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Immunosuppressive and antibacterial activities of dihydromorin and norartocarpetin isolated from *Artocarpus heterophyllus* heartwoods

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ABSTRACT

Objective: To evaluate the immunosuppressive effect on human phagocytes and antibacterial activity of dihydromorin and norartocarpetin isolated from *Artocarpus heterophyllus* heartwoods.

Methods: Dihydromorin and norartocarpetin were isolated from *Artocarpus heterophyllus* heartwoods. A modified Boyden chamber was used to determine the chemotactic activity of human phagocyte. The respiratory burst was evaluated by chemiluminescence assay. Myeloperoxidase (MPO) activity was quantified using a colorimetric assay. The broth microdilution method was performed to assess their antibacterial activity.

Results: Dihydromorin exhibited potent inhibitory effect on the chemotactic activity of polymorphonuclear neutrophils (PMNs) with an IC_{50} value of 5.03 μg/mL. Dihydromorin also inhibited reactive oxygen species production of whole blood cells, PMNs, and monocytes with IC_{50} values of 7.88, 7.59 and 7.24 μg/mL, respectively. Interestingly, dihydromorin also strongly inhibited the MPO activity of PMNs with an IC_{50} value of 5.24 μg/mL, which was lower than indomethacin (24.6 μg/mL). Molecular docking of dihydromorin and crystal structure of MPO showed that dihydromorin had close interaction with key amino acid residues such as Arg239 and Gln91. Antibacterial activity assay showed that only dihydromorin had a strong effect against *Streptococcus pyogenes* with MIC and MBC values of 15.62 and 31.25 μg/mL, respectively.

Conclusions: The results suggest that dihydromorin could be developed as an anti-inflammatory and antibacterial agent.

KEYWORDS: Dihydromorin; Immunosuppressive; Antibacterial; Flavonoid; Myeloperoxidase; *Artocarpus heterophyllus*

1. Introduction

Immunomodulator can be classified as a natural or synthetic substance that is able to modulate both innate and adaptive immune function[1]. The innate immune system is the first line of defense mechanism and it provides a protective response against invading pathogens such as bacteria or fungi[2]. Polymorphonuclear neutrophils (PMNs) are the most abundant white blood cells which play an important role in eliminating foreign material from the body[3]. Pathogenic microorganisms could access tissue and bloodstream through penetration of host barrier including skin and mucous membranes[4]. Invasive pathogens will further release exogenous substances, for instance, formyl peptide along with chemoattractant and pro-inflammatory cytokines[5]. PMNs express various surface receptors that can recognise these pathogenic products and lead to the activation of their protective function. Phagocytes will migrate to the site

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of infection, followed by phagocytosis activity[6]. Through the utilisation of nicotinamide adenine dinucleotide phosphate-oxidase and myeloperoxidase (MPO), PMNs will generate the production of reactive oxygen species (ROS) that is beneficial for microbial killing and degradation[7,8]. However, overproduction of ROS also contributes to the development of various ailments including chronic inflammation[9]. Hence, it needs to find and develop an anti-inflammatory agent that is able to suppress the overactivity of phagocytes.

The natural product is one of the main sources for new anti-inflammatory substances and antimicrobial agents. Plant-derived compounds including flavonoids have been used for the treatment of several diseases. *Artocarpus heterophyllus* (*A. heterophyllus*) Lam. has been used as a traditional medicine in many countries[10]. This plant is also a source of numerous phytochemical constituents such as flavonoid, tannin, and volatile oil[11,12]. Flavonoids isolated from the heartwood of this plant possessed many pharmacological properties including antibacterial, antioxidant, anti-cancer, anti-tyrosinase, and antiinflammation[13-16]. The present study was to isolate active compounds from *A. heterophyllus* heartwoods. Furthermore, the modulating effect of isolated compounds on the activity of human phagocytes as well as their antibacterial activity were also investigated. The results of this study will provide important insight into the biological properties of these isolated compounds.

