

whole orchid *D. alpestre* and its possible antibacterial activity, and also its effects on hemodynamic parameters, myeloperoxidase (MPO) activity as well as mortality rate were evaluated in cecal ligation and puncture (CLP)-induced sepsis in mice.

Materials and Methods

Collection

The whole orchid of *D. alpestre* Royle was purchased from the local flower market of Danang, Vietnam in December 2019 and it was authenticated by Dr Ho Viet Hieu, Duy Tan University, Danang, Vietnam. A voucher specimen (DTU-2019-O141) has been deposited at the Department of Botany, Duy Tan University.

Extraction and Isolation

The whole orchid was dried, powdered (250 g), and extracted three times with ethanol-water (7:3) at 25 °C. All were combined and evaporated under low pressure to obtain a hydroalcoholic extract of *D. alpestre* (Da, 4.1 g), which was preserved at 4 °C in an amber colour bottle²⁴.

Preliminary phytochemical analysis

Preliminary phytochemical analysis upon Da was performed according to the standard practical methods^{25,26}.

Assay for total phenolic content

The total phenolic content of Da was established through the Folin-Ciocalteu reagent²⁷. Initially, 500 µL of Da was mixed with 5 mL of Folin-Ciocalteu reagent 10% (v/v) and 4 mL of sodium bicarbonate solution (1 M). After 15 min of incubation time at room temperature, the absorbance of the produced blue colour was read by spectrophotometer at 750 nm. Ultimately, the total phenolic content of Da was calculated from the calibration curve of gallic acid and expressed as gallic acid equivalent, respectively²⁸.

Antibacterial activity

In vitro antimicrobial activity of Da was performed by the cup-plate method²⁹. The test bacteria used in this study were *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative). Mueller Hinton agar plates procured from American Type Culture Collection, Virginia, United States, were inoculated with 0.5 McFarland standards of mentioned bacteria were used for this assessment. Test strains were inoculated by spread late technique,

and wells were made by sterile cork borer. Accurately 50 µL (100 µg/mL concentration) of Da and standard, streptomycin were poured in each well. After 24 h of incubation at 37 °C, inhibition zones were measured by calibrated scale³⁰.

Animals

Adult male mice (weighting 25±5 mg, age 6-8 weeks) were used in this study. The animals were given food and water *ad libitum* and were housed in the Animal House of Duy Tan University of Medicine and Pharmacy under the standard condition with a temperature of 21±2 °C, the relative humidity of 50±10% and a 12-h light/12-h dark cycle³¹. This study was approved by the Ethics Committee of Duy Tan University of Medicine and Pharmacy (Code: VN.DTU.MP.2020.412).

Acute oral toxicity

Mice were randomly divided into 4 groups (6 mice in each group). The OECD main test 425 (up-and-down dose procedure) was utilized using doses of 175, 550, 1750, and 2000 mg/kg body weight (b.w) of Da. The test animals underwent fasting overnight before administering the extract using oral gavage. The first set of test animals was administered with a dose of 175 mg/kg b.w. When the animal survived after 48 hours, the dose that was given to the next sets of rodents was increased by a factor of 3.2, which was 550 mg/kg b.w. After 48 hours of survival, the next test animal was given 1750 mg/kg b.w then the same cycle was repeated, and the upper bound dose of 2000 mg/kg b.w was given to the test rodents. The testing was ended until the last three animals survived and all of the test animals were observed up to 14 days^{32,33}.

