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*Research article*

## **Bioactive metabolites from endophytic *Bacillus albus* strain AP1 with antioxidant and analgesic activities validated through docking studies**

**Muddasir Khan<sup>1</sup>, Sumera Afzal Khan<sup>1,\*</sup>, Rashid Ali Khosa<sup>2</sup>, Aryan Morita<sup>3</sup>, Mohamad Saifudin Hakim<sup>4,\*</sup>, Ijaz Aziz<sup>5</sup> and Muhammad Hamayun<sup>6</sup>**

<sup>1</sup> Centre of Biotechnology and Microbiology, University of Peshawar, Peshawar, Pakistan

<sup>2</sup> Department of Pharmacology, Mekran Medical College, Turbat, Pakistan

<sup>3</sup> Department of Dental Biomedical Sciences, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia

<sup>4</sup> Department of Biology and Immunology, College of Medicine, Qassim University, Buraydah, Saudi Arabia

<sup>5</sup> Department of Forensic Medicine, Khyber Medical College, Peshawar, Pakistan

<sup>6</sup> Department of Botany, Abdul Wali Khan University, Mardan, Pakistan

\* **Correspondence:** Email: [drsumera@uop.edu.pk](mailto:drsumera@uop.edu.pk); [m.hakim@qu.edu.sa](mailto:m.hakim@qu.edu.sa).

**Abstract:** The present study aimed to investigate the *in vitro* antioxidant and *in vivo* analgesic potential of secondary metabolites produced by endophytic *Bacillus* species inhabiting *Alliaria petiolata* L. and to validate their pharmacological activities through molecular docking against cyclooxygenase-2 (COX-2) and myeloperoxidase (MPO) enzymes. An endophytic bacterial strain was isolated from the stem tissues of *A. petiolata* and identified as *Bacillus albus* strain AP1 using 16S rRNA gene sequencing. Gas chromatography–mass spectrometry (GC-MS) analysis of its secondary metabolites revealed 18 bioactive compounds, with 3,6-bis(2-methylpropyl)-2,5-piperazinedione as the major constituent. The crude extract exhibited notable antioxidant capacity and significant dose-dependent analgesic activity at 30, 60, and 120 mg/kg. Molecular docking, absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis further confirmed 3,6-bis(2-methylpropyl)-2,5-piperazinedione as a promising bioactive compound, displaying high binding affinity toward COX-2 and MPO enzymes, along with favorable drug-like and low-toxicity profiles. These findings suggest that *B. albus* strain AP1 colonizing *A. petiolata* synthesizes pharmacologically valuable metabolites, and 3,6-bis(2-methylpropyl)-2,5-piperazinedione may serve as a potential lead candidate for developing novel antioxidant and analgesic therapeutics.

**Keywords:** natural products; analgesia; antioxidant; 3,6-bis(2-methylpropyl)-2,5-piperazinedione; molecular docking; ADMET analysis

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## 1. Introduction

Endophytic bacteria are microorganisms that reside within plant tissues and confer host resistance to pathogens and abiotic stressors [1]. Notably, such symbionts biosynthesize structurally or functionally related secondary metabolites identical to those of their host's defense compounds, a phenomenon attributed to horizontal gene transfer or convergent evolution [2]. Due to their metabolic diversity and ecological co-adaptation, endophytic bacteria represent a promising yet underexplored reservoir of bioactive natural products with potential pharmaceutical relevance.

Pain is a prevalent global health concern that affects over 1.5 billion individuals worldwide. The prevalence has been estimated to rise 30% by 2040 due to aging populations and inflammatory comorbidities [3]. Even with the development in pharmacotherapy, existing analgesic and antioxidant drugs are hindered by excessive side effects, including gastrointestinal ulcers, cardiovascular toxicities, and dependency [4]. Such limitations have refocused attention toward natural products derived from microbial symbionts, particularly endophytic bacteria, which are prolific producers of structurally diverse secondary metabolites shaped through long-term co-evolution with their host plants [5].

Numerous *Bacillus* strains, ubiquitous residents of plant tissues, have been recognized for their biosynthetic versatility in yielding lipopeptides, polyketides, and cyclic dipeptides [6]. Their ecological adaptability and genetic capacity to synthesize bioactive metabolites under conditions of stress render them untapped reservoirs for novel pain therapeutics [7]. *B. amyloliquefaciens*, isolated from *Ginkgo biloba*, biosynthesizes ginkgolic acid derivatives, a biocontrol agent, which mimics the host plant's anti-inflammatory flavonoids [8]. Similarly, *B. subtilis* strains isolated from *Curcuma longa* have been found to synthesize curcuminoid-like molecules that suppress the production of prostaglandin E2 (PGE2) in macrophages and thus offer a greener alternative over plant extraction methods plagued by low yields and seasonality [9]. Recent advances in metabolomics and genome mining have further unlocked the biosynthetic capabilities of *Bacillus*, including non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) gene clusters responsible for the biosynthesis of bioactive lipopeptides [10]. While these findings are promising, little information is available regarding the mechanistic basis of *Bacillus*-mediated analgesic effects; this represents a significant knowledge gap in the current literature.

Cyclooxygenase enzymes (COX-1 and COX-2) catalyze the rate limiting step of prostaglandin biosynthesis and central actors in inflammation and nociception [11]. Notably, while COX-1 is continuously expressed and governs physiological processes, including gastric mucosal protection, COX-2 can be induced at inflammatory sites, thus rendering COX-2 a valuable target in pharmacological analgesia [12]. Nevertheless, standard nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac and celecoxib, have limited selectivity for COX-2 inhibition, resulting in gastrointestinal bleeding and renal toxicity [13]. This has led to a growing interest in natural- and microbial-derived COX-2 inhibitors with better safety profiles [14].

Computational protocols have become essential strategies to facilitate natural product drug discovery. They can be utilized to rapidly screen metabolite libraries against therapeutic targets, such

as COX-2, to prioritize leads that exhibit tight binding and selectivity [15]. The myeloperoxidase (MPO) is a potential target protein for antioxidant molecular docking because of its role in inflammation and oxidative stress. By identifying compounds that bind to and inhibit MPO, researchers aim to develop new therapeutic approaches for a range of illnesses. Using molecular docking, scientists can predict the molecular interactions between potential antioxidant compounds and MPO, which enables them to develop more effective inhibitors [16].

