

# Efficacy of *Rhamnus frangula* extract against *Acinetobacter baumannii* biofilms: Histopathological evidence from *ex vivo* goat models

RIYA MARIAM RONY VARUGHESE<sup>1</sup>, NAJI NASEEF PATHOOR<sup>2</sup>,  
PRIYADHARSHINI RANGANATHAN<sup>3</sup> and PITCHAIPILLAI SANKAR GANESH<sup>2</sup>

<sup>1</sup>Department of Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, Tamil Nadu 600 077, India; <sup>2</sup>Department of Microbiology, Centre for Infectious Diseases, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, Tamil Nadu 600 077, India; <sup>3</sup>Department of Oral Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, Tamil Nadu 600 077, India

Received November 6, 2024; Accepted February 2, 2025

DOI: 10.3892/wasj.2025.324

**Abstract.** *Acinetobacter baumannii* (*A. baumannii*) is a Gram-negative pathogen notorious for causing severe nosocomial infections, largely due to its ability to form biofilms that enhance antibiotic resistance and complicate treatment strategies. The present study explored the therapeutic potential of *Rhamnus frangula* (*R. frangula*) extract in mitigating *A. baumannii*-induced biofilm formation and related organ damage in goat tissue samples. The efficacy of the extract was assessed using minimum inhibitory concentration (MIC) tests, histopathological evaluations of heart, lung, liver, kidney and spleen tissues, biofilm inhibition assays, and growth curve analysis. The MIC assay demonstrated that *R. frangula* extract effectively inhibited *A. baumannii* growth at a concentration of 5 mg/ml. Histopathological analysis revealed significant reductions in bacterial load and inflammatory infiltrates across all examined organs, with notable improvements, such as decreased myofibril disintegration in cardiac tissue and reduced alveolar damage in lung tissue. Furthermore, at a sub-MIC of 2.5 mg/ml, *R. frangula* extract reduced biofilm formation by 67.26%, while exhibiting no effect on planktonic bacterial growth, highlighting its specific antibiofilm activity. These findings underscore the potential of *R. frangula* extract as a promising natural therapeutic agent for the treatment of

biofilm-associated infections and mitigating tissue damage caused by *A. baumannii*. However, further investigations into its molecular mechanisms and potential clinical applications are warranted to address the escalating challenge of antibiotic resistance and improve infection management strategies.

## Introduction

*Acinetobacter baumannii* (*A. baumannii*), is one of the most potentially hazardous bacteria in the domain of infections linked to healthcare. Once considered to be a generally benign environmental microbe, this Gram-negative coccobacillus has rapidly emerged as a major contributor to nosocomial infections worldwide (1). *A. baumannii* poses significant clinical challenges by driving persistent infections and organ damage, such as endocarditis and pneumonia, particularly in immunocompromised individuals (2). Infections caused by *A. baumannii*, are of significant concern due to their ability to invade and damage vital organs, such as the lungs, liver, kidneys and spleen (3). *A. baumannii* is a pathogen of key concern due to its ability to cause severe infections, particularly in immunocompromised individuals (4).

Biofilm formation by *A. baumannii* poses significant challenges in clinical settings, as it facilitates bacterial adhesion to surfaces, such as heart valves and lung tissue, resulting in persistent and difficult-to-treat infections (5,6). The biofilm matrix serves as a protective barrier, shielding bacteria from the host immune response and antibiotic treatment. In the heart, this can lead to endocarditis, marked by inflammation, tissue necrosis and the development of lesions or abscesses on the heart valves (7). The role of biofilm is pivotal in these processes, as it fosters the chronicity of the infection and complicates pathogen eradication. Similarly, in the lungs, *A. baumannii* biofilms can aggravate pulmonary infections, leading to conditions such as bronchitis, pneumonia and pleuritis. The dense bacterial communities within biofilms are linked to severe pathological changes, including lung tissue consolidation and, in extreme cases, abscess formation (8).

---

Correspondence to: Dr Priyadharshini Ranganathan, Department of Oral Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, 162 Poonamallee High Road, Chennai, Tamil Nadu 600 077, India  
E-mail: priyadharshinir328@gmail.com

**Key words:** *Acinetobacter baumannii*, biofilm, *Rhamnus frangula*, histopathology

These biofilm-related infections often present with respiratory distress, coughing and fever, similar to other bacterial infections, although with heightened resistance to treatment due to the protective nature of the biofilm (9). The ability of *A. baumannii* to form biofilms is closely linked to its virulence factors, which enable it to evade the immune system and persist within the host. The severity of infection, including the extent of tissue damage and clinical outcomes, is influenced by factors, such as the virulence of the strain, the bacterial load and the immune status of the host. Understanding the role of biofilm formation in *A. baumannii* infections is essential for devising more effective treatment strategies, particularly for overcoming the challenges posed by its antibiotic resistance (1). The interplay between biofilm formation and antibiotic resistance is crucial for understanding the pathogenesis of *A. baumannii* infections. Biofilms confer increased resistance to conventional treatments, making it difficult for antibiotics to penetrate and effectively kill the bacterial cells (9). Consequently, there is an urgent need for alternative therapeutic strategies that target both the bacterial cells and their biofilm structures.

Research reveals a strong positive association between biofilm formation and increased antibiotic resistance in *A. baumannii*. Strains of *A. baumannii* exhibit high resistance rates to ciprofloxacin, piperacillin and ceftazidime, with the blaOXA-23-like gene detected in 93% of multidrug-resistant (MDR) isolates (10). Additionally, 50 isolates have been identified as carbapenemase producers. Polymerase chain reaction (PCR) analysis has further demonstrated the presence of key virulence genes: traT (80%) associated with serum resistance, cvaC (34%) linked to colicin V production, and iutA (16%) involved in aerobactin synthesis (11). These findings underscore the critical association between biofilm formation and MDR in *A. baumannii*, highlighting the need for innovative approaches to combat infections caused by this resilient pathogen.

Natural medicine offers a promising and effective treatment option, potentially overcoming these challenges, while providing safer solutions for managing infections. Plants are extensively utilized in traditional medicine across the globe, as plant-derived treatments are considered relatively safe and provide more dependable and effective results. Compared to modern and conventional medicines, plant-based remedies have been reported to have fewer side-effects due to their natural origin (12). Plants produce secondary metabolites and phyto-constituents, including alkaloids, flavonoids, phenols, saponins, sterols, tannins and terpenoids, which can be used therapeutically in plants, humans and animals (13). *Rhamnus frangula* (*R. frangula*), frequently referred to as glossy buckthorn or alder buckthorn, is a small tree or deciduous shrub that is a member of the *Rhamnaceae* family. It is currently found in North America, where it is frequently regarded as an invasive species, having previously been native to Europe, North Africa, and Western Asia. The rich anthraquinone, flavonoid and tannin content of *R. frangula* is primarily responsible for its antimicrobial activity (14). Emodin and frangulin are two examples of anthraquinones that are well-known for having antimicrobial qualities. These substances have the ability to damage microbial cell walls, prevent the synthesis of proteins and obstruct the metabolism of nucleic acids, all of which can

result in cell death. Furthermore, as flavonoids can disrupt cell membranes, chelate metal ions and inhibit bacterial enzymes, they broaden the antimicrobial activity spectrum of the plant (15).

