

# Major Phytochemicals of *Boesenbergia pandurata* Rhizome by LC-HRMS and Virtual Screening of *Staphylococcus aureus* Inhibition

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**Abstract.** The study identified the major phytochemical compounds in the *n*-hexane fraction of the *Boesenbergia pandurata* rhizome and virtual screening study. The rhizome was partitioned with *n*-hexane and analyzed for phytochemicals by LC-HRMS. Virtual screening included prediction activity by PyRx, pharmacokinetics and toxicity with the ADMETLab 3.0. The predicted antibacterial activity of **S1**, **S3**, and **S4** had the best inhibition of the PB2a receptor. The pharmacokinetic profile showed all compounds had excellent Caco-2 permeability, PAMPA, and HIA. **S2** and **S3** showed excellent indicators of drug delivery efficiency in the systemic circulation. All compounds had high plasma protein binding values and optimal distribution volumes in **S1**, **S3**, and **S4**. The blood-brain barrier was not crossed by any of the compounds, and the plasma proteins showed a lower binding affinity. Clearance values were excellent for all compounds, and half-life was intermediate in compounds **S1**, **S3**, and **S4**. Nearly all compounds exhibited a moderate toxicity profile. In summary, compounds **S1**, **S3**, and **S4** from the *n*-hexane fraction demonstrated the best activity and pharmacokinetics, as well as toxicity prediction. Based on the results, the major phytochemicals have the potential for isolation and in vitro determination of antibacterial activity in the future.

**Keywords:** *Boesenbergia pandurata*, major compounds, *Staphylococcus aureus*, virtual screening.

## 1. Introduction

Bacterial infections are the second most prevalent cause of mortality worldwide [1]. Recently, the incidence of multi-drug-resistant (MDR) pathogenic and opportunistic bacteria has been increasingly reported. These bacteria are life-threatening for both hospitalized patients and their caregivers. MDR is multifactorial and has been shown to increase steadily with the

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severity of congenital, dietary, and other clinical infections in humans and animals [2]. *S. aureus* stands out as the most invasive gram-positive bacterium among all staphylococci, implicated in a range of diseases affecting both humans and animals. Additionally, *S. aureus* is known as a primary contributor to bacterial infections acquired in hospital settings worldwide, largely due to its established resistance to penicillin and other antimicrobial agents [3]. Therefore, we urgently need to search and develop new antibacterial compounds and strategies that can reduce MDR. Plant-derived antibacterials containing bioactive compounds are the most attractive source of new therapies. Combining natural products with antibiotics has been indicated to make treatments more effective and lower the risk of bacteria becoming resistant to them. This approach is particularly effective in resensitizing MDR bacteria to antibiotics and in curbing the proliferation of antibiotic resistance [4].

The presence of phytochemicals in plants can interfere with the proteins in bacterial cells and influence immune responses, thereby disrupting bacterial signal transduction and division while promoting apoptosis. The integration of plant extracts with antibiotics presents a viable strategy to address multidrug resistance. Prior studies indicated that bacterial isolates subjected to a combination of extract and cefixime exhibited a fractional inhibitory concentration index (FICI) that demonstrated a notable reduction in bacterial growth and protein levels (5-62%) compared to treatment with either extract or cefixime alone. The use of combinations can reduce the minimum dose required as an effective antibacterial and reduce side effects and treatment costs [5].

*B. pandurata* Roxb. is a plant known as fingerroot and used in the tropics. Previous studies have shown that the rhizomes of fingerroot contain many flavonoids and their prenylated derivatives, especially cyclohexenylcalcone derivatives. Its biological activities include antibacterial, antioxidant, anti-inflammatory, and anti-obesity properties [6]. The antibacterial inhibition zone of ethanol extract was  $21.1 \pm 0.5$  mm, including the strong category with 45% extract concentration on *S. aureus* bacteria. The activity test of the pinostrobin of *n*-hexane extract from fingerroot is MIC 10  $\mu$ M on *S. aureus* bacteria [7]. Although it has several important activities, the fingerroot fraction is less studied regarding its antibacterial potential.

