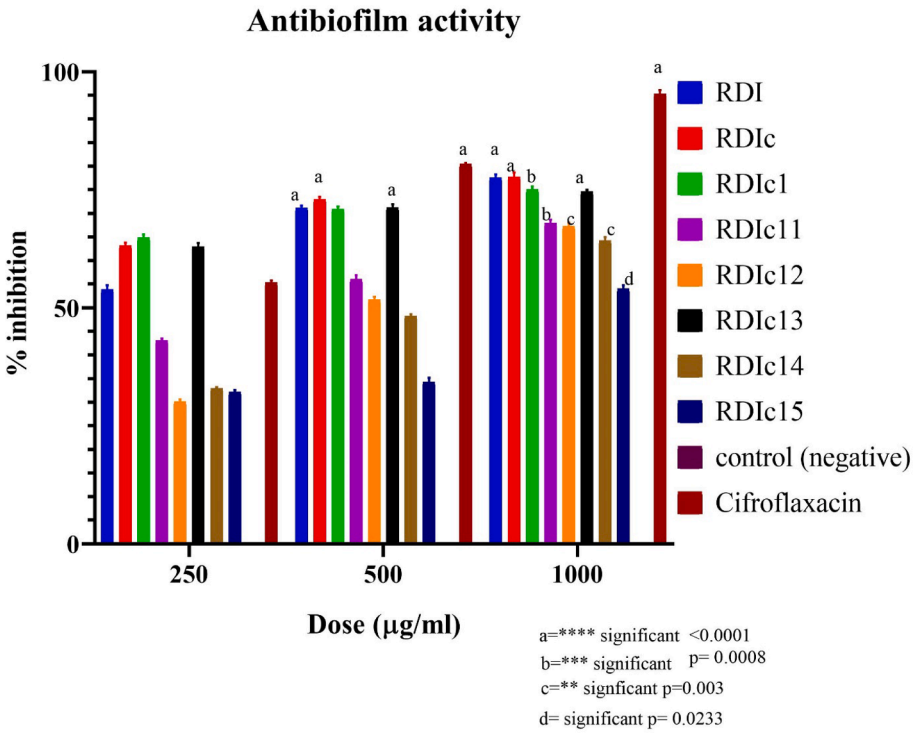

Fig. 3. Antibiofilm activity of *R. dentatus extracts* against *P. aeruginosa*. Data represent as mean ± standard error (n = 3); a, b, c and d represent the level of significance in applied One Way ANOVA Multiple comparison.

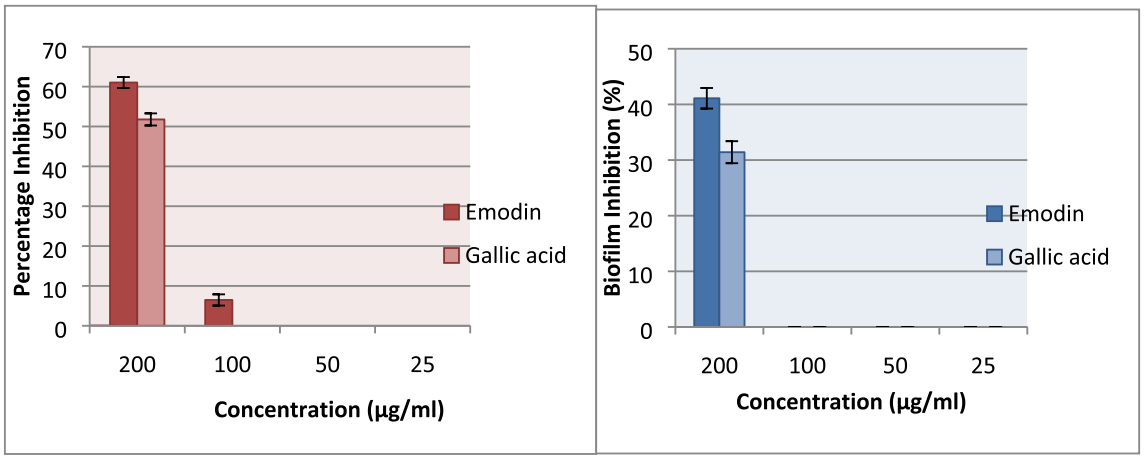

Fig. 4. Antibacterial activity and Antibiofilm potential of Emodin and Gallic acid against *P. aeruginosa*. Data represent as mean ± standard error. (n = 3).

Table 3
Binding energies and root mean squire distance (RMSD) using Auto Dock vina.