Biofilm formation not only supports bacterial survival, but also amplifies pathogenicity by enhancing resistance to antimicrobial treatments (16). In vital organs such as the lungs, heart, liver and kidneys, biofilms are often linked to chronic infections and play a crucial role in disease progression. For instance, in the lungs, biofilms formed by pathogens, such as *A. baumannii* play a critical role in conditions such as cystic fibrosis and chronic obstructive pulmonary disease (COPD), driving persistent inflammation and causing tissue damage. Similarly, biofilm-associated bacteria in the liver and kidneys can induce chronic infections, resulting in marked organ dysfunction and disease progression (17). The role of biofilms in organ damage is largely due to the ability of the bacteria to evade the immune response and persist over long periods of time. This chronic inflammation and infection compromise the integrity of the tissues, leading to scarring, necrosis and functional impairment of the organs. The interplay between biofilm formation and the immune system is crucial in understanding the pathogenesis of these infections and developing more effective treatment strategies to prevent organ damage (18).

The present study aimed to evaluate the antimicrobial and antibiofilm properties of *R. frangula* against *A. baumannii*. Additionally, it sought to identify, characterize, and analyze the subcellular pathological changes in goat organs—an aspect that, to the best of our knowledge, has not been previously explored. As a pioneering investigation, this research examines both the antibiofilm effects of *R. frangula* on *A. baumannii* and the pathological impact of *A. baumannii* infusion in goat organs, utilizing an innovative approach to assess its potential as a therapeutic agent.

## Materials and methods

**Study design and sample collection.** The research study was conducted between March and September, 2024, following approval from the Scientific Review Board of Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India (SRB/SDC/UG-2276/24/GPATH/076). The approval was granted for the use of goat samples in the present study. *R. frangula* herbal powder was sourced from a botanical garden in Chennai, Tamil Nadu, India. To validate the authenticity, purity and quality of the herbal sample, a certified botanist performed a comprehensive examination and verified its identity. The Authentication and Identification Certificate (SVMC/BOT/272/2023-24) issued by Sri Vidya Mandir Arts & Science College (Autonomous), Katteri, Krishnagiri District, Tamil Nadu, India. This certification confirms that the sample was authenticated based on its morphological characteristics. Following authentication, the powder was stored under controlled conditions for subsequent analysis and utilization in the study.

**Sample extraction.** To prepare the extract, 10 g powdered herbal *R. frangula* were combined with 100 ml methanol (Rankem Laboratories, LLC). Throughout the 48-h extraction period, a shaker was used to periodically shake the mixture.

Upon completing the extraction process, the suspension was filtered, the temperature of the water bath was regulated to 50°C, and the methanol solvent was removed from the filtrate. The material was weighed and stored at 4°C for later use after drying.

**Bacterial culture and conditions.** The Malabar Cancer Center in Kerala, India, provided the *A. baumannii* culture samples used in the present study. Luria Bertani (LB) broth (HiMedia Laboratories, LLC) was used to subculture the samples. Subsequently, for 24 h, *A. baumannii* cultures were incubated at 37°C in a shaking incubator with a speed setting of 100 revolutions per minute (rpm). Both LB agar and Nutrient agar exhibited distinctive growth patterns. The laboratory staff at Saveetha Dental College and Hospital in Chennai, Tamil Nadu, India, used the VITEK 2 automated system for preliminary identification to confirm the identity of *A. baumannii*, following the methodology described by Bobenchik *et al* (19).

**Antimicrobial efficacy of *R. frangula*.** The antibacterial activity of *R. frangula* extract was evaluated using the agar well-diffusion method, according to previously established protocols (20). A bacterial culture of *A. baumannii* was uniformly spread onto Mueller Hinton Agar (MHA) plates (HiMedia, Mumbai, India) using a sterile swab moistened with the bacterial suspension. A well with an 8-mm diameter was carefully punched into the MHA medium using a sterile cork borer. Subsequently, 40 µl *R. frangula* extract (prepared in DMSO at a concentration of 20 mg/ml) were added to the well. DMSO alone was used as a negative control. The plates were incubated at 37°C for 24 h. Following the incubation period, the zones of inhibition around the wells were measured in millimeters using a vernier caliper to evaluate the antibacterial activity of the extract.

**Drug susceptibility testing.** The Kirby-Bauer disk diffusion method was employed to evaluate the antimicrobial susceptibility of *A. baumannii*, as previously described (21). A standardized suspension of *A. baumannii* was evenly spread onto MHA plates using a sterile swab. Disks impregnated with a range of antibiotics commonly used against *A. baumannii*, including tetracycline, piperacillin/tazobactam (PIT), cefixime, imipenem, ceftriaxone, cefotaxime and meropenem (all from HiMedia Laboratories, LLC), were placed on the agar surface. The plates were then incubated at 37°C for 24 h. Following incubation, the zones of inhibition around the disks were measured in mm to determine bacterial susceptibility.

**Minimum inhibitory concentration assay (MIC).** *A. baumannii* is a key nosocomial pathogen known for its ability to develop MDR, rendering treatment options limited (22). The present study investigated the antimicrobial activity of *R. frangula* extract against *A. baumannii* using the broth dilution method (20,23). The MIC of the *R. frangula* extract was determined using a 2-fold broth dilution method, with concentrations ranging from 10 to 0.019 mg/ml. Briefly, a standardized *A. baumannii* inoculum ( $1.5 \times 10^8$  CFU/ml) was added to LB broth containing the various extract concentrations, and the samples were incubated at 37°C for 24 h. To monitor color changes and validate the findings, 2,3,5-triphenyl tetrazolium

chloride (TTC) was transferred to each tube following incubation. A level of concentration at which no color change occurred was considered to be the MIC. Further analysis was conducted at the MIC endpoint for histopathological examination.

**Collection of organ samples.** The goat heart, lung, liver, kidney and spleen were carefully sourced from a slaughterhouse in Saidapet, Chennai, Tamil Nadu, India, under the guidance of Professor M. Raman, a veterinary expert from Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India. Upon procurement, the organs were promptly covered with sterile polyethylene to ensure hygiene and prevent any potential contamination. These samples were then stored at a temperature of 4°C to preserve their integrity and prevent bacterial growth. The samples were carefully transported to the laboratory, ensuring that the organs remained undamaged during transit. Upon arrival at the laboratory, the organs were placed under refrigeration conditions at  $-15 \pm 4^\circ\text{C}$  to maintain their quality and suitability for further analysis. This careful handling and storage process ensured that the samples were in optimal condition for subsequent experimental procedures and analysis.