The virtual screening investigations concerning natural products are significant due to their capacity to conserve time and resources during the drug discovery process while also uncovering new opportunities in drug development. Furthermore, these studies can assist in the design of natural products to enhance their bioactivity and minimize toxicity. The study aimed to identify the major phytochemical compounds in the *n*-hexane fraction of the ethanol extract from the *B. pandurata* rhizome, as well as to conduct a virtual screening study. Furthermore, the identification of active compounds with antibacterial properties will be conducted through the LC-HRMS technique, alongside an evaluation of their activity via molecular docking and ADMET studies, aimed at developing new natural medicines for bacterial therapy.

## 2. Material and Methods

### 2.1. Material

*B. pandurata* rhizome purchased at Materia Medika, Batu, Malang, Indonesia. In silico testing used active compound samples obtained from LC-HRMS analysis. Research instruments for *B. pandurata* rhizome fractionation included analytical scales, an autoclave, a hot plate, and a melting point apparatus. Research instruments for in silico screening included the Fujitsu AH544 computer with Core(TM) i7 specifications, CPU @ 2.20 GHz, Nvidia®, 16 GB RAM, ChemBio Draw 2D and 3D programs, PyRX, and Biovia Discovery Studio Visualizer programs. The instrument for phytochemical compound identification by LC (Thermo Scientific™ Vanquish™ UHPLC Binary Pump) and the Orbitrap High-

Resolution Mass Spectrometry system (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ High-Resolution Mass Spectrometer).

## 2.2. Fractionation of Ethanol Extract of *B. pandurata* Rhizome

100 g of *B. pandurata* rhizome was macerated by adding 1 L of ethanol for 3x24 hours. The extract was filtered with Whatman, and the filtrate was concentrated with a rotavapor. The ethanol extract was partitioned by liquid-liquid extraction using *n*-hexane and ethanol. The *n*-hexane fraction was evaporated by rotavapor and tested by LC-HRMS.

## 2.3. Virtual Screening

### 2.3.1. Drug likeness

The main phytochemicals of *B. pandurata* rhizome were determined by drug likeness with the ADMETLab 3.0 website [8].

### 2.3.2. Docking study

Determination of predicted antibacterial activity by molecular docking. The PBP2a receptor (PDB ID: 4DKI) was obtained from the PDB. Molecular docking of the active compound was performed with PyRX 0.8. Validation was performed by docking the native ligand with the PBP2a macromolecule to produce an RMSD value (<2 Å) [8]. The results of the docking were visualized in both 2D and 3D formats utilizing the Biovia Discovery Studio Visualizer software. The outcomes of the molecular docking were saved in .pdbqt format, and the interaction values of binding affinity, RMSD, and amino acid residues were recorded.

### 2.3.3. Pharmacokinetic and toxicity determination

The 3D structure of the active compound was saved in .sdf format and analyzed with the ADMETLab 3.0 website. The parameters determined were absorption, distribution, metabolism, excretion, and toxicity parameters [8].

## 3. Result and Discussion

### 3.1. Major phytochemicals in *n*-hexane fraction of *B. pandurata* rhizome

The combination of non-polar solvent (*n*-hexane) and polar solvent (ethanol) is the main strategy to form two phases. LC-HRMS is a technique used in mass spectrometry in the determination of molecular formulas, parent ions, and fraction ions in plant fractions. In addition, LC-HRMS offers high specificity, sensitivity, and selectivity to low sample amounts in a short time so that many compounds can be obtained. The untargeted LC-HMRS separation affected the molecular properties detected, quality, and intensity of detection [9].

Table 1. Major phytochemicals of the *n*-hexane fraction

No.	Code	Compound	Chemical Formula	Molecular Weight (g/mol)	Retention Time (sec)	Concentration (%)
1.	S1	Pinostrobin	C <sub>16</sub> H <sub>14</sub> O <sub>4</sub>	270.0884	12.111	71.3
2.	S2	Demethoxyyangonin	C <sub>14</sub> H <sub>12</sub> O <sub>3</sub>	228.0783	10.252	9.7
3.	S3	5-Methoxy-7-hydroxyflavanone	C <sub>16</sub> H <sub>14</sub> O <sub>4</sub>	270.0884	8.727	9.3
4.	S4	Pinocembrin	C <sub>15</sub> H <sub>12</sub> O <sub>4</sub>	256.0730	10.294	7.1

Test results in Table 1 showed four compounds with the highest concentrations in the fraction, namely pinostrobin (71.3%), demethoxyyangonin (9.7%), 5-methoxy-7-hydroxyflavanone (9.3%), and pinocembrin (7.1%). The highest concentrations of these compounds are the main contributors to biological activity [9].

