Pharmaceutical Sciences, 2021, 27(2), 162-169
doi:10.34172/PS.2020.69
https://ps.tbzmed.ac.ir/

Research Article

The Effects of Hydroalcoholic Extract of *Arum orientale* on CLP-Induced Sepsis in Rats

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Article Info

Article History:
Received: 12 May 2020
Accepted: 25 August 2020
ePublished: 2 December 2020

Keywords:
-*Arum*
-Anti-bacterial agent
-Sepsis
-Hemodynamics,
-Myeloperoxidase

Abstract

Background: Sepsis is a lethal clinical syndrome that results from dysregulated systemic inflammatory response of the body due to the invasion of pathogens, especially bacteria. Despite advances in medical care and therapy, sepsis is still one of the major causes of death in intensive care units and no decisive medical treatment is available against that. Studies have suggested that some *Arum* species have anti-bacterial properties. The present study investigated the effects of hydroalcoholic extract of *Arum orientale,* on cecal ligation and puncture (CLP) induced sepsis in rats.

Methods: CLP method was used for induction of sepsis in rats. Hydroalcoholic extract of *A. orientale* was injected intraperitoneally with doses of 80 and 640 mg/Kg body weight at times of 0, 1, 3, 6 and 24 h after the surgery. Antibacterial activity, hemodynamic parameters, myeloperoxidase (MPO) activity and survival rate were measured after 72 h.

Results: Hydroalcoholic extract of *A. orientale* showed antibacterial activities as potent as gentamycin against *Escherichia coli.* Administration of the extract with a dose of 80 mg/Kg body weight increased significantly hemodynamic parameters such as mean arterial pressure ($p<0.05$) and decreased optical density (OD) ($p<0.05$) of blood. The extract also increased serum MPO activity ($p<0.01$) and reduced survival rate to 20%.

Conclusion: This study for the first time showed that hydroalcoholic extract of *A. orientale* acts as a double edge sword in the treatment of CLP-induced sepsis. This extract showed anti-bacterial properties and also improved hemodynamic parameters but decreased survival rate, that might be through pro-inflammatory effects.

Introduction

Sepsis is a severe and potentially life-threatening clinical syndrome that globally is the major cause of death from infection in intensive care units.[1,2] Generally, sepsis is defined as a dysregulated systemic inflammatory response to the invasion of pathogenic micro-organisms.[1] Bacteria are the most common cause of sepsis. *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) are the most common bacteria that cause sepsis. However, other pathogenic micro-organisms including fungi and viruses can also cause sepsis.[2,3] The complications of sepsis are varied and involves systemic inflammation, coagulation disorders, immune suppression, and organ dysfunction.[3,4] The cardiovascular system as an important organ system in the body, greatly influenced by sepsis. Severe sepsis causes cardiomyopathy and endothelial dysfunction which result from adverse effects of substances secreted from pathogens and host cells.[5,6] Sepsis also impairs neutrophil migration and its antimicrobial activity. Inadequate migration of neutrophils into the site of infection causes systemic spread of pathogens which result in high rates of mortality.[7]

The initial management of infection in the sepsis requires initiating appropriate and timely antibiotic therapy.[2,4] However, there is no specific therapy or drug against sepsis.[7] Hence, searching to find new medication for the

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management of sepsis is necessary.

Many of the available medicines are derived from herbs, and medicinal plants have long been used to treat various disorders. Species of *Arum* genus are flowering plants from Araceae family which distributed in Western Asia, Mediterranean region, Europe and Northern Africa.[8] One of these species is *A. orientale* that in North West of Iran including Aras district is known as ''Gajab" and its extract from aerial parts locally is used in the treatment of different bacterial infections. In spite of common use of arum species as herbal remedies, there is few studies regarding their chemical constituents.[9] As far, presence of some valuable phytochemical components such as alkaloids, glycosides (flavonoids, saponin and cyanogenic groups), lectins monoterpenes, polyphenols, sesquiterpenes, etc. have detected in different parts of *Arum* species.[9-11] It has been also reported that tubers of *A. orientale* contain glucomannans (A, B and C). Of these glucomannan B was predominant and mainly composed of D-glucose, D-mannose and traces of uronic acid.[12] Although there are no extensive studies with *Arum* species, researchers reported that some of these such as *A. maculatum* and *A. conophalloides* have antibacterial activities.[13-15] It has been also reported that *A. palaestinum* and *A. dioscoridis* have antioxidant effects.[9] It is worth to note, *A. orientale* has not been studied in Iran and only few studies are conducted on this plant hitherto in the world. In the present study, for the first-time phytochemical screening of *A. orientale* 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.