2. Materials and methods

2.1. Chemicals and media

Zymosan A from *Saccharomyses cerevisiae*, luminol (3-aminophthalhydrazide), *N*-formyl-methionyl-leucyl-phenylalanine, phorbol 12-myristate 13-acetate, Hanks Balance Salt Solution, phosphate buffer saline (PBS), ficoll, dimethyl sulfoxide, trypan blue, acetylsalicylic acid (purity 99%), ibuprofen (purity 99%) and indomethacin (purity 99%) were from Sigma (St Louis, MO, USA). Haematoxylin and xylene were from BDH, UK. Lymphoprep® was from Stemcell Technology, USA. MPO activity colorimetric assay kit was from Biovison, UK. Brain heart infusion, Muller-Hinton broth, and agar were from the Becton, Dickinson, and Company (Franklin Lakes, NJ).

2.2. Microorganisms

Streptococcus mutans (DMST 26095), *Streptococcus pyogenes* (*S. pyogenes*) (DMST 17020), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 14990), *Pseudomonas aeruginosa* (DMST 15442) and *Escherichia coli* (*E. coli*) (ATCC 25922) were from the Departement of Medical Sciences, Thailand. *Bacillus subtilis* was from the Faculty of Science, Prince of Songkla University.

2.3. Collection of plant material

The heartwoods of *A. heteropyllus* were collected from Hat Yai District, Songkla Province, Thailand. The voucher specimen (SKP 117 01 08 01) was identified by Dr. Pharkphoom Panichayupakaranant and deposited at the Herbarium of Prince of Songkla University, Thailand.

2.4. Extraction of plant material, isolation, and identification of pure compound

The plant material was dried at 50℃ for 24 h and grounded to powder. The dried powder of heartwood of *A. heterophyllus* was extracted with ethyl acetate (EtOAc) at 110℃ for 1 h. Dihydromorin (1) and norartocarpetin (2) were isolated from the ethyl acetate fraction. Twenty-five gram (25 g) EtOAc extract was fractionated on silica gel column (13 cm in diameter and 6 cm in height) and eluted with the mixture of hexane and EtOAc (100%, 70%, 50% and 30% v/v hexane) followed by mixtures of EtOAc and MeOH (100%, 70% and 50% v/v EtOAc) by vacuum liquid chromatography. According to thin-layer chromatography chromatograms, 13 pooled fractions were obtained. Fraction 5 was then purified by silica gel column (4 cm×50 cm) and a mixture of chloroform and methanol (95:5) to afford compound 1 (18 mg) and compound 2 (25 mg). Isolated compounds were then identified according to their ^{1}H NMR and ^{13}C NMR spectral data.

2.5. Immunomodulatory effect

2.5.1. Isolation of human neutrophil and monocytes

A modified method was used to isolate PMNs[17]. In brief, the mixture of whole blood with an equal volume of PBS and dextran was left for sedimentation at room temperature for 45 min. The supernatant contained plasma cell was then centrifuged using Ficoll-gradient and washed twice with PBS. The isolated PMNs at the bottom of centrifuge tubes were re-suspended in PBS and immediately used for assay within 8 h. The monocyte was isolated from human whole blood as previously described[18]. Briefly, whole blood was mixed with an equal volume of physiological solution and then gently layered on lymphoprep® solution and centrifuged at 400×*g* for 45 min. Mononuclear cells were engaged at the medium interface and transferred to sterile tubes. The cells were then washed with PBS (250×*g* for 10 min) and diluted in PBS. The cells were counted using a Naubauer hemocytometer (Hausser Scientific, USA) under a light microscope (Olympus, USA) and then diluted with

PBS to adjust approximately 1×10^6 cells/mL.

2.5.2. Cell viability

The viability test was determined using the standard trypan blue exclusion method[19]. The cells (1×10^6) were incubated with samples (6.25 and 100 μg/mL) at 37℃ for 2 h. After incubation, the cells were mixed with trypan blue and observed using a light microscope. The blue dye uptake was a signal of cell death. The percent viability was counted from the total cells. The concentration of compounds at which viability was >90% was used for further studies.