### 3.2. Rule of drug-likeness

Drug-likeness indicated the fulfillment of oral permeability and bioavailability based on three rules, including Lipinski, Veber, and Ghose [10]. The requirements for the fulfillment of the three rules are based on the Lipinski rule with  $MW \leq 500$  Da,  $\text{Log P} \leq 5$ ,  $n\text{HBA} \leq 10$ , and  $n\text{HBD} \leq 5$ ; the Veber rule requires  $\text{TPSA} \leq 140 \text{ \AA}^2$  and  $n\text{RB} \leq 10$ ; and the Ghose rule with MR values ranging from 40 to  $140 \text{ cm}^3\text{mol}^{-1}$  [10].

**Table 2.** Drug-likeness of the major phytochemicals

Code	Lipinski				Veber Rule		Ghose Rule
	MW (Da)	Log P	nHBA	nHBD	TPSA ( $\text{\AA}^2$ )	nRB	MR ( $\text{cm}^3/\text{mol}$ )
S1	270.088	3.61	4	1	55.76	2	74.02
S2	228.078	3.18	3	0	33.44	3	66.84
S3	270.088	2.92	4	1	55.76	2	74.02
S4	256.072	3.06	4	2	66.76	1	69.76
AMX	365.404	-0.58	8	5	132.96	5	91.18

MW: Molecular Weight (Da), Log P: Partition coefficient, nHBA: number of Hydrogen Bond Acceptors, (nHBD): number of Hydrogen Bond Donors, TPSA: Topological Polar Surface Area ( $\text{\AA}^2$ ), nRB: number of Rotational Bonds, MR: Molar Refractivity ( $\text{cm}^3/\text{mol}$ ).

Table 2 showed that the molecular weight of the four major phytochemicals was  $\leq 500$  Da, ideal for oral drug development. Log P values of 1-4 indicated moderate lipophilicity. Hydrogen bonds between donors and acceptors are used as drug filters [10]. If the value exceeds 10, there's a decrease in oral bioavailability. The TPSA values had an optimal range for all four compounds, supporting absorption  $>90\%$ . The molar reactivity of the four compounds indicated their ability to react within the body [10]. Based on Lipinski, Veber, and Ghose rules, all major phytochemicals and AMX conform to the rules, implying they exhibit acceptable water solubility and intestinal permeability as candidate compounds for oral drugs and as an initial step in oral bioavailability [10].

### 3.3. Antibacterial prediction

The molecular docking analysis of the four compounds listed in Table 3. A total of four phytochemicals docked with the target at the penicillin-binding protein 2a (PBP2a) receptor. In the Protein Data Bank, the receptor PB2a with ID: 4DKI was chosen based on the type of organism, which is *S. aureus*, the method of X-ray diffraction, and the resolution of 2.9  $\text{\AA}$  [10]. The docking validation procedure involved redocking the co-crystallized ligand and calculating the root mean square deviation (RMSD). The best binding affinity value and the type of amino acid residue binding became the basis for selecting compounds with the best activity [10]. In order, compounds that had binding affinity from the smallest to the largest were S1-S4. S1, S3, and S4 had smaller binding affinity values than AMX, but all compounds had greater binding affinity than the native ligand. The lower binding affinity indicated the stronger stability that the compound could provide. This was also correlated with the free energy required for the compound to impact the target protein, such as the receptor [11].