LasR and Gallic acid Interaction				LasR and emodin Interaction		
Mode	Affinity (kcal/mol)	RMSD Lower Bound (Å)	RMSD Upper Bound (Å)	Affinity (kcal/mol)	RMSD Lower Bound (Å)	RMSD Upper Bound (Å)
1	−6.8	0.000	0.000	−7.0	0.000	0.000
2	−6.7	2.494	2.967	−6.9	1.382	5.470
3	−6.7	2.472	3.751	−6.9	23.206	24.966
4	−6.6	1.574	2.834	−6.7	13.141	15.294
5	−6.2	1.621	3.518	−6.6	27.337	30.027
6	−6.2	1.987	4.284	−6.5	27.659	29.900
7	−6.1	2.148	4.228	−6.5	20.922	22.486
8	−6.1	1.645	4.302	−6.5	1.962	6.200
9	−6.0	1.624	3.149	−6.5	26.351	26.876

facilitated the identification of gallic acid and emodin. These data therefore lead us to the conclusion to check for the antibiofilm effects of gallic acid and emodin that might be the bioactive markers which are contributing to the antibacterial effects of *R. dentatus* being traditionally reported in folk medicine. These analytical characterizations establish a vital link between the chemical components and the subsequent biological activities observed. These findings not only expand our understanding of the chemical composition of the extract but also present a basis for investigating their roles as bioactive markers. The alignment between the historical uses of *R. dentatus* in traditional medicine and the observed antibacterial and antibiofilm activities of its root extract provides a compelling bridge between traditional wisdom and contemporary scientific investigation. These findings validate, in part, the traditional knowledge surrounding *R. dentatus* and provide a scientific basis for its medicinal applications.

To our knowledge, a comprehensive antibacterial and antibiofilm study guided by systemic approaches and bioassays has not yet been documented. Prior antibacterial investigations predominantly focused on leaf extracts, employing the disk diffusion method. Remarkably, the butanol extract displayed notable growth inhibition potential, particularly evident against *Klebsiella pneumoniae*, exhibiting an inhibition of 20 mm (Muhammad et al., 2014). Another study explored the effects on *Bacillus subtilis*, *Escherichia coli*, *S. aureus*, *Micrococcus lutes*, and *P. aeruginosa*. The results highlighted the efficacy of methanol and cold-water extracts against all tested bacterial strains. Notably, the MIC of these extracts against the bacterial strains fell within the range of 0.10 mg/ml (Batool et al., 2019).

The literature validated the antibacterial potential of gallic acid through a variety of mechanisms (Keyvani-Ghamsari et al., 2023). Our findings find support in the work of Zhu et al., who documented the potential of gallic acid as an anti-histaminic and antifungal agent, offering an additional facet to underpin its traditional use in dermatitis treatment (Zhu et al., 2006). Likewise, the presence of emodin or related analogs has been validated by other studies, shedding light on its potential as an antimicrobial agent (Khalil et al., 2022).

Molecular docking serves as a crucial tool for determining the lower binding affinities between ligands and receptors, making it invaluable in the field of drug discovery (Agu et al., 2023). The binding energies for gallic acid and emodin were found to be −6.8 and −7 kcal/mol, respectively. A lower energy value indicates a stronger binding capability. Gallic acid has been documented to exhibit lower binding energies in previous studies, albeit with a different target protein. Nevertheless, these findings confirm the antibacterial potential of gallic acid through molecular docking analysis (Abdelaziz et al., 2022). Similarly, previous studies have confirmed that emodin also possesses the potential to inhibit bacterial growth (Ding et al., 2011). Gallic acid and emodin could be explored further through in-depth mechanistic studies to better understand their antibacterial properties. Additionally, synthesizing derivatives of these compounds may enhance their effectiveness as antibacterial agents.

In our studies, we specifically focused on biomarkers within the water fraction. However, the reported studies suggested that the potential antimicrobial action might also be attributed to non-polar compounds. Exploring this aspect in-depth remains an intriguing avenue for future research. The integration of LC-MS analysis, chemical characterization, and bioactivity assessments has shed light on the potential therapeutic properties of *R. dentatus* root extract. The identification of gallic acid and emodin as key compounds with antibacterial and potentially antibiofilm effects represents a noteworthy contribution to our understanding of this medicinal plant. Further research endeavors should delve into unraveling the mechanisms underpinning these effects, potentially leading to the development of novel treatments for microbial infections and biofilm-associated issues.

Ethical approval

The study was not conducted on animals and humans, so ethical approval not required.