Given the therapeutic need for safer analgesic and antioxidant agents and the unexplored potential of endophytic *Bacillus* species, the goal of this work was to investigate the antioxidant and analgesic efficacy of secondary metabolites generated from *Bacillus* inhabiting *A. petiolata* L. The antioxidant and analgesic potential of these metabolites was further validated through computational analysis against MPO and COX-2 enzymes, two clinically relevant targets in oxidative stress and pain signaling. By integrating biochemical assessment with computational validation, this work seeks to support the discovery of novel microbial-derived compounds with antioxidant and analgesic activities.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Analytical-grade chemicals and reagents were used in the study. These chemicals and reagents were obtained from Thermo Fisher Scientific, Sigma-Aldrich (USA), Merck (Germany), GE Healthcare (UK), and Hicon Pharmaceutical Industries, Peshawar (Pakistan), including nutrient agar and Luria Bertani (LB) media, ethyl acetate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, sodium hypochlorite, crystal violet, Gram's iodine, safranin, and acetone, Whatman No. 1 filter papers, diclofenac sodium, and normal saline.

### 2.2. Bacterial isolation and identification

Healthy plant stem samples of *A. petiolata* L. were gathered from Shamoza, Swat, Pakistan. The plant was verified by a taxonomist of the Department of Botany, Abdul Wali Khan University, Mardan, Pakistan. The samples were rinsed with distilled water after being properly cleaned with tap water to remove any dust or dirt. Subsequently, it was subjected to further surface-sterilization treatment [17]. Four stem segments were placed in triplicate on each nutrient agar plate and incubated at 37 °C for 24–48 h. The produced bacterial colonies were purified by sub-culturing on nutrient agar medium plates. The bacterial isolates were initially characterized based on colony morphology and Gram staining. Preliminary screening for antioxidant and analgesic activities was then performed, and the most bioactive isolate was selected for molecular identification using 16S rRNA gene sequencing followed by phylogenetic analysis [18].

### 2.3. Characterization of secondary metabolites

A previously described procedure [19] was used to culture the selected bacteria with slight modifications. Bacteria were cultivated in 250 mL of LB broth medium, followed by incubation with continuous shaking at 200 rpm and 37 °C for up to seven days. Sterile Whatman filter paper was

used to extract cell-free supernatant following culture. Ethyl acetate was added in a 1:1 (v/v) ratio, and the mixture was then rapidly stirred for 5–10 min. After splitting it into two layers, the supernatant was gathered and concentrated using a rotary evaporator.

Secondary metabolites present in the crude extract were subsequently identified using gas chromatography–mass spectrometry (GC-MS) using an Agilent 7890B GC system coupled with an Agilent 5977A mass selective detector and the NIST-17 mass spectral library. Separation was performed on an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; 5% phenyl-methylpolysiloxane stationary phase). High-purity helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injection port temperature was maintained at 250 °C, and injections (1 µL) were performed in split mode (10:1 ratio). The GC oven temperature program was initiated at 60 °C (held for 2 min), ramped to 280 °C at a rate of 10 °C/min, and held at 280 °C for 10 min (total run time: 34 min). The MS transfer line and ion source temperatures were set to 280 °C and 230 °C, respectively, with electron impact (EI) ionization at 70 eV, acquiring in full-scan mode (m/z 50–550) [20].

#### 2.4. *In vitro* antioxidant activity

The antioxidant activity of the extract (1 mg/mL) was evaluated using the DPPH free radical scavenging assay [21]. The reaction mixture containing DPPH solution and methanol (without bacterial extract) served as the negative control, whereas ascorbic acid served as the positive control. The absorbance at 517 nm was measured using a spectrophotometer. The percentage of DPPH scavenged is calculated as follows:

$$((\text{blank solution absorbance} - \text{test sample absorbance}) / \text{blank solution absorbance}) \times 100$$

#### 2.5. *In vivo* experiment

##### 2.5.1. Ethical approval

All procedures involving animals were conducted in accordance with internationally accepted guidelines for the care and use of laboratory animals. The institutional animal research ethics board at the University of Peshawar, Pakistan, approved the study protocol (Approval No. REB-05/07; June 2025). All efforts were made to minimize animal pain, suffering, and the number of animals used.

##### 2.5.2. Animals

A total of 30 Swiss albino mice (20–30 g), 5 females and 25 males, were obtained from the Veterinary Research Institute (VRI), Peshawar, Pakistan. The study was conducted in the Department of Pharmacy, University of Peshawar, Pakistan. A week before the experiments, animals were housed in polycarbonate cages under controlled environmental conditions (temperature:  $22 \pm 3$  °C, relative humidity:  $55\% \pm 10\%$ , 12/12 h light/dark cycle) with desired access to standard feed and water. Animal husbandry and routine care strictly followed VRI guidelines. Animals were monitored at least twice daily throughout the study for general health, activity, food or water intake, grooming behavior, posture, respiration, and signs of distress.

### 2.5.3. Acute oral toxicity analysis

The Organization for Economic Co-operation and Development (OECD) limit test was used to determine the acute oral toxicity of the secondary metabolite crude extract. The toxicity investigation involved five female mice, since female mice are generally less aggressive than males, facilitating stable group housing and minimizing stress-related variability during the experiment. Female mice have also been widely used in acute toxicity studies in accordance with established laboratory practices and experimental guidelines [22]. First, a single dose of 1000 mg/kg was administered orally to a single animal, followed by monitoring for 48 h. Then, when there was no death, the other animals received the same dosage. Over the course of a 14-day observation period, animals were observed for any clinical symptoms (such as changes in fur and skin condition, irritability, tremors, altered respiration, reduced locomotor activity, loss of appetite, and diarrhea), changes in body weight, and survival [22].

### 2.5.4. Humane endpoints

Animals that exhibited loss of more than 20% body weight, inability to move, labored breathing, inability to access food or water, continuous tremors, seizures, extreme distress, or severe dehydration or unresponsiveness were selected for removal from the study and immediate euthanasia without delay.