**Table 3.** Molecular docking of the major phytochemicals

No.	Code	Binding affinity (kcal/mol)	RMSD ( $\text{\AA}$ )	Type of bond of amino acids	Category of bond
1.	S1	-7.4	1.662	Conventional hydrogen bond (Lys A:406; Ser A:403; Asn A:464)	Hydrogen

2.	S2	-6,4	1.588	Conventional hydrogen bond (Lys A:406; Ser A:403; Asn A:462)	Hydrogen
3.	S3	-6.7	1.889	Conventional hydrogen bond (Thr A:399)	Hydrogen
4.	S4	-7.1	1.699	Conventional hydrogen bond (Arg A:399)	Hydrogen
5.	AMX	-6,6	1.662	Conventional hydrogen bond (Asp A:221; Gly A:374; Glu A:379)	Hydrogen
6.	NL	-7.9	1.793	Conventional hydrogen bond (Lys A:406;Asn A:462; Arg A:346; Ser A:403; Asn A:464; Ser A:365); Pi-Anion (Lys A:247; Asp A:367; Glu A:379); Pi-Alkyl (Pro A:370; Val A:217);	Hydrogen and hydrophobic

All compounds had RMSD values  $< 2 \text{ \AA}$ ; the RMSD value threshold value was less than  $2 \text{ \AA}$ . Visualization from the Biovia Discovery Studio Visualizer software showed the presence of conventional hydrogen bonds generated in all four test compounds. Such bonds illustrated the basis for solubilization in biomolecular structures. The solubilization process of the test compounds was significantly affected by the hydrogen bonds.

### 3.4. Toxicity prediction

#### 3.4.1 Toxicity

In the toxicity parameter, there were indicators of hERG blockers that could induce LQTS, arrhythmia, and TdP in Table 4 [12].

**Table 4.** Toxicity profile of *B. pandurata* n-hexane fraction (S1-S4)

Parameter	Code				
	S1	S2	S3	S4	AMX
hERG Blockers	0.146	0.267	0.245	0.145	0.028
DILI	0.538	0.524	0.588	0.566	0.903
Ames Toxicity	0.734	0.647	0.634	0.666	0.114
Rat oral acute toxicity	0.497	0.273	0.686	0.743	0.017
FDAMD	0.548	0.659	0.759	0.799	0.001
Skin sensitization	0.664	0.868	0.839	0.901	1.000
Carcinogenicity	0.526	0.597	0.411	0.301	0.049
Eye corrosion	0.029	0.593	0.201	0.139	0.003
Eye irritation	0.990	0.985	0.991	0.995	0.782
Respiratory toxicity	0.213	0.490	0.796	0.877	0.008

hERG: human Ether-a-go-go Related Gene, DILI: Drug-Induced Liver Injury, FDAMDD: FDA Maximum Daily Dose.

The four phytochemicals and **AMX** were predicted to act as hERG blockers, causing cardiovascular toxicity. DILI is a major factor in drug failure in clinical trials. The four phytochemicals and **AMX** caused moderate liver damage. Ames toxicity was used to determine the mutagenic potential of compounds, and bacteria were used in this test. The Ames toxicity prediction was **S2-S4** as moderate toxicity and **S1** as cardiotoxicity compared with **AMX** (low toxicity). These findings meant that all phytochemical compounds caused mutagenicity in the body. The FDA recommended threshold values for the maximum daily

dose in humans. Phytochemical compounds have moderate toxicity (**S1**, **S2**) and can be toxic (**S3**, **S4**) based on the FDA [10]. All phytochemicals have moderate to high sensitivity on the skin, causing allergic contact dermatitis. The carcinogenicity of all phytochemicals was moderate compared to **AMX**, which disrupted cell metabolism. **S1**, **S3**, **S4**, and **AMX** didn't cause eye corrosion, while **S2** had a moderate effect. All phytochemicals and **AMX** acted as eye irritants. All phytochemical compounds exhibit moderate to toxic respiratory toxicity, which is the primary reason for drug withdrawal [10].