2.5.3. Chemotaxis assay

Chemotaxis assay was performed using a modified 48-wells Boyden chamber as described in our previous work[17]. In brief, 25 μL of chemoattractant was added at the lower part of the chamber. Then, 5 μL of samples (final concentrations of samples were 10, 5, 2.5, 1.25 and 0.625 μg/mL) were added at the upper part of the chamber which contains 45 μL PMNs (1×10^6 cells/mL). Ibuprofen was used as a positive conrol. The Boyden chamber was then incubated for 1 h at 37℃. Percentage (%) inhibition was quantified using the following formula:

(Distance of untreated cell–Distance of treated cell)/(Distance of untreated cell)×100%

2.5.4. Chemiluminescence assay

Luminol-based chemiluminescence assay was used to determine ROS production during respiratory burst as previously described[17]. In brief, samples at final concentrations (12.5, 6.25, 3.13, 1.56 and 0.78 μg/mL) were incubated with whole blood or PMNs/monocytes (1×10^6/mL), opsonized zymosan and luminol (7×10^{-5} mol/L). Aspirin was selected as a positive control. The results were observed as reading per luminometer unit (RLU). Percentage (%) inhibition was quantified based on the following formula:

(RLU control–RLU sample)/(RLU control)×100%

2.5.5. MPO activity assay

The test was performed using an MPO colorimetric assay kit (Biovision) as described in our previous study[20]. Briefly, activated neutrophil was incubated with the reaction mix (MPO buffer assay: MPO substrate, 4:1) for 30 min. After that, 2 μL of stop solution was added to each well and incubated for 10 min, followed by 50 μL of TNB standard solution. Indomethacin was used as a postive control. The reading was observed at 412 nm using a microplate reader.

2.6. Determination of minimum inhibitory and bactericidal concentration

The slightly modified broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)[21]. Briefly, two-fold dilutions of each sample were prepared in 96-wells plate. Bacteria suspensions were prepared in normal saline and adjusted approximately 1×10^8 CFU/mL to 0.5 McFarland standards. The suspension was diluted in normal saline to reach 1×10^6 CFU/mL and added into each well. The plate was incubated at 37℃ for 24 h. Ampicillin was used as the positive control, while dimethyl sulfoxide was used as the negative control. The MIC and MBC were then observed.

2.7. Molecular docking simulation

The target compound was docked to the crystal structure of the MPO enzyme from Brookhaven Protein Data Bank (PDB:3ZS0). Autodock4 (version 4.2.6) was employed for docking simulation, while its calculation has been done by AutoDock tools 1.5.6 and MGL tools 1.5.6 packages. In docking calculations, the receptor-ligand poses obtained were ranked using an energy-based scoring function and the Lamarckian-Genetic Algorithm (LGA) was employed in this docking study. The highest binding activities were calculated and the protein-ligand interactions were analysed using Discovery Studio Visualizer.

2.8. Statistical analysis

Each experiment was carried out in triplicate and presented as mean ± SEM. Data were analysed using one-way ANOVA followed by Tukey's test for multiple comparisons and $P < 0.05$ was considered as a significant difference. The IC_{50} values were calculated using Graph PAD prism 6 analysis software.

2.9. Ethical statement

The use of human blood was approved by the Human Ethical Committee, Faculty of Medicine, Universiti Kebangsaan Malaysia (approval number FF/2012/Ibrahim/23-May/432-May 2012-August 2013).

3. Results

3.1. Identification of pure compounds

Compounds 1 and 2 (Table 1) were identified as dihydromorin (1) and norartocarpetin (2) (Figure 1) by comparing their spectral data with the data in the literature[15,22].

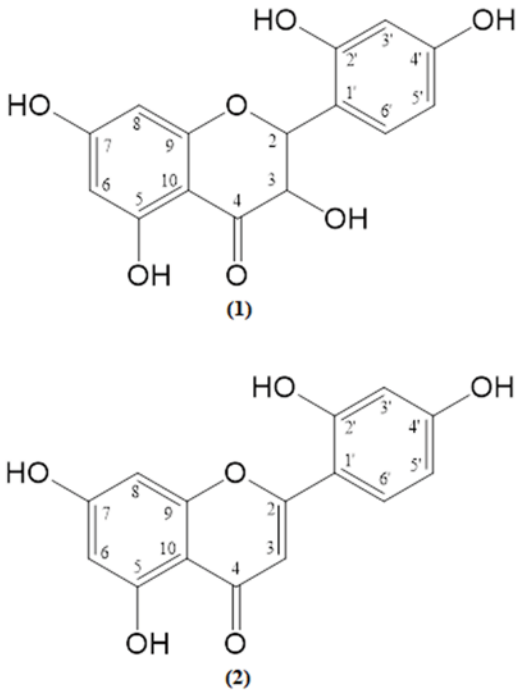

Figure 1. Chemical structures of flavonoids, dihydromorin (1), and norartocarpetin (2).