Cecal ligation and puncture (CLP)-induced sepsis

CLP model³⁴ was used for the induction of sepsis. At the beginning of the experiment, mice were randomly divided into 4 groups (6 mice in each group). Mice in group 1 (normal control) underwent midline abdominal incision without CLP. Mice in group 2 (CLP) underwent midline abdominal incision with cecal ligation (50%) and were punctured to induce polymicrobial sepsis. Mice in groups 3 and 4 received 100 mg/Kg b.w (as a low dose) and 200 mg/Kg b.w (as a high dose) of Da intraperitoneal (i.p.) at 0, 1, 3, 6, and 24 h after CLP operation. Blood samples were obtained from the portal vein. Exactly 0.5 mL of blood samples were transferred into

laboratory tubes containing pre-autoclaved nutrient broth medium (Sigma-Aldrich, Germany) and put in an incubator at 37 °C. The remaining blood samples decanted gently into collection plastic tubes, then centrifuged at 3000 rpm for 5 min. Then serum was obtained, aliquoted into microtubes, and stored at -20 °C for biochemical analysis.

Later, mice were anaesthetized by i.p. injection of ketamine (60 mg/Kg b.w) and xylazine (10 mg/Kg b.w). Then, the abdominal region of animals was shaved and sterilized by betadine. The cecum was exposed through a midline abdominal incision, ligated (50%) with 3/0 silk suture, and then punctured with a sterile 18-gauge needle. The cecum was gently squeezed and after a drop of cecal contents was discharged, the cecum was repositioned into the abdominal cavity. The abdominal wall and skin were closed with 3/0 silk suture. After the surgery, mice received 3 mL warm 0.9% normal saline subcutaneously (s.c) for fluid resuscitation. After mice recovered from anaesthesia, they had free access to food and water.

Animal survival rate

In addition to monitoring the animals for three days, animals' survival rate was reported after 72 h³⁵.

Hemodynamic parameters

For measurements of hemodynamic parameters such as arterial blood pressure (ABP), mean arterial blood pressure (MAP), developed pressure (DP) and heart rate (HR), a polyethylene cannula connected to a pressure transducer that prefilled with heparinized normal saline solution was cannulated into the right common carotid artery³⁵.

Myeloperoxidase measurement

The activity of MPO³⁶, an abundant enzyme of neutrophils, was assessed as previously described with minor modification. Briefly, 1 mL of the serum was mixed with 1 mg of hexadecyltri methylammonium bromide (HTAB). Then sonicated for 5 min and centrifuged at 3000 rpm for 10 min at 4 °C. Exactly 0.1 mL of supernatant was mixed with 2.9 mL of 50 mM phosphate buffer (pH 6.0), containing 0.167 mg/mL O-Dianisidine dihydrochloride and 1% hydrogen peroxide. Then the mixture was incubated for 5 min at room temperature. After adding 0.1 mL of 1.2 M HCl, the change in absorbance was measured at 460 nm using a spectrophotometer.

Results

Phytochemical analysis

Results of the preliminary phytochemical screening of Da showed the presence of alkaloids, coumarins, phenanthrenes, sesquiterpenoids, flavonoids, polyphenols, and tannins, among the tested class of compounds. Besides, anthraquinones, bibenzyls, fluorenones, saponins, and cardiac glycosides were found to be absent in the extract.

Additionally, total phenolic content was ascertained via the absorbance of Da and the equation obtained from the standard curve of gallic acid through the Folin-Ciocalteu method. As a consequence, the total phenolic value for Da was equivalent to 51.6±0.8 mg of gallic acid per 100 g of dried plant material.

Acute oral toxicity

Da was non-toxic up to 2000 mg/kg body weight of tested mice. There were no significant changes that occurred in the behaviour pattern of the tested animals. No mortality was noted for 14 days. These results present that the extract is non-toxic up to 2000 mg/kg b.w, and the low (1/20th) and high (1/10th) dosage was fixed as 100 and 200 mg/Kg b.w, respectively.

Antibacterial activity

The results of *in vitro* antimicrobial activity of Da revealed that it has antibacterial activity against both selected bacteria (*E. coli* and *S. aureus*). As can be seen in Table 1, Da was as potent as streptomycin against *E. coli* and showed the same inhibitory effect. As shown in Fig. 1, the optical density of blood (OD) significantly ($P < 0.01$) increased in the CLP group compared with the normal control group. On the other hand, the administration of Da (at both doses) to the septic mice significantly ($P < 0.05$) decreased OD in the blood of animals compared with the CLP group.