### 2.5.5. Euthanasia procedure

According to the American Veterinary Medical Association (AVMA) guidelines, euthanasia was performed using CO<sub>2</sub> inhalation, followed by cervical dislocation, to ensure death and minimize suffering. No animal died spontaneously during the experiment; and no unplanned deaths occurred.

### 2.5.6. Analgesic activity

The evaluation of analgesic activity was performed in accordance with globally recognized standards and Eddy's hot plate test procedure [23,24] with minor modifications. Male albino mice were prevented from eating 2 h prior to the test, since male mice show more stable pain-response patterns without hormonal fluctuations [24]. Mice were subjected to a pre-test on a hot plate maintained at  $55 \pm 1$  °C. Mice with delay durations longer than 15 s were eliminated. Each group consisted of five mice, and intraperitoneal administrations of the dosages (prepared in normal saline) were used, as presented in Table 1. Following treatment, behaviors like lifting, clicking, leaping, or licking forepaws for 30, 60, and 90 min were observed. To avoid tissue damage, the cutoff time was set at 30 s.

**Table 1.** Intraperitoneal dosage treatment model to assess the analgesic activity of the secondary metabolite crude extract.

| Groups | Number of mice | Treatment                                  | Concentration (mg/kg) |
|--------|----------------|--------------------------------------------|-----------------------|
| I      | 5              | Normal saline (NS)                         | 10                    |
| II     | 5              | Diclofenac sodium as a standard drug (STD) | 20                    |
| III    | 5              | Secondary metabolites (SM1)                | 30                    |
| IV     | 5              | Secondary metabolites (SM2)                | 60                    |
| V      | 5              | Secondary metabolites (SM3)                | 120                   |

Note: Doses of the secondary metabolites (30, 60, and 120 mg/kg) were selected to evaluate dose-dependent analgesic activity and were chosen based on acute toxicity findings.

#### 2.5.7. Animal monitoring and humane care

Animals were continuously monitored during the analgesic activity for signs of distress or prolonged discomfort. Any mouse showing abnormal pain reactions, severe distress, or inability to perform normal behaviors (grooming, locomotion, orientation) was immediately removed from the test and managed according to humane endpoints. No procedure involved prolonged or unrelieved pain, and no mortality occurred in the analgesic experiment.

#### 2.6. Statistical analysis

Each experiment was performed in triplicate ( $n = 3$  independent observations per group), and statistical analyses were performed using the individual replicate values rather than the calculated group means. Data are presented as means. For comparisons of multiple treatment groups with a single reference/control group, one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparisons test was used. For comparisons involving two groups only, an unpaired two-tailed t-test was applied. Statistical significance was considered at  $p < 0.05$ . All statistical analyses were performed using GraphPad Prism version 10.4.1.

#### 2.7. Molecular docking analysis of COX-2 and MPO enzymes

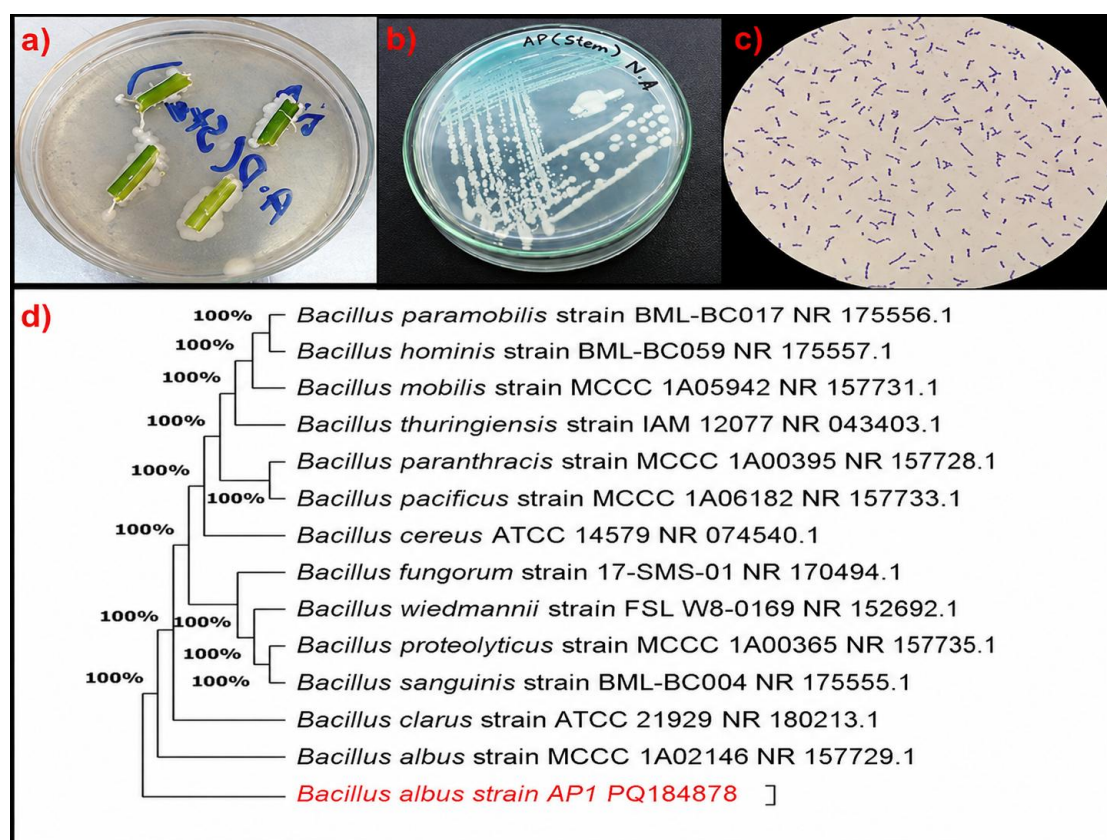
The current study used molecular docking, a computational approach that predicts the affinity of the interaction between a ligand and a receptor protein. The 3D structure of the selected GC-MS-identified secondary metabolite (ligand) was obtained from the PubChem website. The Protein Data Bank (PDB) website provided the 3D structure of the target COX-2 and MPO enzymes. The active site grid and box dimensions for the COX-2 and MPO enzymes were determined based on previous studies, while the 3D structures of already known inhibitors, including celecoxib (CID: 2662) for COX-2 and quercetin (CID:5280343) for MPO, were utilized as positive controls [25–27]. The ligand and target protein were prepared using BIOVIA Discovery Studio 2021 software; for molecular docking, the AutodockTools-1.5.7 software was used [25,26]. For docking validation, the root mean square deviation (RMSD) value was calculated using PyMOL 3.1 software. The compound was further screened for pharmacokinetics ADMET (absorption, distribution, metabolism, excretion, and toxicity) characteristics using the online tool

<http://biosig.unimelb.edu.au/pkcsml/prediction> (accessed on March 1, 2025) [27].