3.2. Cells viability

The cell viability test was conducted using the trypan blue exclusion method. The result showed that compounds at 50 μg/mL were non-toxic against isolated cells after 2 h incubation.

3.3. Chemotaxis

Dihydromorin at 10 μg/mL inhibited the migration of PMNs towards chemoattractant with percentage inhibition of 57% (Figure 2). This compound showed strong inhibition activity with an IC_{50} value of 5.03 μg/mL, but lower than ibuprofen (1.52 μg/mL). Norartocarpetin did not show any inhibition effect on chemotaxis activity.

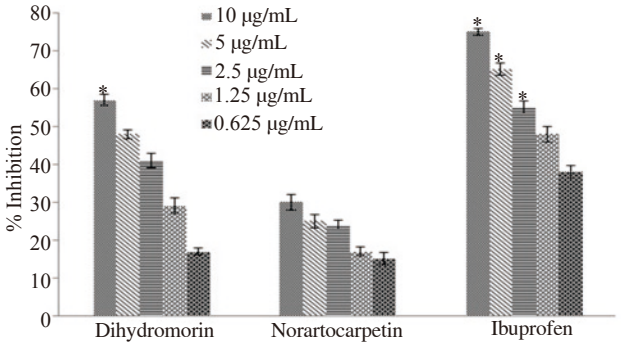

Figure 2. Percentage inhibition of polymorphonuclear neutrophil migration by flavonoids and ibuprofen. Data are mean ± SEM (n=3). $^{*}P < 0.05$ indicates significant difference compared with the respective control determined by one-way ANOVA followed by Tukey's test.

3.4. ROS production

Preliminary screening on whole blood cells showed that dihydromorin inhibited ROS production in a dose-dependent manner (Figure 3). Dihydromorin revealed strong activity on whole blood cells, PMNs and monocytes with IC_{50} values of 7.88, 7.59 and 7.24 μg/mL, respectively. This compound possessed lower activity compared to aspirin with IC_{50} values of 2.50, 2.11 and 1.82 μg/mL, respectively. In contrast, norartocarpetin was not active in inhibiting ROS production.

3.5. MPO activity

Dose-dependent inhibition of MPO activity of dihydromorin and positive control was observed. However, norartocarpetin did not show any inhibition effect. As shown in Figure 4, dihydormorin exhibited strong inhibitory activity with an IC_{50} value of 5.24 μg/mL which was lower than indomethacin with an IC_{50} value of 24.6 μg/mL.

Table 1. NMR spectral data (500 MHz, $CDCl_3$) for flavonoids (δ in ppm).

Position	Dihydromorin		Norartocarpetin	
	δH (in Hz)	δC	δH (in Hz)	δC
2	5.38 (d, 1H, *J*=11.5 Hz)	79.97		165.79
3	4.77 (d, 1H, *J*=11.5 Hz)	72.50	7.12 (s, 1H)	108.32
4		198.87		184.37
5		168.51		163.09
6	5.86 (d, 1H, *J*=2.5 Hz)	97.13	6.18 (d, 1H, *J*=2.5 Hz)	99.82
7		164.93		164.18
8	5.90 (d, 1H, *J*=2.5 Hz)	96.19	6.41 (d, 1H, *J*=2 Hz)	94.83
9		165.26		163.33
10		101.89		105.18
1'		115.53		110.74
2'		158.55		159.47
3'	6.34 (d, 1H, *J*=3 Hz)	103.68	6.41 (d, 1H, *J*=2 Hz)	104.17
4'		160.10		160.32
5'	6.35 (d, 1H, *J*=3 Hz)	107.89	6.44 (dd, 1H, *J*=9.5, 2.5 Hz)	109.09
6'	7.21 (d, 1H, *J*=9Hz)	130.83	7.75 (d, 1H, *J*=8.5 Hz)	130.98