Hemodynamic responses

It was observed that the MAP significantly decreased from 125.0±3.5 mm of Hg in the normal

Table 1 — Antibacterial screening test of hydroalcoholic extract of *D. alpestre*

| Sample | Zone of inhibition (mm)* | |
|--------------|--------------------------|----------------|
| | <i>S. aureus</i> | <i>E. coli</i> |
| Da | 22.0±0.2 | 20.0±0.1 |
| Streptomycin | 22.5±0.1 | 19.5±0.1 |

*mean±SD values (n = 3)

control group to 72.0 ± 12.3 mm of Hg in the CLP group ($P < 0.05$). There was a significant increase ($P < 0.05$) in the MAP of mice treated with Da at 100 and 200 mg/Kg b.w to 85.0 ± 5.3 and 109.0 ± 3.3 mm of Hg. It was found that arterial blood pressure (ABP) decreased from 138.0 ± 4.0 mm of Hg in the normal control group to 82.0 ± 6.7 mm of Hg ($P < 0.001$) in the CLP group. Treatment with Da at 100 and 200 mg/Kg b.w increased the arterial blood pressure ($P < 0.001$) significantly. Developed pressure (DP) decreased, and HR increased significantly ($P < 0.05$) in the CLP group and Da treated groups showed no significant change (Table 2).

Serum MPO activity

Animals in the CLP group showed a significant ($P < 0.05$) increase in MPO activity compared with the normal control group. The treatment of mice with Da (at both doses) decreased markedly ($P < 0.01$) the enzyme activity compared with the CLP group (Fig. 1).

Survival rate

To examine the effects of Da on survival rates, the animals were monitored for 72 h after CLP surgery. There was no death of mice in the normal control group after 72 h, and the survival rate was $100.0 \pm 0.0\%$. At 72 h, the survival rate decreased in the CLP group to $42.50 \pm 3.67\%$ compared with the normal control group. Treatment of septic mice with Da with doses of 100 and 200 mg/Kg b.w decreased

survival rate to 72.84 ± 5.34 and $92.50 \pm 3.50\%$, respectively, at 72 h (Fig. 1).

Discussion

The present study showed that Da improved hemodynamic parameters with potent antibacterial activity. In addition, Da unexpectedly decreased the inflammatory response and mortality rate in mice with polymicrobial sepsis. Besides, preliminary phytochemical screenings showed that Da contains alkaloids, coumarins, phenanthrenes, sesquiterpenoids, flavonoids, polyphenols, and tannins. Also, a good amount of total phenolic content was observed in Da.

The results showed that Da has antibacterial activity against *E. coli* and *S. aureus* (Table 1). As these bacteria are the predominant cause of sepsis, this edible flower can have beneficial effects against sepsis¹. The antibacterial activity of *D. alpestre* might be related to its phytochemical contents because the antibacterial activities of alkaloids, flavonoids, and phenolic acids have been reported in several studies^{37,38}.

As mentioned, sepsis causes cardiac and endothelial dysfunction^{6,7}. The hemodynamic monitoring in this study showed attenuation of hemodynamic parameters in the CLP group. This event can lead to misbalancing in tissues oxygen supply/demand and accelerates the process of septic shock. For this reason, apart from antibiotic therapy,

Fig. 1 — Effect of hydroalcoholic extract of *D. alpestre* (Da) on (A) OD at 600 nm (B) MPO activity. Values are mean \pm SEM ($n = 6$). * $P < 0.05$, as compared with the normal control group; ** $P < 0.01$, as compared with CLP group using one-way ANOVA with Student-Newman-Keuls *post hoc test*.