CRediT authorship contribution statement

Imran Khan: Methodology, Writing – original draft. **Uzma Khan:** Supervision, Formal analysis. **Wajiha Khan:** Supervision, Resources, Project administration. **Aljawharah Alqathama:** Formal analysis, Writing – review & editing. **Muhammad Riaz:** Methodology, Writing –

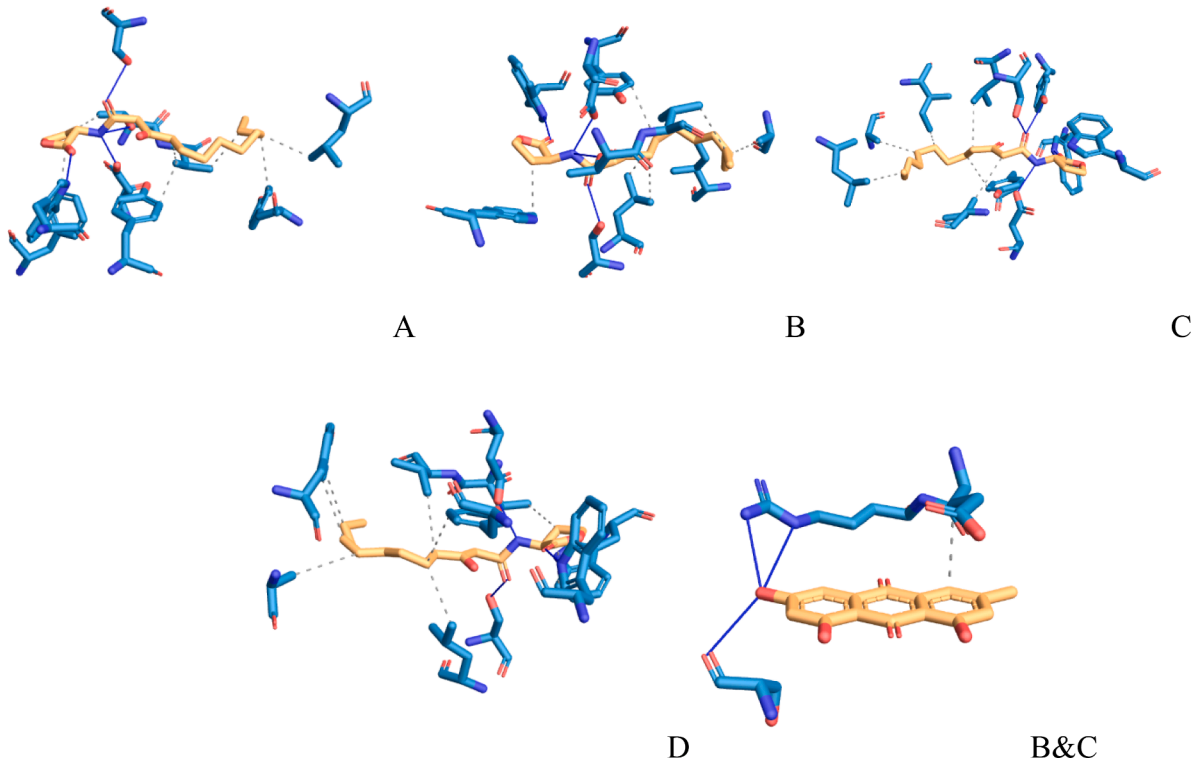

Fig. 5. Emodin and LasR protein interactions with Chain A, B, C, D, B & C, Blue = protein, orange color = ligand (emodin), dotted line = hydrophobic interactions, visualized using Protein Ligand Interaction Profiler (Adasme et al., 2021).