**Infusion of *R. frangula* extract and bacteria into goat organs.** At the end point of MIC of 5 mg/ml, *R. frangula* extract was introduced into a sterile container with brain heart infusion (BHI) (HiMedia Laboratories, LLC) medium. A 20-µl aliquot of *A. baumannii* was added to freshly prepared BHI broth, followed by incubation at 37°C for 18 h. Following this, a wedge-shaped incision (3 mm) was made in the grossed piece of the goat heart, lung, liver, kidney and spleen, which was subsequently immersed in BHI broth. The previously incubated 20 µl of the bacterial culture was inoculated into the broth. The container was incubated at 37°C for 24 h, alongside a control group consisting of untreated organs. After 24 h, the organs were processed to assess histopathological changes.

**Histopathology of bacterial infused organs.** Hematoxylin and eosin (H&E) staining is a widely utilized method in histopathology to visualize cellular components of tissues. Herein, to preserve tissue structure and to prevent degradation, tissue samples were fixed in 10% formalin (HiMedia Laboratories, LLC) for at least 24 h. The fixed tissues were then dehydrated through a series of increasing ethanol concentrations (70, 80 and 100%) to remove water. Subsequently, the tissues were treated with xylene to eliminate alcohol and prepare them for paraffin infiltration. The tissues were then immersed in molten paraffin wax, embedded in paraffin blocks and allowed to solidify.

Thin tissue sections were obtained using a microtome, cutting them to a thickness of 4-5 µm. These sections were then floated on a water bath to flatten them before being placed on glass slides. To remove the paraffin wax, the slides were immersed in xylene, followed by rehydration through decreasing concentrations of ethanol (100, 80 and 70%) and a rinse in distilled water. The slides were then stained with hematoxylin (Sigma-Aldrich, USA) at room temperature ( $25 \pm 2^\circ\text{C}$ ) for 5-10 min, rinsed in tap water, and differentiated in acid alcohol (1% HCl in 70% ethanol). Following another rinse in tap water, the slides were 'blued' by immersion in a weak

alkaline solution (e.g., 0.1% ammonia water) or tap water until the nuclei appear blue. The slides were then stained with eosin (MilliporeSigma) at room temperature ( $25\pm 2^\circ\text{C}$ ) for 1-3 min and briefly rinsed in tap water. Dehydration is performed again by passing the slides through increasing concentrations of ethanol (70, 80 and 100%) to remove water, followed by immersion in xylene to eliminate ethanol and prepare for mounting. A drop of mounting medium was applied to the slide, and a coverslip was placed over the tissue sections. After allowing the sections to dry, the stained sections were examined under a light microscope (CX23, Olympus Corporation) to observe the cellular and tissue structures (24).

Further experimental analysis was conducted to evaluate the antibiofilm activity and growth curve of *R. frangula* extract at sub-MIC concentrations against *A. baumannii*.

**Biofilm assay.** To evaluate the effects of *R. frangula* extract on *A. baumannii* biofilm formation, a crystal violet staining assay was employed as previously described by Venkatraman *et al* (25). A microtiter plate containing 180  $\mu\text{l}$  fresh LB medium was first filled with an overnight culture of *A. baumannii* (20  $\mu\text{l}$ ). Subsequently, *R. frangula* extract was added at concentrations ranging from 2.5 to 0.004 mg/ml, which is after the MIC point. For 48 h, the mixture was incubated at  $37^\circ\text{C}$ . Following incubation, the biofilm that stuck to the surface was stained for 2 min at room temperature using a 0.1% crystal violet solution (HiMedia Laboratories, LLC), and the planktonic cells were eliminated by rinsing with sterile distilled water at room temperature. The crystal violet-bound biofilm was then eluted with 200  $\mu\text{l}$  70% ethanol after a 10-min incubation. The absorbance of the eluted crystal violet was measured at 520 nm using a UV-Vis spectrophotometer (JASCO UV/Vis, India). Biofilm inhibition (%) was calculated using the following formula:  $(\text{Control OD } 520 \text{ nm} - \text{treated OD } 520 \text{ nm})/\text{control OD } 520 \text{ nm} \times 100$ .

***A. baumannii* growth analysis.** *A. baumannii* bacterial development was examined using *R. frangula* extract at 1.25 mg/ml doses or the extract. The culture was incubated at  $37^\circ\text{C}$  for up to 24 h, and the cell density was measured at OD 600 nm every hour.

**Statistical analysis.** All experiments were performed in triplicate, and the biofilm assay (crystal violet assay), and growth curve demonstrated statistical significance. The data were analyzed using one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) test, performed with GraphPad Prism 10.1.0 software (Dotmatics). A P-value  $<0.05$  was considered to indicate a statistically significant difference.

## Results

**Antimicrobial activity of *R. frangula*.** The antimicrobial potential of *R. frangula* extract was evaluated using the agar well diffusion method against *A. baumannii*. The results revealed a distinct zone of inhibition measuring 16 mm (Fig. 1), indicating a significant level of antimicrobial efficacy against this pathogen. This suggests that *R. frangula* extract possesses the capability to inhibit the growth of *A. baumannii*, which is particularly relevant given the MDR nature of the pathogen.

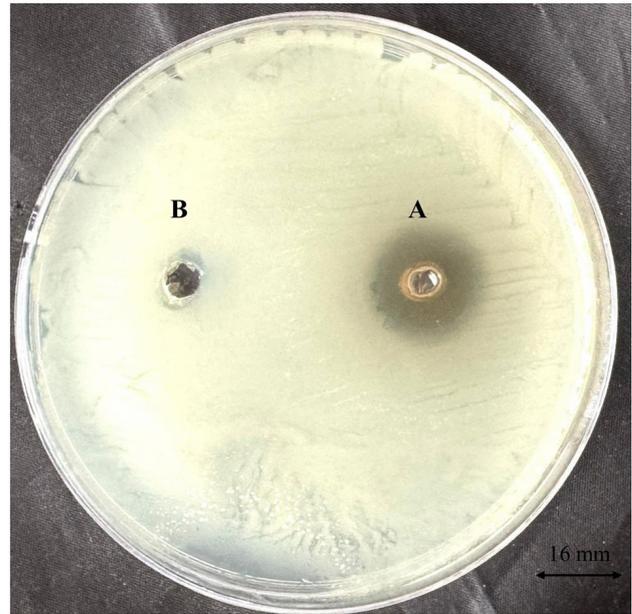


Figure 1. Antimicrobial activity of *R. frangula* against *A. baumannii*. (A) The antimicrobial activity of *R. frangula* extract is demonstrated by a zone of inhibition measuring 16 mm in diameter against *A. baumannii*. (B) DMSO serves as the control, showing no inhibition zone. *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

**Antibiotic sensitivity patterns.** The antibiotic resistance patterns of *A. baumannii* were assessed using a panel of different antibiotic discs. The findings indicated that the majority of the antibiotics tested were ineffective against *A. baumannii*, highlighting a notable level of MDR (Table I). This extensive resistance complicates treatment options and underscores the urgent need for new therapeutic strategies.