### 3. Results

#### 3.1. Bacterial isolation

Among the eight bacterial isolates from stem samples of *A. petiolata* L. (Figure 1a), *Bacillus albus* strain AP1 was primarily characterized by colony morphology (creamy-white, smooth, and circular) (Figure 1b) and Gram staining (Gram-positive rods) (Figure 1c). The phylogenetic analysis of the 16S rRNA gene confirmed a 100% gene sequence identity with *B. albus* (Figure 1d).



**Figure 1.** a) *A. petiolata* L. stem samples on nutrient agar and appearance of bacterial growth after 24 h. b) Creamy-white, smooth, and circular colonies of *B. albus* strain AP1 were observed. c) Gram-positive rod staining of isolated *B. albus* strain AP1. d) Phylogenetic analysis of strain AP1, showing 100% 16S rRNA gene sequence identity with *B. albus* strains, constructed using MEGA 11 software by the neighbor-joining method [18].

#### 3.2. Secondary metabolite extraction and GC-MS analysis

The AP1 strain was cultivated to produce secondary metabolites, followed by GC-MS analysis. The crude extract revealed the existence of 18 compounds. The most abundantly detected compound was 3,6-bis(2-methylpropyl)-2,5-piperazinedione (58.89%) (Table 2).

**Table 2.** GC-MS profile of secondary metabolites presents in crude extracts prepared from *A. petiolata* L.–associated *B. albus* strain AP1.

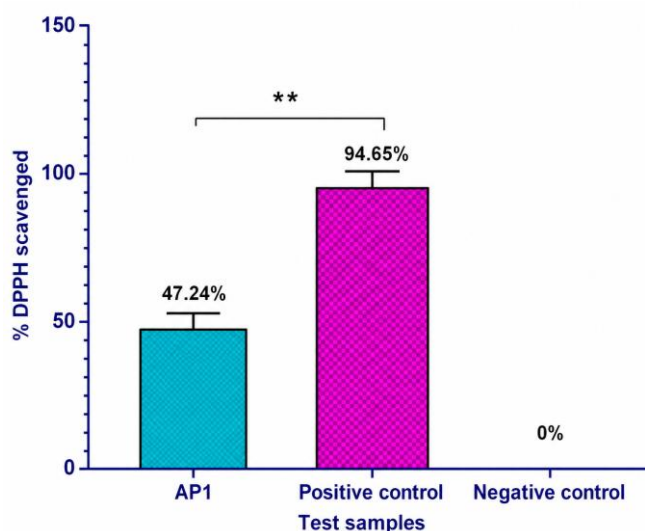
| Compound name                                                 | Retention time | Relative abundance (%) | Molecular formula                                             | Molecular weight (g/mol) |
|---------------------------------------------------------------|----------------|------------------------|---------------------------------------------------------------|--------------------------|
| Thymol                                                        | 10.310         | 1.96                   | C <sub>10</sub> H <sub>14</sub> O                             | 150.2                    |
| 2-methoxy-3-(2-propenyl)-phenol                               | 11.959         | 3.05                   | C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>                | 164.2                    |
| Pentadecane                                                   | 19.995         | 1.78                   | C <sub>15</sub> H <sub>32</sub>                               | 212.4                    |
| 3,4-diethyl-1,1'-biphenyl                                     | 20.237         | 0.97                   | C <sub>16</sub> H <sub>18</sub>                               | 210.3                    |
| 1,1'-oxybis-decane                                            | 20.950         | 1.67                   | C <sub>20</sub> H <sub>42</sub> O                             | 298.5                    |
| 4-ethyl-tetradecane                                           | 21.116         | 1.08                   | C <sub>16</sub> H <sub>34</sub>                               | 226.4                    |
| 4-methyl-pentadecane                                          | 21.252         | 1.18                   | C <sub>16</sub> H <sub>34</sub>                               | 226.4                    |
| 2-methyl-octadecane                                           | 21.364         | 0.93                   | C <sub>19</sub> H <sub>38</sub> O                             | 282.5                    |
| 3-methyl-heptadecane                                          | 21.533         | 1.16                   | C <sub>18</sub> H <sub>38</sub>                               | 254.5                    |
| Hexadecane                                                    | 22.141         | 2.15                   | C <sub>16</sub> H <sub>34</sub>                               | 226.4                    |
| (Z)-3-methyl-2-decene                                         | 22.340         | 8.35                   | C <sub>11</sub> H <sub>22</sub>                               | 154.2                    |
| Pentadecanoic acid                                            | 22.612         | 3.75                   | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>                | 242.4                    |
| Cis-1,1,2,6-tetramethylsilacyclohexane                        | 23.021         | 1.16                   | C <sub>9</sub> H <sub>20</sub> Si                             | 156.3                    |
| 4-methyl-hexadecane                                           | 23.341         | 1.01                   | C <sub>17</sub> H <sub>36</sub>                               | 240.5                    |
| Hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-a]pyrazine-1,4-dione | 24.318         | 6.49                   | C <sub>11</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub> | 210.2                    |
| <i>n</i> -Hexadecanoic acid                                   | 25.354         | 2.58                   | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>                | 256.4                    |
| Methyl ester, (Z)-6-octadecenoic acid                         | 28.044         | 1.84                   | C <sub>19</sub> H <sub>36</sub> O <sub>2</sub>                | 296.5                    |
| 3,6-bis(2-methylpropyl)-2,5-piperazinedione                   | 29.317         | 58.89                  | C <sub>12</sub> H <sub>22</sub> N <sub>2</sub> O <sub>2</sub> | 226.3                    |

### 3.3. *In vitro* antioxidant activity

The secondary metabolites (1 mg/mL) exhibited high antioxidant capacity in comparison to the control, as it scavenged 47.24% ± 4.48% of DPPH radicals, which was statistically significant ( $p < 0.05$ ), as shown in Figure 2.