### 3.4.2 Tox21 pathway

Some phytochemicals affect AR, AR-LBD, NR-Aromatase, ER-LBD, PPAR gamma, HSE, and p53 inactivation toxicity listed in Table 5. There was no AR, AR-LBD, and NR-Aromatase toxicity that interferes with the production, metabolism, and physiological function of testosterone, especially in men [12]. There was no toxicity in ER-LBD, thereby improving female reproduction. The absence of disruption in peroxisome proliferator-activated receptors (PPAR) and SR-HSE results in proper glucose and lipid metabolism and prevents DNA damage and other cellular stress. p53 inactivation occurs, leading to genetic mutations [12].

**Table 5.** TOX21 Pathway parameters of *B. pandurata* n-hexane fraction (S1-S4)

Parameter	Code				
	S1	S2	S3	S4	AMX
NR-AhR	+	+++	+++	++	---
NR-AR	---	---	--	-	---
NR-AR-LBD	---	---	---	---	---
NR-Aromatase	---	--	--	--	---
NR-ER	++	+++	+++	+++	---
NR-ER-LBD	---	--	--	--	---
NR-PPAR-gamma	---	---	---	---	---
SR-ARE	--	+++	-	-	++
SR-ATAD5	---	+++	-	--	---
SR-HSE	---	---	---	---	---
SR-MMP	+	+++	++	++	---
SR-p53	---	-	--	---	---

NR-AhR: Aryl hydrocarbon Receptor, NR-AR: Androgen Receptor, NR-AR-LBD: Androgen Receptor Ligand Binding Domain, NR-ER: Estrogen Receptor, NR-ER-LBD: Estrogen Receptor Ligand Binding Domain, NR-PPAR gamma: Peroxisome Proliferator-Activated Receptor Gamma, SR-ARE: Antioxidant Response Element, SR-ATAD5: ATPase family AAA Domain-containing protein 5, SR-HSE: Heat Shock Factor Response Element, SR-MMP: Mitochondrial Membrane Potential.

All compounds have an aryl hydrocarbon receptor, an estrogen receptor, and a mitochondrial membrane potential. That meant the compounds had an adaptive response to environmental changes and estrogen receptor disruption, a boost to ATP synthesis in the mitochondria [12]. Only **S2** has ATPase family AAA domain-containing protein 5 as a protein that plays a role in cell division. **S2** and **AMX** possess antioxidant response elements that are crucial for the repair mechanisms associated with oxidative stress [12].

### 3.5. Pharmacokinetic prediction

Pharmacokinetic parameter predictions were determined based on absorption, distribution, metabolism, and excretion parameters in Table 6.

**Table 6.** Pharmacokinetic profile of *B. pandurata* n-hexane fraction (S1-S4)

Parameters	Code				
	S1	S2	S3	S4	AMX
<b>Absorption</b>					
Caco-2 Permeability	-4.854	-4.645	-4.827	-4.971	-5.810
MDCK Permeability	-4.754	-4.568	-4.735	-4.773	-5.220
PAMPA	---	---	---	---	+++
Pgp inhibitor	+++	+++	++	+++	---
Pgp substrate	---	---	---	---	--
HIA	---	---	---	---	---
F20%	---	---	---	---	---
F30%	+	-	---	++	---
F50%	+++	--	+	+++	---
<b>Distribution</b>					
PPB (%)	98.3	97.8	98.1	97.6	31.2
VD <sub>ss</sub>	0.287	-0.072	0.088	0.104	-0.578
BBB penetration	---	---	--	--	---
Fu (%)	1.4	1.6	1.4	2.0	66.6
<b>Metabolism</b>					
CYP1A2 inhibitor	++	+++	---	---	---
CYP1A2 substrate	+++	---	---	+	---
CYP2C19 inhibitor	+++	+++	+++	+++	---
CYP2C19 substrate	---	---	---	---	+++
CYP2C9 inhibitor	+++	++	+++	+++	---
CYP2C9 substrate	+	+++	---	---	---
CYP2D6 inhibitor	---	---	---	---	---
CYP2D6 substrate	---	++	---	---	---
CYP3A4 inhibitor	-	---	++	--	---
CYP3A4 substrate	---	---	---	---	---
<b>Excretion</b>					
CL plasma	5.459	9.288	8.446	7.132	2.932
T1/2	1.045	0.828	1.126	1.030	1.269

Caco-2: Caucasian colon adenocarcinoma cell lines (log cm/s), MDCK: Madin-Darby Canine Kidney (cm/s), Pgp: Para-glycoprotein, HIA: Human Intestinal Absorption, F: bioavailability, PPB: Plasma Protein Binding, VD<sub>ss</sub>: Volume of Distribution steady state (L/kg), BBB: Blood Brain Barrier, Fu: Fraction unbound in plasma, CYP: Cytochrome P450, CL: Clearance (ml/min/kg), T1/2: half-life.