**Bactericidal activity at the MIC level.** The inhibitory effect of *R. frangula* extract was assessed using a 2-fold serial dilution method, with concentrations ranging from 10 to 0.019 mg/ml. The results revealed that the growth of *A. baumannii* was effectively inhibited at a concentration of 5 mg/ml (Table II). Subsequently, the potential antibiofilm properties of *R. frangula* at sub-MIC concentrations were further investigated. This additional analyses aimed to explore whether lower, non-lethal concentrations of the extract could disrupt or prevent biofilm formation, which is a key factor in the persistence and resistance of *A. baumannii* in clinical settings.

**Findings of the histopathological analysis.** As evidenced herein, the infiltration of the bacterium *A. baumannii* leads to the marked disintegration of myofibrils. This bacterial presence triggers the degeneration of myofibers, which is accompanied by a dense inflammatory infiltrate. The extensive inflammatory response exacerbates the damage to muscle tissue, resulting in the breakdown of myofibril structure and contributing to overall muscle degeneration. This complex pathological process underscores the aggressive nature of *A. baumannii* infection and its detrimental effects on muscle integrity (Fig. 2). As illustrated in Fig. 2D, the cardiac tissue treated with 5 mg/ml *R. frangula* extract exhibited a gradual

Table I. Antibiogram of *Acinetobacter baumannii* against several antibiotics.

Sample no.	Antibiotics	<i>Acinetobacter baumannii</i>
1	Tetracycline	10±0.8
2	PIT	11±0.8
3	Cefixime	R
4	Imipenem	R
5	Ceftriaxone	R
6	Cefotaxime	R
7	Meropenem	R

R, resistant.

reduction in bacterial load and a corresponding decrease in inflammatory cell infiltration.

An H&E-stained section of the lung tissue revealed diffuse alveolar damage accompanied by marked inflammatory infiltrate. Following the infusion of the bacterium *A. baumannii*, there was a marked increase in alveolar septal thickening. This pathological alteration indicated severe lung tissue injury, characterized by widespread damage to the alveolar structures and intensified inflammatory response, leading to the thickening of the alveolar walls. The histopathological findings highlight the extensive impact of *A. baumannii* infection on lung tissue architecture and function (Fig. 3). As illustrated in Fig. 3D, the lung tissue treated with the endpoint MIC of *R. frangula* extract at 5 mg/ml exhibited a reduction in bacterial load, along with a noticeable decrease in inflammatory cell infiltration.

The hepatocytes demonstrated vacuolar degeneration accompanied by the infiltration of *A. baumannii* (Fig. 4A). Following treatment with 5 mg/ml *R. frangula* extract, there was a marked decrease in the bacterial load (Fig. 4B). The spleen exhibited widespread degeneration of the germinal centers, accompanied by infiltration of *A. baumannii* (Fig. 5A). This was associated with a significant bacterial load and a dense inflammatory infiltrate. However, following treatment with 5 mg/ml *R. frangula* extract, there was a notable reduction in the bacterial load (Fig. 5B).

The spleen revealed generalized degeneration in the kidney tubules, with certain regions of Bowman's capsule showing infiltration by *A. baumannii* and a chronic inflammatory response (Fig. 6A). Additionally, areas of the kidney and Bowman's capsule exhibited vacuolar degeneration. Following treatment with 5 mg/ml *R. frangula* extract, there was a notable decrease in bacterial load, indicating a therapeutic effect on the infected tissues (Fig. 6B).

**Inhibition of biofilm formation in *A. baumannii*.** The inhibitory effect of *R. frangula* extract on the ability of *A. baumannii* to form biofilms was investigated using the static microtiter plate method, with 0.1% crystal violet staining to quantify biofilm biomass. The results revealed that *R. frangula* extract significantly inhibited biofilm formation ( $P < 0.05$ , significant difference compared to the untreated control) with spectrophotometric analysis revealing 67.26%

Table II. Minimum inhibitory concentration of *R. frangula* extract for the inhibition of *A. baumannii* growth (final concentration of 5 mg/ml).

Sample no.	Two-fold dilution concentration (mg/ml)	Growth measured <sup>a</sup>
1	10	-
2	5	-
3	2.5	+
4	1.25	+
5	0.62	+
6	0.312	+
7	0.156	+
8	0.078	+
9	0.039	+
10	0.019	+

<sup>a</sup>The growth measured refers to the presence (+) or absence (-) of visible growth in the microbial culture following exposure to the respective 2-fold dilution concentrations (mg/ml). *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

inhibition at 2.5 mg/ml and 58.22% inhibition at 1.25 mg/ml (Fig. 7).

**Analysis the growth of *A. baumannii*.** The growth curve analysis was applied both with and without the *R. frangula* extract. Fig. 8 demonstrates that at a dose of 2.5 mg/ml, *R. frangula* extract did not inhibit the growth of *A. baumannii*. Spectrophotometric measurements showed no appreciable difference between the treated and control bacterial cells at 600 nm. These findings suggest that *R. frangula* extract does not inhibit the planktonic growth of *A. baumannii* at 2.5 mg/ml also effectively reduces its ability to form biofilms.

## Discussion

*A. baumannii* is considered one of the most hazardous bacteria associated with healthcare-related infections. Its marked ability to acquire antibiotic resistance and persist in diverse environments poses a significant challenge in clinical settings. In the present study, a series of *in vitro* experiments demonstrated that *R. frangula* extract effectively reduced *A. baumannii* biofilm formation, highlighting its potential as a therapeutic agent. Similarly, Zhou *et al* (26) demonstrated that *P. aeruginosa* three quorum sensing (QS) systems were inhibited by hordenine, an herbal extract made from sprouting barley.