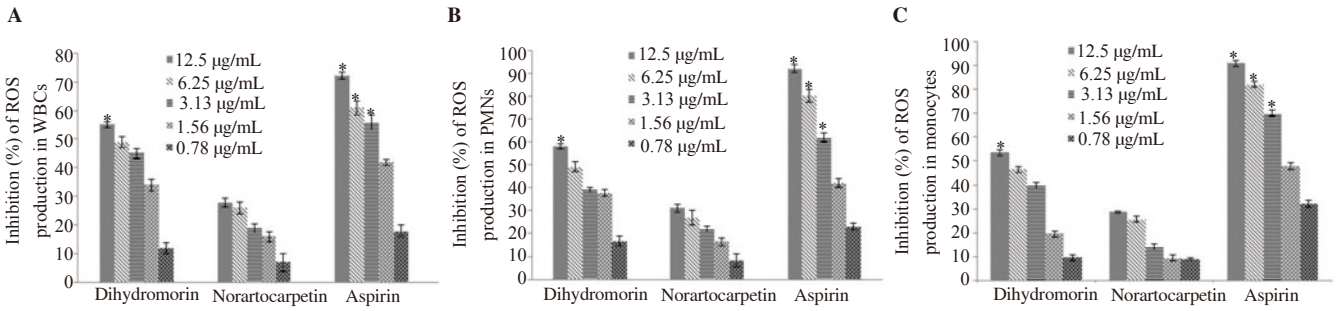

Figure 3. Percentage inhibition by flavonoids and aspirin of reactive oxygen species (ROS) production in white blood cells (WBC) (A), polymorphonuclear neutrophils (PMNs) (B) and monocytes (C). Data are mean ± SEM (n=3). $^{*}P < 0.05$ indicates significant difference compared with the respective control determined by one-way ANOVA followed by Tukey's test.

3.6. Molecular docking

The possible binding interactions of dihydromorin with MPO active site, the protein crystal structure of MPO (PDB Code: 3ZS0) was retrieved from Brookhaven Protein Data Bank. According to the docking results, the compound dihydromorin was found to have close interaction with key amino acid residues such as Arg239 and Gln91 (Figure 5).

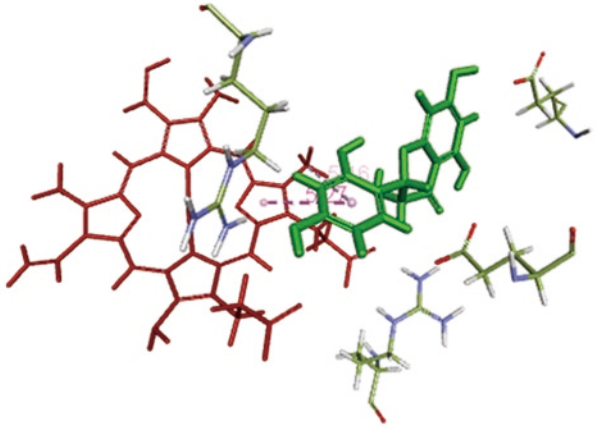

Figure 5. Docking model for compound dihydromorin (green) bound to the heme plane close to the pyrrole ring (red).

3.7. MICs and MBCs

Dihydromorin showed a strong effect against *S. pyogenes* with an MIC value of 15.62 μg/mL (MBC value of 31.25 μg/mL). While norartocarpetin only revealed moderate activity against Gram-positive bacteria. As shown in Table 2, both compounds did not exhibit any activity against Gram-negative, including *E. coli* and *Pseudomonas aeruginosa*.

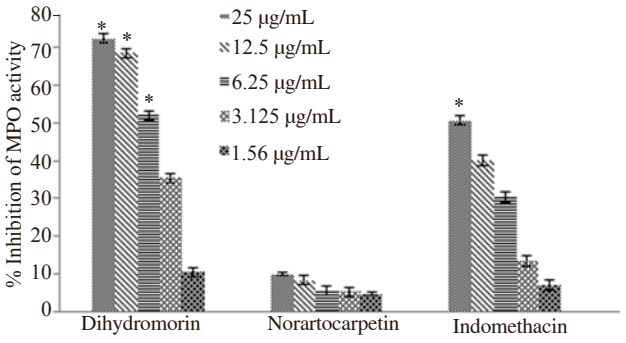

Figure 4. Percentage inhibition of myeloperoxidase (MPO) activity of flavonoids and indomethacin in polymorphonuclear neutrophils. Data are mean ± SEM (n=3). $^{*}P$<0.05 indicates significant difference compared with the respective control determined by one-way ANOVA followed by Tukey's test.