Materials and Methods

Plant extraction

The aerial part of *Arum orientale* was collected from the mountains of Aras district, West Azerbaijan, Iran in May 2017. Voucher samples were maintained for reference in the Herbarium of School of Pharmacy, Urmia University of Medical Sciences (No: HUPS-506). The entire plant materials were washed and kept under shade at room temperature till the whole parts became well dried followed by powdering using a grinder (Moulinex, France). Then 130 g of plant powder was dispersed in ethanol 70% (2.5L × 3) for 24 h at room temperature in order to perform maceration extraction. Whatman filter paper was used to filter the extract and eventually ethanol was evaporated by using a rotary evaporator (Heidolph, Germany), at 45°C under vacuum. The yield of extraction in maceration procedure was 17.3% w/w for hydroalcoholic *A. orientale* extract.

Qualitative phytochemical screening

Preliminary phytochemical analysis upon the hydroalcoholic *A. orientale* extract was performed according to the standard practical methods as had been noted by Harborne,[16] Sofowora,[17] and Trease and Evans.[18] Assays for presence of alkaloids, anthraquinones, coumarins, flavonoids, saponins, cardiac glycosides and tannins were conducted.

Assay for free radical scavenging activity

The *in vitro* antioxidant activity of the *A. orientale* hydroalcoholic extract was evaluated through scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals from the bleaching of the purple colored solution of DPPH, a routinely practiced method.[19,20] The stock concentration of the *A. orientale* extract (1mg/mL) followed by serial dilutions were prepared to achieve different concentrations of the extract. Same volume of the extract dilutions and DPPH solution 0.08% (W/V) were mixed and placed at room temperature for 30 min. Subsequent to incubation, absorbance of each solution was measured versus a blank sample at 517 nm (Shimadzu 2100 spectrophotometer, Japan). After all, inhibition percentages, R (%), of the DPPH free radicals by different concentrations of the extract were calculated as follows:

R (%) = 100 × [(absorbance of blank – absorbance of sample) / absorbance of blank].

Furthermore, RC_{50} value for the *in vitro* antioxidant activity of the *A. orientale* extract was calculated according to the linear regression of a plot where the abscissa represented extract concentrations and the ordinate represented inhibition percentages. It is of note to mention that the testing was carried out in triplicate over and above the same process was carried out for quercetin as the positive control.

Assay for total phenolic content

Total phenolic content of the *A. orientale* hydroalcoholic extract were established through Folin-Ciocalteu reagent.[21] This assay is based on electron transfers from phenolic to phosphomolybdic/phosphotungstic acid complexes, developing blue complexes in an alkaline medium that could be measured by spectrophotometer at 760 nm.[22] Initially, five hundred microliters of the extract was mixed with 5 mL of Folin-Ciocalteu reagent 10% (v/v) in distilled water diluted and 4 mL of sodium bicarbonate solution (1M). After 15 min of incubation time for the mixture at room temperature, absorbance of the produced blue color was read by spectrophotometer. Ultimately, the total phenolic content of the *A. orientale* hydroalcoholic extract was calculated from the calibration curve of Gallic acid and expressed as Gallic acid equivalent, respectively.

Antibacterial activity measurements

In vitro antimicrobial activity of the extract was performed by Agar disk-diffusion testing method that is the most common method for routine evaluation of primary antimicrobial screening tests. The procedure was performed according to NCCLS (National Committee for Clinical Laboratory Standards) methodology.