### 3.4. *In vivo* acute oral toxicity analysis

The secondary metabolite crude extract, at a dose of 1000 mg/kg, did not cause any death or poisoning signs, as presented in Table 3. These results led to the selection of dosages (approximately 30, 60, and 120 mg/kg) to further assess the dose-dependent therapeutic potential of the secondary metabolite crude extract.



**Figure 2.** Antioxidant activity of *B. albus* strain AP1–derived secondary metabolite crude extract, tested at a 1 mg/mL concentration. Each column shows the mean % DPPH scavenged for different treatment groups. \*\* $p < 0.01$ . Error bars represent the standard error of the mean (SEM). Positive control: Ascorbic acid; negative control: DPPH.

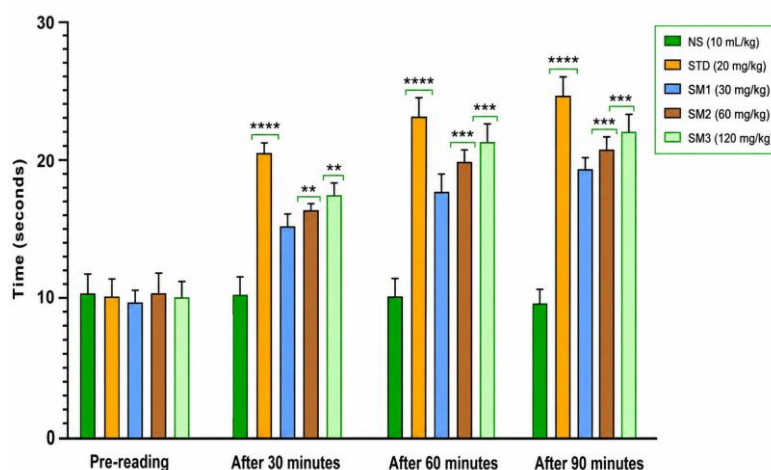
**Table 3.** Acute oral toxicity of secondary metabolite crude extract at a dose of 1000 mg/kg.

| Mouse ID | Clinical symptoms observed* | Body weight day 0 (g) | Body weight day 14 (g) | % weight change | Survival (yes/no) |
|----------|-----------------------------|-----------------------|------------------------|-----------------|-------------------|
| B1       | No                          | 28                    | 28                     | 0               | Yes               |
| B2       | No                          | 30                    | 30                     | 0               | Yes               |
| B3       | No                          | 23                    | 23                     | 0               | Yes               |
| B4       | No                          | 32                    | 32                     | 0               | Yes               |
| B5       | No                          | 30                    | 30                     | 0               | Yes               |

\*: Including changes in fur and skin condition, irritability, tremors, altered respiration, reduced locomotor activity, loss of appetite, and diarrhea.

### 3.5. *In vivo analgesic study*

The analgesic efficacy of the secondary metabolite crude extract from *B. albus* strain AP1 was investigated at doses of 30, 60, and 120 mg/kg. After 30 min, the mean latency time of 12.6 s at 30 mg/kg was inconsequential compared to the normal saline (NS) group (10.4 s) and standard drug (STD, 20 mg/kg) group (20.8 s). However, after 60 and 90 min, mean latency times of 16.4 and 18 s were significant ( $p < 0.01$ ), indicating a delayed analgesic effect. At 60 mg/kg, mean latency times of 13.4, 20, and 20.8 s at 30, 60, and 90 min, respectively, were significant ( $p < 0.05$ ) compared to NS (10.2, 10.8, and 9.6 s), and approached the effect of STD (21, 23.4, and 25.6 s). At the highest dose of 120 mg/kg, mean latency times of 14.6, 21.6, and 21.4 s at 30, 60, and 90 min, respectively, were significant ( $p < 0.05$ ) relative to NS, and showed analgesic activity comparable to STD, particularly at 60 and 90 min (Figure 3). These results indicate that the secondary metabolite crude extract exhibits dose-dependent analgesic activity, with the highest dose approaching the efficacy of the standard reference drug.



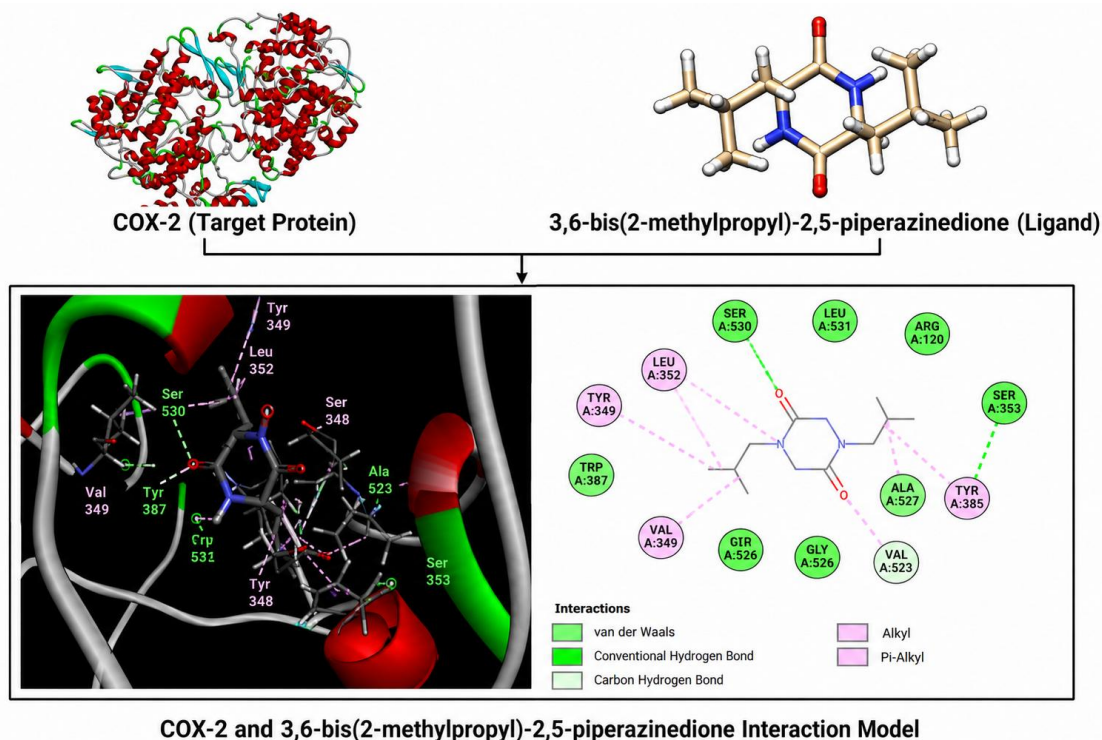
**Figure 3.** Analgesic effect of *B. albus* strain AP1-derived secondary metabolite crude extract tested at 30, 60, and 120 mg/kg concentrations. Each column shows the mean latency time (seconds) for different treatment groups. Error bars represent the standard error of the mean (SEM). Data are presented as mean ( $n = 3$  independent observations). Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple-comparisons test, with the control group as the reference group. ns: not significant; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$  versus the control group. NS: normal saline; STD: standard; SM: secondary metabolite extract.