In the present study, at a MIC of 5 mg/ml, the initial results demonstrated that the bactericidal activity of *A. baumannii* was inhibited by *R. frangula* extract. This observation is consistent with the findings of a previous study demonstrating that methanolic extracts of *Cuminum cyminum* can inhibit Gram-negative bacteria at MIC levels (27). Additionally, it has been noted that aloe vera gel extract is more effective against Gram-positive than Gram-negative bacteria, with

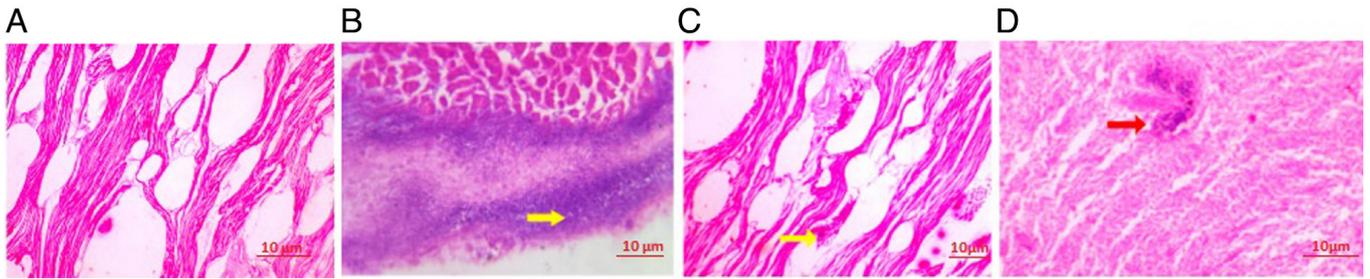


Figure 2. (A) Control sample. (B) Heart tissue sample with infiltration of *A. baumannii* (yellow arrow), exhibiting chronic inflammatory infiltrate (x40 total magnification). (C) Degeneration of cardiac muscles (yellow arrow) (x40 total magnification). (D) Cardiac tissue treated with 5 mg/ml *R. frangula* extract exhibited a gradual decrease in bacterial load and inflammatory infiltrate (red arrow) (x40 total magnification). *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

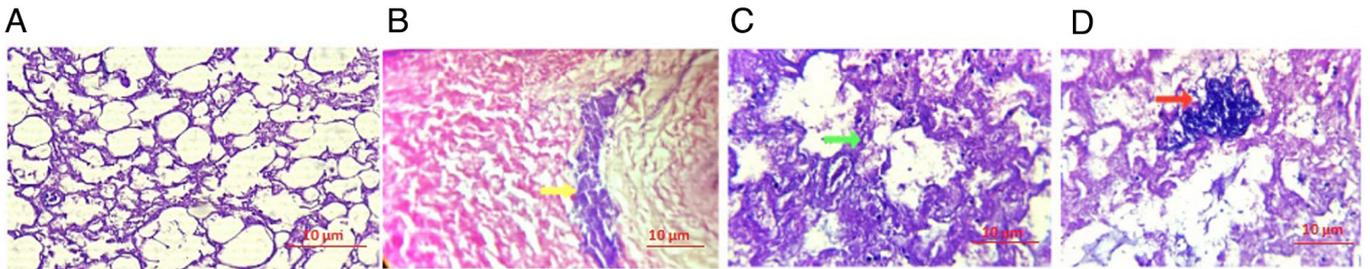


Figure 3. (A) Control sample. (B) Lung tissue with inflammatory infiltrate (yellow arrow). (C) Diffuse alveolar septal thickening post infiltration of *A. baumannii* with degeneration of bronchial epithelium (green arrow) (x40 total magnification). (D) Lung tissue treated with 5 mg/ml *R. frangula* extract exhibited a decreased bacterial load and inflammatory infiltrate (red arrow) (x40 total magnification). *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

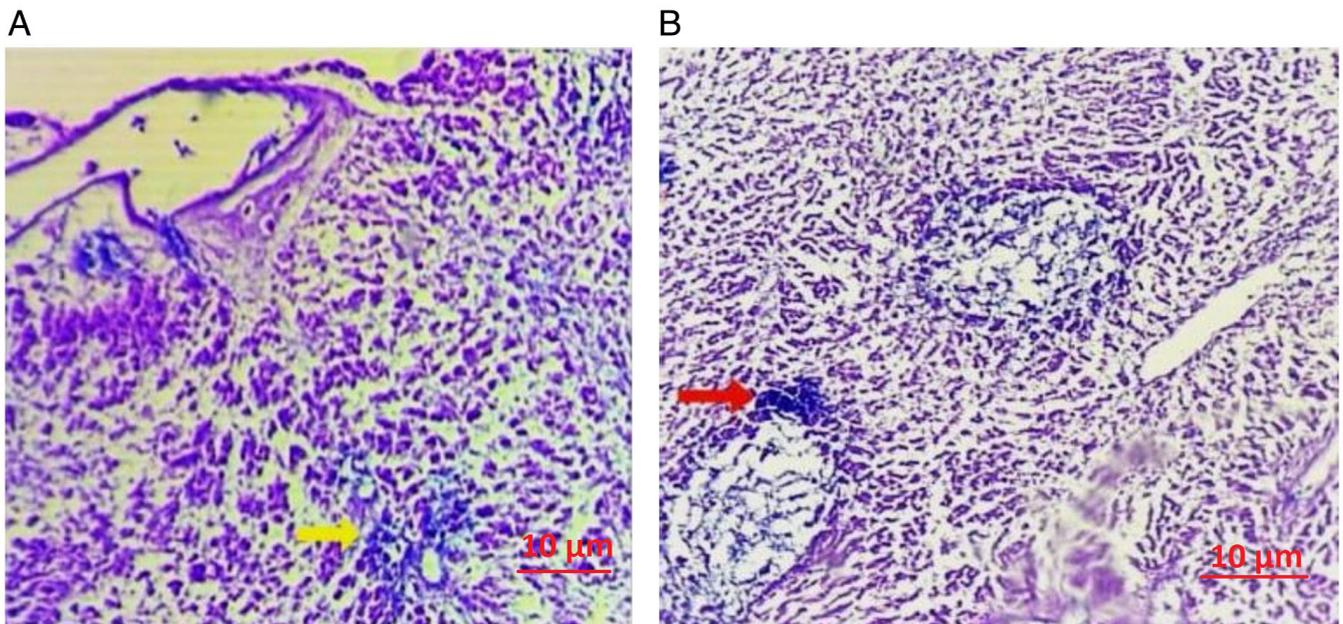


Figure 4. (A) Hepatocytes exhibiting vacuolar degeneration with infiltration of *A. baumannii* (yellow arrow) (x40 total magnification). (B) Liver tissue treated with 5 mg/ml *R. frangula* extract exhibited a gradual decrease in bacterial load (indicated by red arrows) under x40 total magnification. *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

ethanol and methanol extracts exhibiting the highest activity, while acetone extract exhibited the least inhibition (28).