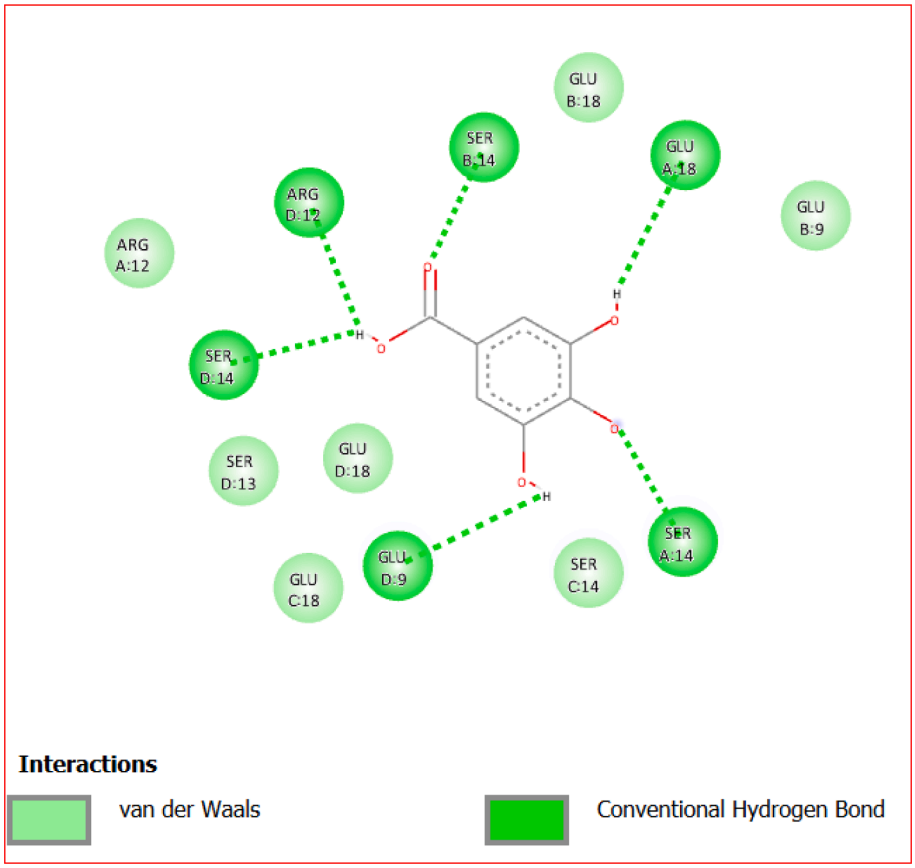

Fig. 6. Protein (LasR) and ligand (gallic acid) interaction.

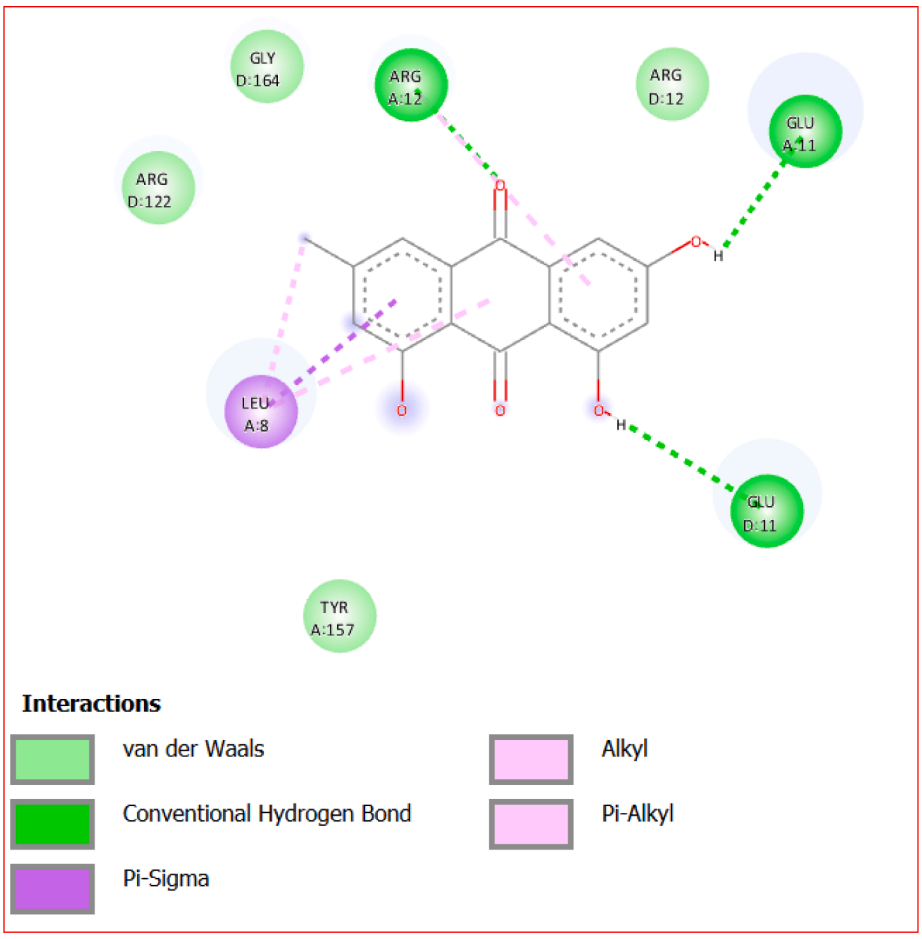

Fig. 7. Protein (LasR) and ligand (emodin) interaction.

review & editing. **Rizwan Ahmad:** Formal analysis, Visualization. **Mohammad Mahtab Alam:** Formal analysis, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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