### 3.6. Computational analysis of secondary metabolites against COX-2 and MPO enzymes

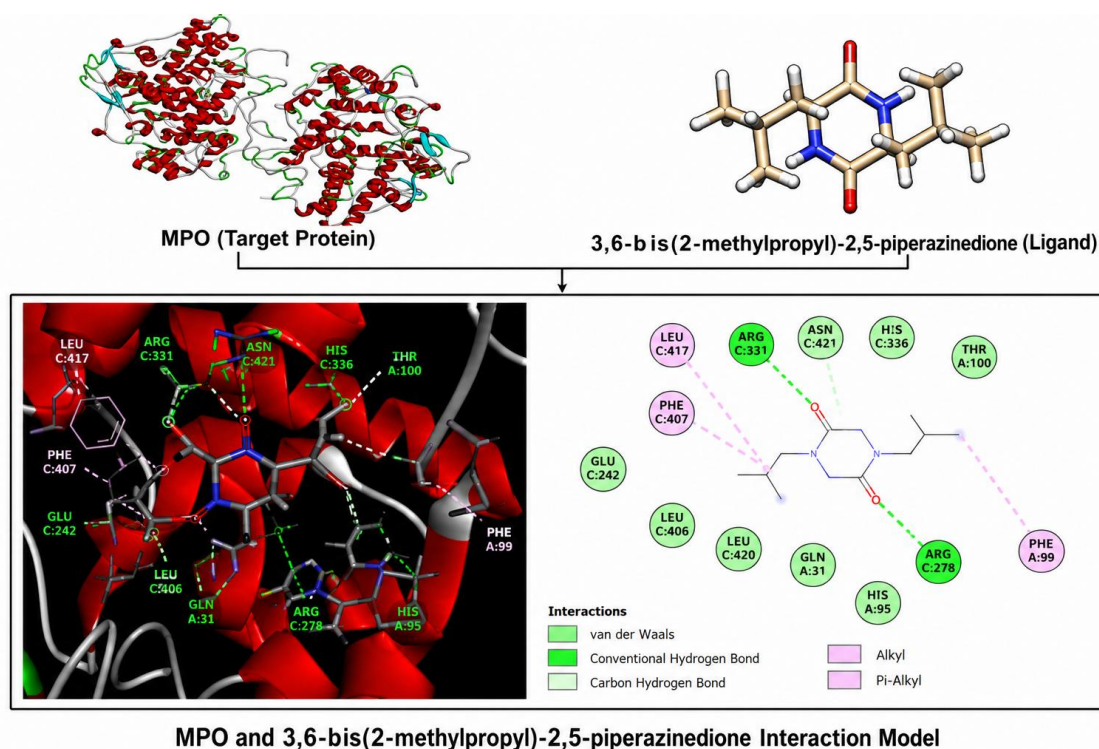
COX-2 and MPO enzymes were selected as the target proteins in our current study. The Protein Data Bank website provided the 3D structures of COX-2 (PDB ID: 5IKR) and MPO (PDB ID: 4C1M). The secondary metabolite 3,6-bis(2-methylpropyl)-2,5-piperazinedione, found at a high concentration in the crude extract, was chosen as the ligand. The PubChem webserver was used to obtain its three-dimensional structure (CID: 192731). The ligand was docked against COX-2 and MPO enzymes to validate the extract's analgesic and antioxidant activities.

The metabolite 3,6-bis(2-methylpropyl)-2,5-piperazinedione was found to have a high binding affinity toward COX-2 (-8.7) and MPO (-6.7) (Figures 4 and 5). It showed potential alkyl and pi-alkyl bonds with the COX-2 enzyme's tyrosine (A: 348 and 355), leucine (A: 352), and valine (A: 349) residues. Additionally, the valine (A: 523) residue exhibited van der Waals interactions, while serine (A: 353 and 530), arginine (A: 120), glycine (A: 526), tryptophan (A: 387), leucine (A: 531), and tyrosine (A: 385) residues exhibited hydrogen bonds (Figure 4). It also exhibited alkyl and pi-alkyl interactions with phenylalanine (A: 99 and C: 407) and leucine (C: 417), hydrogen bonds with arginine (C: 239 and 333), and van der Waals interactions with leucine (C: 406 and 415), glutamic acid (C: 242), glutamine (A: 91), histidine (A: 95 and C: 336), asparagine (C: 421), and threonine (A: 100) amino acid residues of the MPO enzyme (Figure 5).

These binding affinities and interaction results indicate predictive inhibition of COX-2 and MPO by 3,6-bis(2-methylpropyl)-2,5-piperazinedione when compared with positive controls. RMSD values were also lower than the threshold of 2 Å, as presented in Table 4. In addition, the 3,6-bis(2-methylpropyl)-2,5-piperazinedione ADMET features were established, as presented in Table 5.



**Figure 4.** Molecular docking protein and ligand complex model of human COX-2 enzyme and 3,6-bis(2-methylpropyl)-2,5-piperazinedione.



**Figure 5.** Molecular docking protein and ligand complex model of MPO enzyme and 3,6-bis(2-methylpropyl)-2,5-piperazinedione.