The results of the absorption parameters showed that all phytochemicals had good Caco-2 permeability and %HIA but poor MDCK values. These data indicated that all phytochemicals had specific absorption mechanisms in human enterocytes but not in MDCK cells [13]. Caco-2 permeability values were more relevant because MDCK cells had greater physiological similarity to human intestinal epithelium than MDCK cells [12]. The excellent PAMPA values of all phytochemicals indicated excellent permeability through passive diffusion. High PAMPA and Caco-2 values indicate that the absorption of all phytochemicals was dominated by passive diffusion [13]. All phytochemicals were Pgp substrates rather than inhibitors, meaning that phytochemicals could be pumped out of the cell (active efflux) without inhibiting Pgp function, which affects the efflux of other phytochemicals [14]. Based on the absorption parameters, it was determined that all phytochemicals had good absorption.

The results of the distribution parameters showed that all phytochemicals had very high %PPB values (97-98%) and low Fu values (1.4-2.0%), indicating that the concentration of bound phytochemicals was very high compared to the free/active form in plasma. The

excellent  $V_{dss}$  indicates that phytochemicals distributed very well to tissues [8]. Crossing the BBB would have caused an SSP effect, which is not beneficial for peripheral drugs [14]. Based on the distribution parameters, it was determined that all phytochemicals had satisfactory distribution.

Phytochemicals are substrates for enzymes whose concentrations in the blood change by inducing or inhibiting enzymes during metabolism [8]. All phytochemicals were inhibitors of CYP2C19 and 2C9 but not CYP2D6 inhibitors and CYP3A4 substrates. CYP2C19 and CYP2C9 are important enzymes that break down a number of drugs, including clopidogrel, warfarin, and some NSAIDs [12]. Blocking these two enzymes raises the risk of drug interactions by raising the levels of their substrates in the blood, which means that doses need to be watched [13]. The absence of CYP2D6 inhibitors provides significant clinical benefits, as it has extensive genetic polymorphism and metabolizes many psychoactive drugs (such as antidepressants and antipsychotics) [13]. In addition, because CYP3A4 does not extensively metabolize phytochemicals, they are less susceptible to induction/inhibition. This enhances their pharmacokinetic predictability, as phytochemical levels would not fluctuate due to interactions with the highly common CYP3A4 inhibitors or inducers [14]. Based on metabolic parameters, it was determined that all phytochemicals have advantageous metabolism.

Excretion parameters showed that all phytochemicals had moderate CL plasma and half-life ( $T_{1/2}$ ). This profile indicated favorable and balanced excretion conditions, allowing steady-state to be achieved within a reasonable time, maintaining sufficiently stable therapeutic concentrations in plasma, and allowing dosing at convenient intervals (e.g., once or twice daily) [12]. This aspect enhances patient adherence and minimizes fluctuations in drug concentration, which could potentially diminish efficacy or heighten toxicity. However, caution should be exercised in cases of hepatic dysfunction, as such situations may reduce clearance and prolong  $T_{1/2}$  significantly [13].

#### 4. Conclusion

The conclusion of the research was that the major phytochemicals in the *n*-hexane fraction of the ethanol extract of *B. pandurata* rhizome include pinostrobin, demethoxyyangonin, 5-methoxy-7-hydroxyflavanone, and pinocembrin. Pinostrobin, 5-methoxy-7-hydroxyflavanone, and pinocembrin had the best activity and pharmacokinetics, as well as toxicity prediction. Based on these findings, an isolation method for the four major phytochemicals using *n*-hexane or a combination of phases needs to be developed and tested in vitro on *S. aureus*.

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