The test bacteria used in this study were from American Type Culture Collection including *E. coli* (AT CC 8739) and *S. aureus* (ATCC 6538) that were purchased from Iranian Biological Resource Center and Iranian Research

Organization for Science and Technology respectively. Gentamycin was obtained from Darou Pakhsh Pharmaceutical MFG Co. (Iran). Mueller Hinton agar plates inoculated with 0.5 McFarland standards of mentioned bacteria were used for this assessment. Sterile filter paper discs were impregnated with *A. orientale* extract (80 and 640 mg/mL) and placed on the top of Mueller-Hilton agar plates.[23,24] Paper discs loaded with Gentamycin 10 mg/mL were used as positive control. After 48 h incubation at 37°C, inhibition zones were measured by caliper.

Animals

Adult male Wistar rats (weighting 250 ± 10 mg, age 8-10 weeks) were used in this study. The animals were given food and water *ad libitum* and were housed in the Animal House of Urmia University of Medical Sciences under standard condition with temperature of 21±2 °C, relative humidity of 50±10% and a 12-h light/12-h dark cycle. This study was performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and was approved by the Ethics Committee of Urmia University of Medical sciences (code: IR.UMSU.REC.1395.394).

Experimental design

At the beginning of the experiment, rats were randomly divided into 4 groups (10 rat each group). Rats in group 1 (sham), underwent midline abdominal incision without CLP. Rats in group 2 (CLP), underwent midline abdominal incision with cecal ligation (50%) and puncture to induce polymicrobial sepsis. Rats in groups 3 and 4 received 80 mg/Kg body weight (as a low dose) and 640 mg/Kg body weight (as a high dose) of *A. orientale* extract intraperitoneally at 0, 1, 3, 6 and 24 h after CLP operation. Regarding the dose selection, since there was no study with this plant, we conducted a large pilot study to get the toxic dose of *A. orientale* hydroalcoholic extract in animals. Besides, we performed a project to study the effects of *A. orientale* in myocardial infarction (MI) (unpublished data). Therefore, according to the mentioned studies, we chose low and high doses to investigate the possible effects of this plant on sepsis.

CLP surgery

CLP model was used for the induction of sepsis. Briefly, rats were anesthetized by intraperitoneal (*i.p*) injection of ketamine (60 mg/Kg body weight) and xylazine (10 mg/Kg body weight). Then, abdominal region of animals was shaved and sterilized by betadine. The cecum was exposed through a midline abdominal incision and ligated (50 %) with 3.0 silk suture 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, rats received 3 mL warm 0.9% normal saline subcutaneously (*s.c*) for fluid resuscitation. After rats recovered from anesthesia, they had free access to food and water.[25]

Sampling

Blood samples were obtained from portal vein. 0.5 mL of blood samples were transferred into laboratory tubes containing pre-autoclaved nutrient broth medium (Sigma-Aldrich, Germany) and put in incubator at 37°C. The remaining of blood samples, decanted gently into collection plastic tubes, then centrifuged at 3000 rpm for 5 min. Then serum was obtained, aliquoted into micro tubes and stored at -20°C for biochemical analysis.

Animal survival rate

In addition to monitoring the animals during three days, animals' survival rate was reported after 72 h.[26,27]

Hemodynamic Parameters

72 h after the surgery, the animals were anesthetized by an *ip* injection of a mixture of ketamine (60 mg/Kg body weight), xylazine (10 mg/Kg body weight), and when the rats no longer responded to external stimuli a standard limb lead II ECG (Powerlab system; AD Instruments, Australia) was recorded and the changes in ECG pattern were examined. 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 pressure transducer that prefilled with heparinized normal saline solution was cannulated into the right common carotid artery. All parameters were continuously recorded using a Power lab system (AD Instruments, Australia).[28]

MPO measurement

The activity of MPO, an abundant enzyme of neutrophils, was assessed as previously described[29] with minor modification. Briefly, 1 mL of the serum was mixed with 1 mg of hexadecyltrimethylammonium bromide (HTAB). Then sonicated for 5 min and centrifuged at 3000 rpm for 10 min at 4 ˚C 0.1 mL of supernatant was mixed with 2.9 mL of 50 mM phosphate buffer (pH 6.0), that 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 (Cecil 9000, UK).