**Table 4.** Binding affinities and RMSD values of 3,6-bis(2-methylpropyl)-2,5-piperazinedione against COX-2 and MPO enzymes.

| Target proteins | Compounds (ligands)                         | Binding affinities (kcal/mol) | RMSD values (Å) |
|-----------------|---------------------------------------------|-------------------------------|-----------------|
| COX-2           | 3,6-bis(2-methylpropyl)-2,5-piperazinedione | −8.7                          | 1.30            |
|                 | Celecoxib (positive control)                | −8.4                          | 0.77            |
| MPO             | 3,6-bis(2-methylpropyl)-2,5-piperazinedione | −6.7                          | 1.43            |
|                 | Quercetin (positive control)                | −7.6                          | 0.26            |

**Table 5.** Predicted ADMET features of 3,6-bis(2-methylpropyl)-2,5-piperazinedione.

| Property     | ADMET features                    | Predicted results (unit)                  |
|--------------|-----------------------------------|-------------------------------------------|
| Absorption   | Water solubility                  | −2.03 (log mol/L)                         |
|              | Caco2 permeability                | 1.236 (log Papp in 10 <sup>−6</sup> cm/s) |
|              | Intestinal absorption (human)     | 74.608%                                   |
|              | Skin permeability                 | −3.07 (log Kp)                            |
|              | P-glycoprotein substrate          | No                                        |
|              | P-glycoprotein I inhibitor        | No                                        |
|              | P-glycoprotein II inhibitor       | No                                        |
| Distribution | VDss (human)                      | −0.231 (log L/kg)                         |
|              | Fraction unbound (human)          | 0.42 (Fu)                                 |
|              | BBB permeability                  | −0.076 (log BB)                           |
|              | CNS permeability                  | −2.887 (log PS)                           |
| Metabolism   | CYP2D6 substrate                  | No                                        |
|              | CYP3A4 substrate                  | No                                        |
|              | CYP1A2 inhibitor                  | No                                        |
|              | CYP2C19 inhibitor                 | No                                        |
|              | CYP2C9 inhibitor                  | No                                        |
|              | CYP2D6 inhibitor                  | No                                        |
|              | CYP3A4 inhibitor                  | No                                        |
| Excretion    | Total clearance                   | 1.275 (log mL/min/kg)                     |
|              | Renal OCT2 substrate              | No                                        |
| Toxicity     | AMES toxicity                     | Yes                                       |
|              | Max. tolerated dose (human)       | 0.449 (log mg/kg/day)                     |
|              | hERG I inhibitor                  | No                                        |
|              | hERG II inhibitor                 | No                                        |
|              | Oral rat acute toxicity (LD50)    | 1.844 (mol/kg)                            |
|              | Oral rat chronic toxicity (LOAEL) | 2.508 (log mg/kg_bw/day)                  |
|              | Hepatotoxicity                    | No                                        |
|              | Skin sensitization                | No                                        |
|              | <i>T. pyriformis</i> toxicity     | 0.535 (log µg/L)                          |
|              | Minnow toxicity                   | 1.551 (log mM)                            |

#### 4. Discussion

*Bacillus* species are among the most frequently reported endophytic bacteria in a wide range of plant species. For example, studies reported their association with *Commelina communis* [28], *Brassica chinensis* L. [29], and *Musa acuminata* [30]. Presently, *B. albus* strain AP1 was isolated from stem samples of *A. petiolata* L., which belong to the family *Brassicaceae*. *Bacillus* species associated with *A. petiolata* L. remain largely unexplored. Al Farraj and Elshikh [31] reported *Brassica nigra*–associated *B. xiamenensis*, and Nagah et al. [19] reported *B. napus* L.–associated *B. vallismortis*. Moreover, *B. pseudomycoides* was also reported as an endophyte by Priyanto et al. [32].

Endophytic bacteria demonstrate a variety of metabolic features. They produce abundant, distinctive, and physiologically active secondary metabolites, which benefit both host plants and bacteria [33]. In this work, the *B. albus* strain AP1 secondary metabolite crude extract showed the presence of 18 compounds, with 3,6-bis(2-methylpropyl)-2,5-piperazinedione being predominant (58.89%) among all secondary metabolites. This compound mostly refers to cyclo(*L*-leucyl-*L*-leucyl), a member of the diketopiperazine class of cyclic dipeptides, which are bioactive secondary metabolites found in a variety of bacterial taxa [34]. Previously, this compound was also reported as a secondary metabolite of *B. amyloliquefaciens* [35]. Its antitumor, anti-inflammatory, antiparasitic, and antimicrobial properties have been extensively studied [36–38].

The presence of various compounds in the crude extract showed similarity with previously reported *Bacillus* secondary metabolites, such as pentadecane [39], 4-ethyl-tetradecane [40], 3-methyl-heptadecane [41], (*Z*)-3-methyl-2-decene [42], pentadecanoic acid [43], 4-methyl-hexadecane [44], hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-*a*]pyrazine-1,4-dione [45], *n*-Hexadecanoic acid [46], methyl ester, (*Z*)-6-octadecenoic acid [20], and 3,6-bis(2-methylpropyl)-2,5-piperazinedione. However, thymol, 2-methoxy-3-(2-propenyl)-phenol, 3,4-diethyl-1,1'-biphenyl, 1,1'-oxybis-decane, 4-methyl-pentadecane, 2-methyl-octadecane, hexadecane, cis-1,1,2,6-tetramethylsilacyclohexane, and hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-*a*]pyrazine-1,4-dione were reported for the first time in the present study as *B. albus* strain AP1 secondary metabolites.