4. Discussion

This study was conducted to determine the immunosuppressive effect of flavonoid compounds from *A. heterophyllus* heartwoods extract on the innate immune response of human phagocytes. The first step of phagocytosis that we assessed in this study is chemotactic activity. Chemotaxis is the migration of cells towards a gradient of chemoattractant and it plays a crucial role to attract neutrophil to the site of infection[23]. *N*-formyl-l-methionyl-l-leucyl-l-phenylalanine from bacteria was used as a chemoattractant that is able to stimulate neutrophil migration through intracellular activation[24]. In certain conditions, neutrophil can rapidly be activated and finally lead to inflammation[25]. The inhibition of chemotactic may be beneficial to overcome this problem. We found that dihydromorin shows a strong inhibitory effect on chemotaxis. This result is in accordance with previous work in which flavonoid compounds isolated from *A. heterophyllus* heartwoods including artocarpin exhibited strong activity in inhibiting the migration of neutrophils[20].

Table 2. MIC and MBC values of flavonoid compounds and standard drug (μg/mL).

Bacteria	Dihydromorin		Norartocarpetin		Ampicillin	
	MICs	MBCs	MICs	MBCs	MICs	MBCs
Streptococcus mutans	31.25	31.25	125.00	125.00	0.50	0.50
Streptococcus pyogenes	15.62	31.25	31.25	31.25	0.50	0.50
Bacillus subtilis	62.50	125.00	250.00	na	0.50	0.50
Staphylococcus aureus	62.50	125.00	125.00	250.00	0.50	0.50
Staphylococcus epidermidis	31.25	62.50	250.00	na	0.50	0.50
Pseudomonas aeruginosa	na	na	na	na	31.25	62.50
Escherichia coli	na	na	na	na	0.25	0.50

na: no activity.

PMNs are the most abundant type of white blood cells in mammals and play an essential role in response to infections. This cell expresses many receptors involved in the recognition of foreign materials. In response to invading pathogens, neutrophil will phagocyte and lead to the activation of the nicotinamide adenine dinucleotide phosphate in generating ROS production and release some proteolytic enzymes[26]. ROS produced during phagocytic activity is a key element to eliminate foreign materials including bacterial and fungi. However, the overproduction of oxidative stress also contributes to the development of several ailments. In our study, inhibition of ROS was calculated using a chemiluminescence assay. Luminol which has small molecular weight was used to detect the level of intracellular ROS during respiratory burst[27]. Dihydromorin exhibited strong activity in inhibiting ROS production of human phagocytes. It has been known that flavonoid compounds display an anti-inflammatory effect by modulating neutrophil's oxidative burst[28]. Kanashiro and colleagues reported that flavonoids from *Lychnophora* sp. including 5- and 7- dihydroxylation were able to inhibit ROS production[29]. Recently, pilloin isolated from *Aquilaria sinensis* had potent anti-inflammatory activity by suppressing ROS elevation in macrophage[30].

MPO is a heme enzyme found in neutrophil which has a crucial role as an antibacterial agent[31]. Upon activation, neutrophil will release MPO into the phagolysosomal compartment and extracellular environment[32]. Furthermore, this enzyme utilizes hydrogen peroxide to produce reactive oxygen intermediate including hypochlorous acid (HOCl) which kills bacteria during infection. MPO plays an important role as anti-inflammatory agents only in selected conditions. In contrast, the free radical produced will also promote inflammation and cause membrane damage[33]. Therefore, the inhibition of MPO activity is probably useful for the treatment of chronic ailments, including inflammation. Interestingly, in this study, dihydromorin showed anti-MPO activity which is stronger than indomethacin as a positive control. This result was in agreement with our previous study, in which flavonoid artocarpin had potent anti-MPO activity[20]. Moreover, another study also reported that eriodictyol was able to inhibit MPO activity[34]. Molecular docking was then performed in order to know the interaction of compound on protein target *via* computational study. Dihydromorin had close interaction with two key amino acid residues. The result was in agreement with the X-ray structure of salicylhydroxamic acid, a known MPO inhibitor that showed similar interactions. Furthermore, compound dihydromorin could bind to the active site of MPO with B-ring oriented to the heme plane and close to the D pyrrole ring in a way similar to that for the salicylhydroxamic acid[35,36]. The docking results obtained further support the strong inhibition activity of MPO demonstrated by dihydromorin.