Furthermore, in the present study, *R. frangula* extract inhibited QS-regulated biofilm formation in *A. baumannii* in a

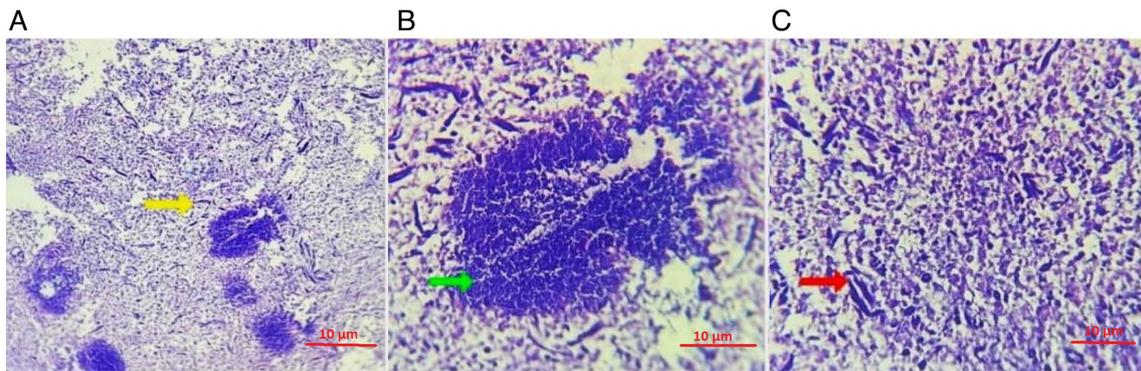


Figure 5. (A) Spleen exhibiting a generalized area of degeneration of germinal center with infiltration of *A. baumannii* (yellow arrow) (x10 magnification). (B) Bacterial load with dense inflammatory infiltrate (green arrow) (x40 total magnification). (C) Decreased bacterial load post-treatment with 5 mg/ml *R. frangula* extract (red arrow) (x40 total magnification). *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

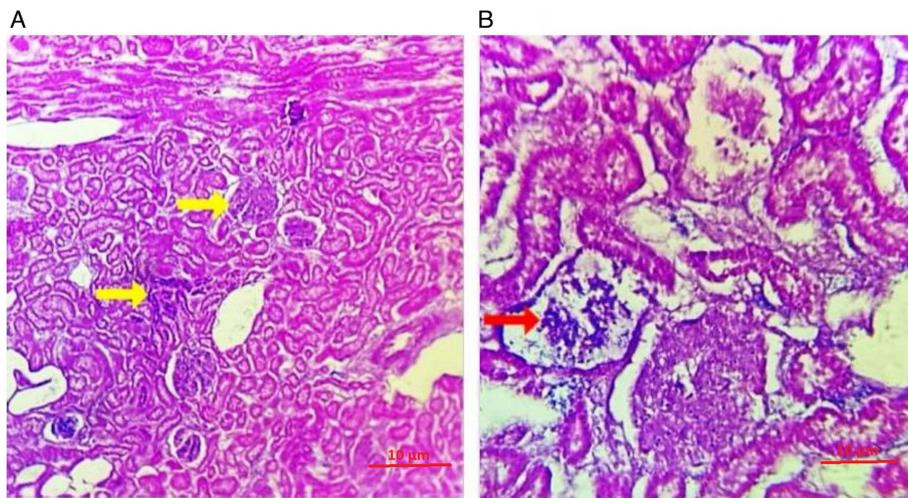


Figure 6. (A) Spleen exhibiting areas of degeneration of kidney tubules and areas of Bowman's capsule showing *A. baumannii* with chronic inflammatory infiltrate (yellow arrow) (x10 total magnification). (B) Areas exhibiting vacuolar degeneration of kidney and Bowman's capsule along with decreased bacterial load post-treatment with 5 mg/ml of *R. frangula* extract (red arrow) (x40 total magnification). *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

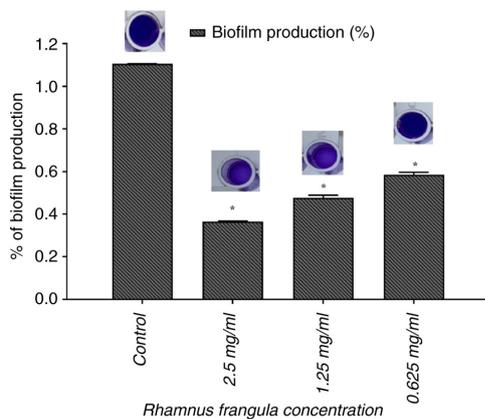


Figure 7. Crystal violet biofilm inhibition assay. Graphical representation: 67.26, 58.22 and 47.37% of biofilm inhibition in *A. baumannii* when treated with 2.5, 1.25 and 0.625 mg/ml of *R. frangula* extract, respectively. Statistical analysis was performed using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test ( $P < 0.05$ , significant difference compared to the untreated control). The x value refers to the concentration of *R. frangula* extract in mg/ml (presented on the x-axis), and the y value refers to the % of biofilm production (presented on the y-axis). *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

concentration-dependent manner at sub-MIC concentrations. Specifically, the crystal violet assay revealed that a concentration of 2.5 mg/ml *R. frangula* extract markedly decreased biofilm formation without influencing planktonic cell growth (Figs. 7 and 8). Miyasaki *et al* (29) also reported that specific compounds in herbal extracts, such as flavones, tannins, and phenolic compounds, are generally known for their anti-*Acinetobacter* activity.

The discussion on biofilm formation and its pathological implications in vital organs highlights the crucial role of biofilms in chronic infections. Biofilm formation is a key survival mechanism for bacteria, enhancing their resistance to antimicrobial treatments and the immune system (30). The ability of bacteria to form biofilms, particularly in organs such as the lungs, liver and kidneys, contributes to persistent infections that are challenging to eradicate, often leading to prolonged inflammation and organ damage (31). Infections caused by *A. baumannii*, a notorious biofilm producer, exemplify this phenomenon, particularly in the lungs where biofilms are involved in severe respiratory diseases such as cystic fibrosis and COPD (32).