Statistical analysis

All results were presented as mean±SEM and were analyzed using one-way analysis of variance (ANOVA) to make comparisons between the groups. If the ANOVA analysis indicated significant differences, a Student–Newman–Keuls *post-hoc test* was used for pair wise comparison. The level of significance was considered at $p<0.05$.

Results

Phytochemical analysis

Results of the preliminary phytochemical screenings have been represented in Table 1. *A. orientale* hydroalcoholic extract possesses different sorts of phytochemicals including alkaloids, polyphenols, flavonoids, tannins, and saponins among the tested class of compounds. Besides, anthraquinones, coumarins and cardiac glycosides were found to be absent in the extract.

Considering results of the *in vitro* free radical scavenging activity of *A. orientale* extract, calculated RC_{50} value for extract and standard of quercetin, were established as 869.1±5.3 µg/mL and 4.5±0.6 µg/mL, respectively (Table 1). Based on our findings, *A. orientale* extract compared to the standard of quercetin revealed fairly negligible *in vitro* antioxidant activity. Additionally, total phenolic content was ascertained via absorbance of the plant extract and the equation obtained from the standard curve of Gallic acid through Folin-Ciocalteu method. As a consequence, the total phenolic value for hydroalcoholic extract of *A. orientale* was equivalent to 51.6±0.8 mg of Gallic acid per 100 g of dried plant material (Table 1).

Antibacterial activity of hydroalcoholic extract of *A. orientale*

The results of *in vitro* antimicrobial activity of the extract revealed that plant extract has antibacterial activity against both selected bacteria (*E. coli* and *S. aureus*). As shown in Figure 1 and Table 2, the plant extract shows an inhibitory effect against *E. coli* and *S. aureus*. As shown in Figure 2, optical density of blood (OD) significantly ($p<0.01$) increased in CLP group compared with sham group. In

Table 1. Phytochemical analysis, DPPH radical scavenging capacity and total phenolics content of *A. orientale* hydroalcoholic extract.

Alkaloids	Anthraquinones	Coumarins	Flavonoids	Saponins	Tannins	Cardiac glycosides	Total phenols[c]	DPPH (RC_{50}[d])
+[a]	-[b]	-	+	+	+	-	51.6 ± 0.8 mg/g	869.1 ± 5.3 µg/mL

a) +: detected content
b) -: not detected content
c) Mean ± S.D.: Standard deviation (n=3)
d) RC_{50}: Concentration of the extract that can inhibit 50% of DPPH scavenging activity

Table 2. Antibacterial screening test of A. orientale hydroalcoholic extract and positive control as inhibition zone diameters (mm±SD).

Tested agent	Inhibition zones (mm)	
	E. coli	*S. aureus*
Extract 80 mg/mL	14.6 ± 0.5	16.6 ± 0.5
Extract 640 mg/mL	16.3 ± 0.5	17.6 ± 0.5
Gentamycin (10 mg/mL)	16.3 ± 0.5	19.3 ± 0.5