Table 2 — Effects of hydroalcoholic extract of *D. alpestre* on hemodynamic parameters in CLP-induced sepsis after 72 h

| Sample | Hemodynamic parameters (mm of Hg)* | | | |
|--------------------|------------------------------------|------------------------------|------------------|-----------------------------|
| | Mean arterial pressure | Arterial blood pressure | Heart rate | Developed pressure |
| Normal Control | 125.0 \pm 3.5 | 138.0 \pm 4.0 | 208.0 \pm 6.7 | 40.0 \pm 1.7 |
| CLP | 72.0 \pm 12.3 ^a | 82.0 \pm 6.7 ^b | 240.0 \pm 10.5 | 24.0 \pm 5.0 ^a |
| Da (100 mg/Kg b.w) | 85.0 \pm 5.3 ^c | 111.0 \pm 6.5 ^d | 229.0 \pm 5.7 | 29.0 \pm 3.0 |
| Da (200 mg/Kg b.w) | 109.0 \pm 3.3 ^d | 122.0 \pm 4.5 ^c | 221.0 \pm 4.0 | 32.0 \pm 3.5 |

*mean \pm SEM values ($n = 6$); one-way ANOVA with Student-Newman-Keuls *post hoc test* was used for wise pair comparison where ^a $P < 0.05$, ^b $P < 0.001$ vs normal control group; ^c $P < 0.05$, ^d $P < 0.001$ compared with CLP group.

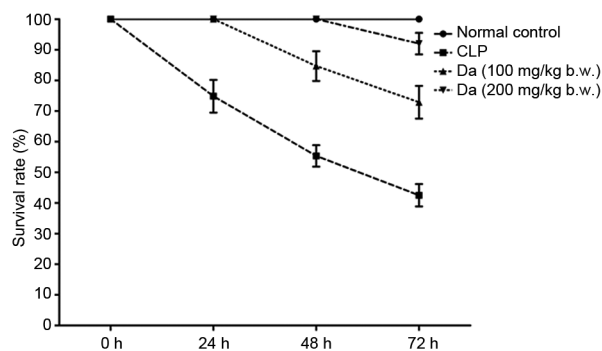


Fig. 2 — Effect of hydroalcoholic extract of *D. alpestre* (Da) on survival rate after 72 h (n=6).

hemodynamic stability is essential in the management of sepsis⁸. Administration of Da to septic mice increased mean arterial and mean blood pressure (Table 2). Therefore, the administration of medications extracted from *D. alpestre* might be useful for the treatment of sepsis.

MPO, the major enzyme in azurophilic granules of neutrophils, is a marker of inflammation initiation in plasma. Thus, increased MPO activity indicates the onset of the inflammatory response and neutrophil infiltration due to the induction of microbial sepsis³⁶. Our results showed that MPO activity increased in the serum of animals with CLP-induced poly-microbial sepsis in comparison to normal animals. Administration of Da decreased MPO activity, which shows the anti-inflammatory effect of *D. alpestre* when compared to CLP group (Fig. 1). Evidence related to the pro-inflammatory activities of *D. alpestre* is poor. Also, the present study demonstrated a reduction in survival rate in CLP-induced sepsis in mice (Fig. 2). The reason for high mortality rates in sepsis is the excessive release of cytokines, which results in a hyper-inflammatory state.

Conclusion

To conclude, the results of the present study is the first report of orchid *D. alpestre*, as a double edge sword in the treatment of CLP-induced sepsis in mice. The key phytochemicals responsible for this activity claimed to be alkaloids, flavonoids, polyphenols, and tannins. The results provide evidence that supports the Ayurvedic uses of *D. alpestre*. Also, these findings suggest that orchid *D. alpestre* can take an account as a good natural source of remedial medicine for sepsis. Hence, the results of the current study remain useful for further research to identify the potential bioactive molecules from *D. alpestre*.

Conflict of interest

No conflict of interest between any of the authors.

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