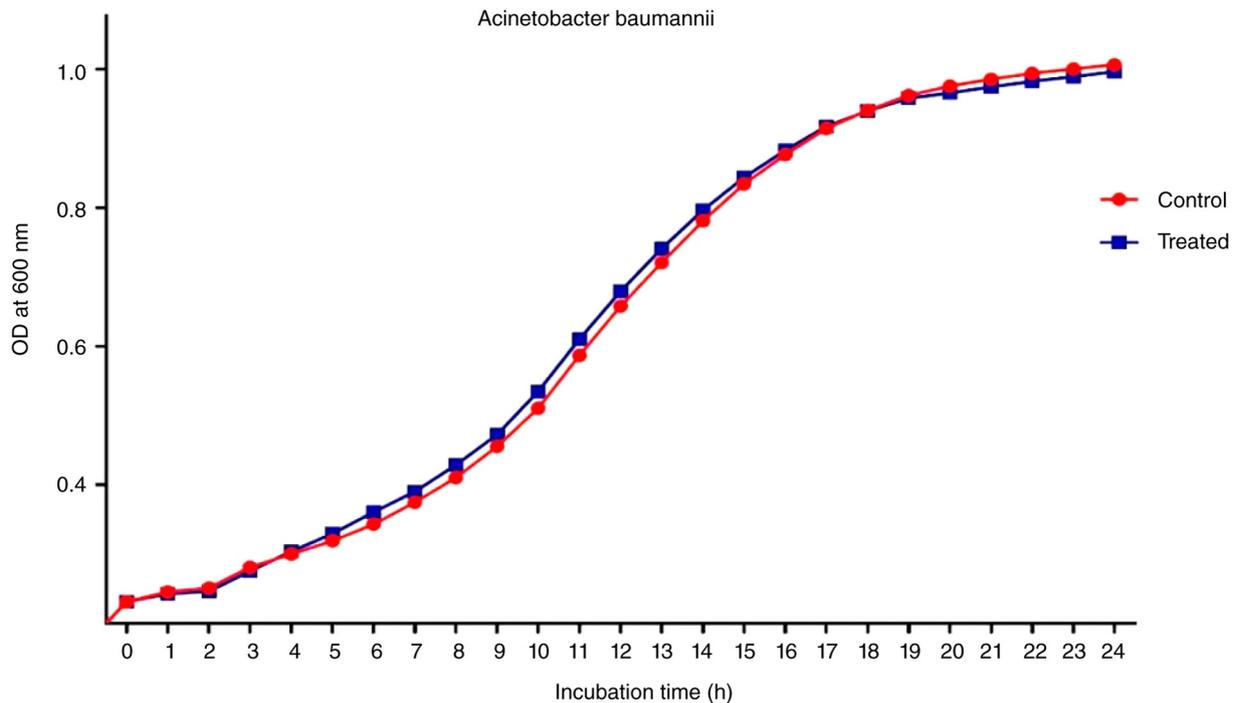


Figure 8. Analysis of the growth curve. *A. baumannii* grown both in the absence (control) and with 2.5 mg/ml *R. frangula* extract. *A. baumannii*, strain of *Acinetobacter baumannii*; *R. frangula*, *Rhamnus frangula*.

In the present study, *A. baumannii* infiltrations induced notable pathological changes, including disintegration of myofibrils in cardiac muscle and extensive damage to alveolar structures in the lungs, which were effectively mitigated with 5 mg/ml *R. frangula* extract. These findings are consistent with those of previous research, demonstrating that biofilm-producing bacteria exacerbate tissue damage by triggering chronic inflammatory responses, which further deteriorate tissue architecture (33). The persistent presence of bacterial biofilms hinders the effectiveness of immune clearance, allowing the bacteria to persist and cause scarring, necrosis and the functional impairment of vital organs over time (34).

An innovative feature of the present study lies in its use of *ex vivo* goat models to evaluate the effects of *R. frangula* extract on *A. baumannii* biofilms, an approach that has not been extensively explored in previous research to date, at least to the best of our knowledge. This biologically relevant platform bridges the gap between *in vitro* findings and potential *in vivo* applications, providing a more comprehensive assessment of the extract's efficacy. Furthermore, the holistic evaluation of antimicrobial and antibiofilm activity, along with its impact on histopathological changes across multiple organ systems (heart, lungs, liver, kidney and spleen), underscores the therapeutic potential of the extract and its ability to preserve tissue integrity.

The present study opens several promising avenues for future research. The *ex vivo* goat organ model provides valuable insight into the potential of *R. frangula* extract for the management of *A. baumannii*-associated infections. Expanding this research to include human clinical settings will enable a more comprehensive evaluation of the extract's

efficacy and safety. Rigorous clinical trials could further establish its therapeutic potential and bridge the gap to clinical applications. Additionally, the present study investigated only a single isolate of *A. baumannii*, which may limit the broader applicability of the findings. Future studies are thus required to involve a diverse range of clinical isolates to ensure the consistency and generalizability of the results. Moreover, the findings of the present study on biofilm inhibition create opportunities to delve deeper into the molecular mechanisms behind this activity. A more detailed exploration could illuminate the mode of action of the extract, paving the way for innovative strategies, such as combining *R. frangula* with conventional antibiotics to enhance therapeutic outcomes.

Taken together, the findings of the present study provide compelling evidence of the therapeutic potential of *R. frangula* extract against *A. baumannii* biofilms, while also recognizing its inherent strengths and limitations. These findings contribute to the ongoing efforts to combat antibiotic-resistant infections and underscore the importance of further studies to fully explore and realize the clinical applicability of this natural remedy.

In conclusion, the results of the present study demonstrate the potential of *R. frangula* extract as a beneficial treatment choice for *A. baumannii* infections. The present study demonstrates the complex role of biofilms in promoting bacterial persistence and tissue damage, while also shedding light on the potential of natural compounds to mitigate these effects. Further investigations are required however, to on the molecular mechanisms underlying the biofilm-inhibitory properties of *R. frangula*, contributing to the development of novel therapeutic strategies for biofilm-associated infections.

## Acknowledgements

Not applicable.

## Funding

No funding was received.

## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

RMRV collected, managed the data and participated in the writing of the manuscript. PR and NNP participated in writing the proposal, performing data collection and in the writing of the manuscript. PR and PSG were involved in data curation, data analysis and in revising the manuscript. PR, NNP and PSG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Scientific Review Board of Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India (SRB/SDC/UG-2276/24/GPATH/076). The approval was granted for the use of goat samples.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