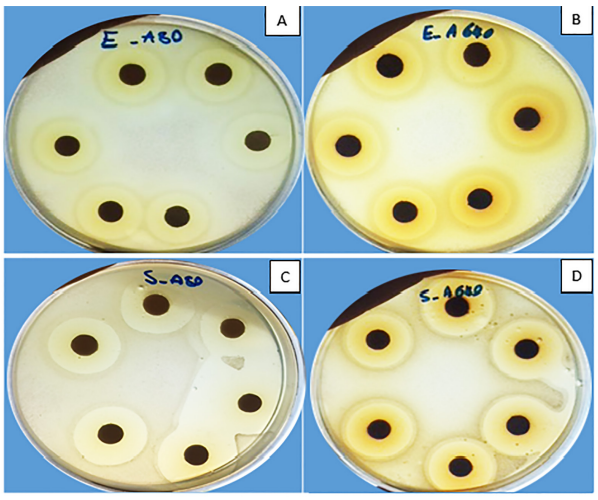

Figure 1. Growth inhibition of *E.coli* (A and B) and *S. aureus* (C and D) caused by *A. orientale* hydroalcoholic extract (80 and 640 mg/mL respectively).

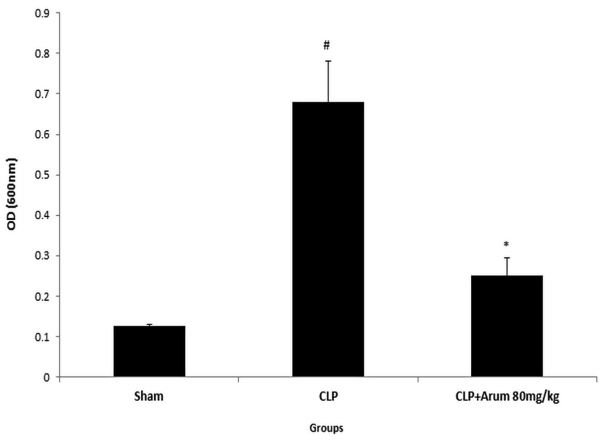

Figure 2. The effect of hydroalcoholic extract of A. orientale on OD at 600 nm. Values are mean±SEM (n=6). #p<0.01, as compared with sham group; *p<0.05, as compared with CLP group using one-way ANOVA with Student-Newman-Keuls post hoc test. CLP: Cecal ligation and puncture; Arum: A. orientale

other hand, administration of *A. orientale* to the septic rats significantly ($p<0.05$) decreased OD in the blood of animals compared with CLP group.

The effect of hydroalcoholic extract of A. orientale on the electrocardiogram and hemodynamic responses in CLP-induced sepsis

The electrocardiogram showed normal pattern in the sham group. CLP group demonstrated distorted and abnormal pattern. In addition, it can be seen that treatment with 80 mg/Kg body weight of *A. orientale* amended pattern of ECG near to normal (Figure 3). It was observed that mean arterial pressure (MAP) was significantly decreased from 121±8 mmHg in sham group to 72±15 mmHg in the CLP group ($p<0.05$). There was a significant increase ($p<0.05$) in the mean arterial blood pressure (MAP) of rats treated with *A. orientale* 80 mg/Kg body weight to 107±6 mmHg. It was found that arterial blood pressure (ABP) from 140±3 mmHg in the sham group decreased to 89±7 mmHg ($p<0.001$) in the CLP group. Treatment with *A. orientale* 80 mg/Kg body weight increased significantly the arterial blood pressure ($p<0.001$). Developed pressure (DP) decreased and HR increased significantly ($p<0.05$) in CLP group and *A. orientale* treated group showed no significant change (Table 3).

The effect of hydroalcoholic extract of A. orientale on serum MPO activity in CLP-induced sepsis

Animals in CLP group showed a significant ($p<0.05$) increase in MPO activity compared with sham group. The treatment of rats with *A. orientale* extract increased markedly ($p<0.01$) the enzyme activity compared with CLP group (Figure 4).