Antibacterial resistance caused by the overuse of synthetic antibiotics has become an emerging public health problem worldwide. It has been reported that aminoglycoside antibiotics were able to induce biofilm formation in Gram-negative bacteria including *E. coli*. Besides, isolates nosocomial can produce β-lactamase that hydrolyzes ampicillin derivate[37]. One appealing strategy to overcome this problem is the use of alternative compounds derived from nature. In this study, dihydromorin and norartocarpetin isolated from the active fractions of *A. heterophyllus* were assessed for their antibacterial activity against various pathogenic bacteria. On the basis of the microdilution method, dihydromorin revealed its potency as an antibacterial compound. This compound was able to inhibit the growth of tested bacterial. In this study, the antibacterial effect against Gram-positive bacteria was better than Gram-negative in which *S. pyogenes* is shown as a more susceptible strain. Flavonoids have been considered to have a major antibacterial activity due to their ability to interact with bacterial cell walls. Moreover, this compound also inhibits the nucleic acid synthesis and energy metabolism in bacteria. Reportedly, flavonoid compounds isolated from *A. heterophyllus* have shown their antibacterial activity against both Gram-positive and Gram-negative bacteria[14].

The results of this study also indicated the structure-activity relationship of the flavonoids. The presence of one hydroxyl group on C-3 in ring C, as well as the saturation of C-2 and C-3 of dihydromorin, contributed to its immunosuppression effect particularly on chemotaxis, ROS production, and MPO activity. It has been reported that the lack of a double bond on C-2 and C-3 in ring C of flavonoid may enhance its scavenging activity. Moreover, the substitution of one hydroxyl group in ring C increased superoxide scavenging activity[38]. This result denoted that the inhibition of phagocytosis activity may be associated with its antioxidant effect[39].

Zheng and the team reported that the presence of the hydroxyl group in the aromatic ring of flavonoid will increase its biological activity including anti-tyrosinase[40]. In the case of antibacterial activity, the lack of double bond C-2, C-3 in ring C and the presence of hydroxyl moiety on C-3 contributed to its antibacterial activity. This result might be related to the hydrophobic feature of flavonoid, which causes outer membrane permeabilization[41]. In addition, Tsuchiya and colleagues reported that tetrahydroxy flavanones show strong antibacterial activity against methicillin-resistant *Staphylococcus aureus*. The substitution of the hydroxyl group on C-3 enhanced the antibacterial activity[42].

5. Conclusion

In conclusion, two flavonoid compounds, named dihydromorin and norartocarpetin have been isolated from the active fraction of *A. heterophyllus* heartwoods extract. Dihydromorin exhibited strong inhibition effect at different steps of phagocytosis of human phagocytes, such as chemotaxis, respiratory burst, and MPO activity. Dihydromorin had close interaction with key amino acid residues in MPO. Between these compounds, dihydromorin showed strong antibacterial activity against *S. pyogenes* and only demonstrated mild activity against *Streptococcus mutans*, *Bacillus subtilis*, and *Staphylococcus aureus*. The finding provided insight regarding the potency of dihydromorin as an immunosuppressive and antibacterial agent. Nevertheless, further experiments are still needed to determine other immunomodulatory responses as well as the mechanism of antibacterial action.

Conflict of interest statement

We declare that there is no conflict of interest.

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Authors' contributions

AWS conducted research and drafted the manuscript. IJ is the leader who edited the manuscript. PP contributed to the idea and reviewed the manuscript. MFFMA contributed to the molecular docking study. EPR is a member who contributed to the design.

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