- Ibrahim S, Al-Saryi N, Al-Kadmy IMS and Aziz SN: Multidrug-resistant *Acinetobacter baumannii* as an emerging concern in hospitals. *Mol Biol Rep* 48: 6987-6998, 2021.
- Pathoor NN, Ganesh PS and Gopal RK: Microbiome interactions: *Acinetobacter baumannii* biofilms as a co-factor in oral cancer progression. *World J Microbiol Biotechnol* 40: 398, 2024.
- Gedefie A, Demsis W, Ashagrie M, Kassa Y, Tesfaye M, Tilahun M, Bisetegn H and Sahle Z: Biofilm formation and its role in disease pathogenesis: A review. *Infect Drug Resist* 14: 3711-3719, 2021.
- Müller C, Reuter S, Wille J, Xanthopoulou K, Stefanik D, Grundmann H, Higgins PG and Seifert H: A global view on carbapenem-resistant *Acinetobacter baumannii*. *mBio* 14: e0226023, 2023.
- Girija ASS: *Acinetobacter baumannii* as an oro-dental pathogen: A red alert!! *J Appl Oral Sci* 32: e20230382, 2024.
- Loehfelme TW, Luke NR and Campagnari AA: Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. *J Bacteriol* 190: 1036-1044, 2008.
- Pai L, Patil S, Liu S and Wen F: A growing battlefield in the war against biofilm-induced antimicrobial resistance: Insights from reviews on antibiotic resistance. *Front Cell Infect Microbiol* 13: 1327069, 2023.
- Smiline Girija SA: Hijacking the epigenetic mechanisms of *A. baumannii*. *Mol Biol Res Commun* 13: 51-53, 2024.
- Pathoor NN, Viswanathan A, Wadhwa G and Ganesh PS: Understanding the biofilm development of *Acinetobacter baumannii* and novel strategies to combat infection. *APMIS* 132: 317-335, 2024.
- Mohajeri P, Farahani A, Feizabadi MM and Norozi B: Clonal evolution multi-drug resistant *Acinetobacter baumannii* by pulsed-field gel electrophoresis. *Indian J Med Microbiol* 33: 87-91, 2015.
- Mohajeri P, Sharbati S, Farahani A and Rezaei Z: Evaluate the frequency distribution of nonadhesive virulence factors in carbapenemase-producing *Acinetobacter baumannii* isolated from clinical samples in Kermanshah. *J Nat Sci Biol Med* 7: 58-61, 2016.
- Nasim N, Sandeep IS and Mohanty S: Plant-derived natural products for drug discovery: Current approaches and prospects. *Nucleus (Calcutta)* 65: 399-411, 2022.
- Kalinowska M, Gołębiewska E, Świdorski G, Męczyńska-Wielgosz S, Lewandowska H, Pietryczuk A, Cudowski A, Astel A, Świsłocka R, Samsonowicz M, *et al*: Plant-derived and dietary hydroxybenzoic acids-a comprehensive study of structural, anti-/pro-oxidant, lipophilic, antimicrobial, and cytotoxic activity in MDA-MB-231 and MCF-7 cell lines. *Nutrients* 13: 3107, 2021.
- Greenleaf J, Karimzadeh R and Park YL: Spatial patterns of *Frangula alnus* (Rosales: Rhamnaceae): Implications for invasive plant management. *Biology (Basel)* 12: 1393, 2023.
- Górniak I, Bartoszewski R and Króliczewski J: Comprehensive review of antimicrobial activities of plant flavonoids. *Phytochem Rev* 18: 241-272, 2019.
- Lahiri D, Nag M, Ray RR and Ghosh S (eds): *Biofilm-Associated Antimicrobial Resistance and Its Recovery*. CRC Press, Boca Raton, p391, 2023.
- Kumar S, Chandra N, Singh L, Hashmi MZ and Varma A (eds): *Biofilms in Human Diseases: Treatment and Control*. Springer Nature, p318, 2019.
- Labis V, Gaiduk I, Bazikyan E, Khmelenin D, Zhigalina O, Dyachkova I, Zolotov D, Asadchikov V, Kravtsov I, Polyakov N, *et al*: The role of metal nanoparticles in the pathogenesis of stone formation. *Int J Mol Sci* 25: 9609, 2024.
- Bobenchik AM, Deak E, Hindler JA, Charlton CL and Humphries RM: Performance of Vitek 2 for antimicrobial susceptibility testing of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* with Vitek 2 (2009 FDA) and CLSI M100S 26th edition breakpoints. *J Clin Microbiol* 55: 450-456, 2017.
- Soni M, Naseef Pathoor N, Viswanathan A, Veeraragavan GR and Sankar Ganesh P: Exploring the antimicrobial and anti-biofilm activities of *Artocarpus heterophyllus* Lam. against *Pseudomonas aeruginosa* PAO1. *World Acad Sci J* 6: 50, 2024.
- Hudzicki J: Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. American Society for Microbiology, Washington, DC, pp55-63, 2009.
- Howard A, O'Donoghue M, Feeney A and Sleator RD: *Acinetobacter baumannii*: An emerging opportunistic pathogen. *Virulence* 3: 243-250, 2012.
- Peer Mohammed S, Pathoor N, Veeraragavan G and Ganesh P: Unlocking the antibiofilm and anti-virulence potential of *Pithecellobium dulce* against *Chromobacterium violaceum* CV12472. *World Acad Sci J* 7: 14, 2024.
- Bancroft JD and Gamble M (eds): *Theory and practice of histological techniques*. 6th edition. Elsevier Health Sciences, p744, 2008.
- Venkatraman M, Sankar Ganesh P, Senthil R, Akshay J, Veera Ravi A, Langeswaran K, Vadivelu J, Nagarajan S, Rajendran K and Shankar EM: Inhibition of quorum sensing and biofilm formation in *Chromobacterium violaceum* by fruit extracts of *Passiflora edulis*. *ACS Omega* 5: 25605-25616, 2020.
- Zhou JW, Luo HZ, Jiang H, Jian TK, Chen ZQ and Jia AQ: Hordenine: A novel quorum sensing inhibitor and antibiofilm agent against *Pseudomonas aeruginosa*. *J Agric Food Chem* 66: 1620-1628, 2018.
- Sybiya Vasantha Packiavathy IA, Agilandeeswari P, Musthafa KS, Karutha Pandian S and Veera Ravi A: Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against gram-negative bacterial pathogens. *Food Res Int* 45: 85-92, 2012.
- Lawrence R, Tripathi P and Jeyakumar E: Isolation, purification and evaluation of antibacterial agents from *Aloe vera*. *Braz J Microbiol* 40: 906-915, 2009.

29. Miyasaki Y, Rabenstein JD, Rhea J, Crouch ML, Mocek UM, Kittell PE, Morgan MA, Nichols WS, Van Benschoten MM, Hardy WD and Liu GY: Isolation and characterization of antimicrobial compounds in plant extracts against multidrug-resistant *Acinetobacter baumannii*. PLoS One 8: e61594, 2013.
30. Hu J, Shuai W, Sumner JT, Moghadam AA and Hartmann EM: Clinically relevant pathogens on surfaces display differences in survival and transcriptomic response in relation to probiotic and traditional cleaning strategies. NPJ Biofilms Microbiomes 8: 72, 2022.
31. Li XZ, Elkins CA and Zgurskaya HI: Efflux-mediated antimicrobial resistance in bacteria: mechanisms, regulation and clinical implications. Springer, New York, NY, p848, 2016.
32. Grygiel I, Bajrak O, Wójcicki M, Krusiec K, Jończyk-Matysiak E, Górski A, Majewska J and Letkiewicz S: Comprehensive approaches to combatting *Acinetobacter baumannii* biofilms: From biofilm structure to phage-based therapies. Antibiotics (Basel) 13: 1064, 2024.
33. Mendhe S, Badge A, Ugemuge S and Chandi D: Impact of biofilms on chronic infections and medical challenges. Cureus 15: e48204, 2023.
34. Kostakioti M, Hadjifrangiskou M and Hultgren SJ: Bacterial biofilms: Development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. Cold Spring Harb Perspect Med 3: a010306, 2013.



Copyright © 2025 Rony Varughese et al. This work is licensed under a Creative Commons Attribution 4.0 International (CC BY 4.0) License.