The secondary metabolite crude extract derived from the *B. albus* strain AP1 exhibited significant ( $p < 0.05$ ) analgesic activity. The existence of bioactive compounds in the crude extract is in agreement with the previously documented analgesic effects of pentadecane [47], *n*-Hexadecanoic acid [48], 2-methoxy-3-(2-propenyl)-phenol [49], and thymol [50]. A notable limitation of this study is the preliminary nature of the safety evaluation. Although oral administration of the extract at 1000 mg/kg produced no overt mortality or gross clinical signs over 14 days, this assessment relied solely on visible observations and body weight changes. The absence of serum biochemical markers, organ coefficients, and histopathological analysis limits our ability to detect subclinical organ toxicity. Furthermore, a route-of-administration mismatch exists: toxicity was screened orally, whereas analgesic activity was evaluated via intraperitoneal administration. Because these routes differ significantly in absorption kinetics, bioavailability, and first-pass metabolism, the oral toxicity data cannot directly establish the safety of the intraperitoneal doses used in the efficacy assays. Finally, the sex discrepancy using female mice for toxicity and male mice for analgesia introduces a biological confounding variable, given well-documented sex differences in drug metabolism and nociception. Consequently, these findings should be viewed as preliminary proof-of-concept. Future studies employing consistent administration routes, comprehensive dual-sex cohorts, and full

toxicological panels are required to confirm safety.

The extract of *B. albus* secondary metabolites also exhibited statistically significant ( $p < 0.05$ ) antioxidant capacity against DPPH radicals. The hydrogen-donating and radical-scavenging properties of the bioactive compounds present in the crude extract, which aid in neutralizing reactive oxygen species (ROS) and preventing oxidative stress, may be responsible for the extract's antioxidant activity [51]. Moreover, the presence of these compounds in the extract was also in line with previously reported antioxidant potential, including hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-a]pyrazine-1,4-dione [52], *n*-Hexadecanoic acid [53], and methyl ester, (*Z*)-6-octadecenoic acid [54]. Moreover, the antioxidant activity of the crude extract was evaluated using the DPPH radical scavenging assay at a single concentration (1 mg/mL), which provides only a preliminary indication of free radical scavenging capacity. Consequently, the present study does not establish a dose-dependent antioxidant response or determine the IC<sub>50</sub> value, which would allow quantitative comparison of antioxidant potency.

Furthermore, antioxidant activity is a multifaceted phenomenon, and reliance on a single assay does not comprehensively represent the antioxidant potential of the extract, as different assays measure distinct mechanisms of antioxidant action. Therefore, the antioxidant findings should be interpreted as preliminary evidence of antioxidant activity rather than a complete characterization. Future studies will include concentration-dependent analysis for IC<sub>50</sub> determination, together with complementary antioxidant assays, such as 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC), to provide a more comprehensive assessment of the antioxidant properties of microbial metabolites.

COX-2 enzyme is a useful target in pharmaceutical analgesia, as it is activated during inflammatory activity [12], while the MPO enzyme is a reasonable target protein for antioxidant molecular docking [16]. Therefore, our study aims to validate the analgesic and antioxidant activities of the extract to identify the best secondary metabolites with high binding affinities to COX-2 and MPO enzymes. Notably, molecular docking is only a computational prediction and does not represent actual binding or inhibition. The effects of the crude extract are likely the result of additive or synergistic actions of multiple constituents. Our findings showed that 3,6-bis(2-methylpropyl)-2,5-piperazinedione has a high binding affinity toward COX-2 and MPO enzymes. Similarly, previous studies also documented a possible interaction between 3,6-bis(2-methylpropyl)-2,5-piperazinedione and COX-2 [55,56], as well as MPO [57,58] enzymes. The established ADMET features of 3,6-bis(2-methylpropyl)-2,5-piperazinedione have shown low toxicity and high intestinal absorption. Safety is an essential aspect in drug development, in addition to the efficient docking result [59]. Low toxicity and efficient distribution to the intended site are essential characteristics of the therapeutic agent during drug development [60].

Our finding is noteworthy as it is among the first reports of *B. albus* producing 3,6-bis(2-methylpropyl)-2,5-piperazinedione and other bioactive compounds, indicating a unique biosynthetic potential of this bacterial species. The findings suggest that 3,6-bis(2-methylpropyl)-2,5-piperazinedione is one of the potential bioactive constituents contributing to the antioxidant and analgesic activities of the crude extract. However, the observed biological effects likely result from the combined action of multiple metabolites present in the extract, and further bioactivity-guided isolation and validation studies are required to confirm the contribution of individual compounds. Given its high relative abundance and established bioactivities,

the molecule shows promise for use in natural product-based drug discovery, especially in the development of antioxidant and analgesic treatments. Its precise pharmacological potential may be clarified through additional studies, focusing on purification, structure–activity correlations, and mechanistic studies. Through additional purification, these activities may also be enhanced and brought closer to those of conventional antioxidants and analgesics.

## 5. Conclusions

The secondary metabolites derived from the *B. albus* strain AP1 associated with *A. petiolata* L. were explored for their analgesic and antioxidant effects. The secondary metabolite extract revealed the existence of 18 bioactive compounds, with 3,6-bis(2-methylpropyl)-2,5-piperazinedione being identified as the predominant constituent. The crude extract showed significant dose-dependent analgesic and antioxidant effects, although additional concentration-dependent studies and complementary antioxidant assays are required to comprehensively evaluate its antioxidant potential. The computational study of 3,6-bis(2-methylpropyl)-2,5-piperazinedione secondary metabolite demonstrated a high binding affinity and pharmacological compatibility with COX-2 and MPO enzymes. Furthermore, ADMET profiling indicated favorable properties, suggesting its potential as a drug-like candidate. These computational findings call for additional purification, followed by *in vitro* and *in vivo* validation to understand its mode of action. Our findings expand the chemical space of analgesics and antioxidants and highlight the value of *in silico* screening in identifying bioactive compounds that have hitherto been overlooked in pharmacological research.

## Author contributions

Conceptualization, M.K., S.A.K. and M.S.H.; methodology, M.K. and M.H.; software, M.K., R.A.K. and I.A.; validation, S.A.K. and M.H.; formal analysis, M.K.; investigation, M.K.; resources, S.A.K.; data curation, M.K.; writing—original draft preparation, M.K., A.M. and M.S.H.; writing—review and editing, M.K., A.M. and M.S.H.; visualization, M.K., R.A.K. and I.A.; supervision, S.A.K., M.S.H. and M.H.; project administration, S.A.K. and M.H. All authors have read and agreed to the published version of the manuscript.

## Use of Generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

## Data availability statement

The obtained 16S rRNA sequence was submitted to NCBI GenBank nucleotide database with accession number: PQ184878.

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### Conflict of interest

The authors declare no conflicts of interest in this paper

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