The effect of hydroalcoholic extract of A. orientale on survival rate in CLP-induced sepsis

To examine the effects of *A. orientale* on survival rates, the animals were monitored during 72 h after CLP surgery. There was no death of rats in the sham group after 72 h and survival rate was 100 %. Survival rate decreased in CLP group to 47% compared with sham group (100%). Treatment of septic rats with *A. orientale* extract with doses

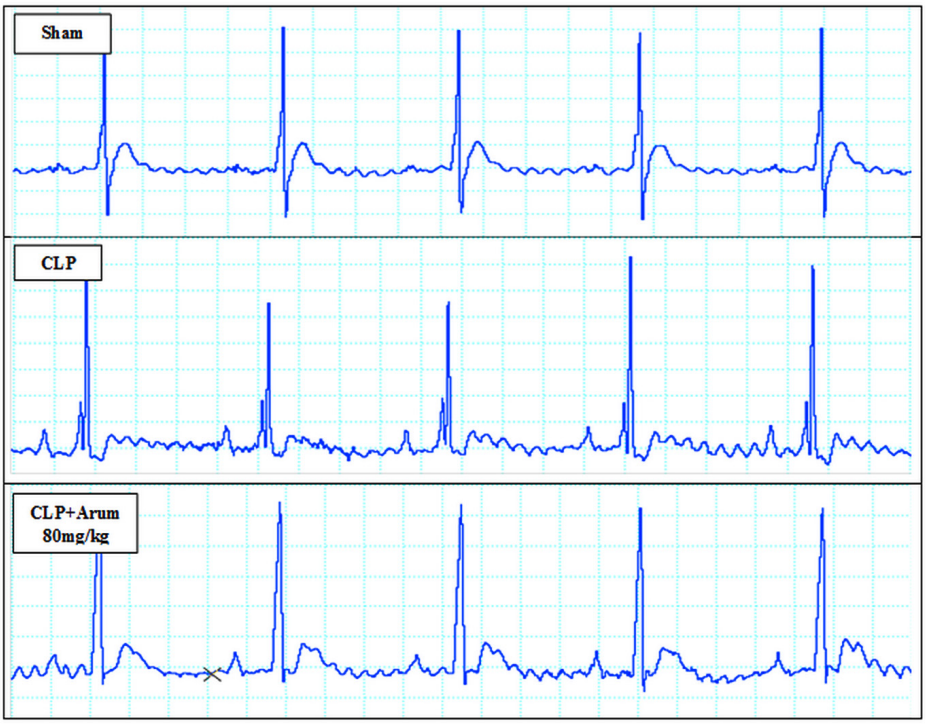

Figure 3. Representative of ECG pattern and changes (recorded from limb lead II) in sham, CLP and CLP+Arum 80mg/Kg body weight groups CLP: Cecal ligation and puncture; Arum: *A. orientale*. (n = 10).

Table 3. Effects of hydroalcoholic extract of *A. orientale* on hemodynamic parameters in CLP-induced sepsis after 72 h.

Group	Mean arterial pressure (mm Hg)	Arterial blood pressure (mm Hg)	Heart rate (bpm)	Developed pressure (mm Hg)
Sham	121±8	140±3	207±10	38±1
CLP	72±15#	89±7a	236±7*	27±4#
Arum 80 mg/Kg body weight	107±6*	121±4***	227±5	28±3

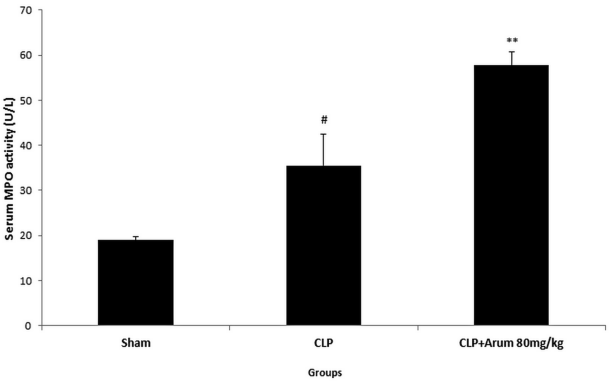

Figure 4. The effect of hydroalcoholic extract of *A. orientale* on MPO activity. Values are mean±SEM (n=6). *#p<0.05*, as compared with sham group; ***p<0.01*, as compared with CLP group using one-way ANOVA with Student-Newman-Keuls *post hoc test*. CLP: Cecal ligation and puncture; Arum: *A. orientale*

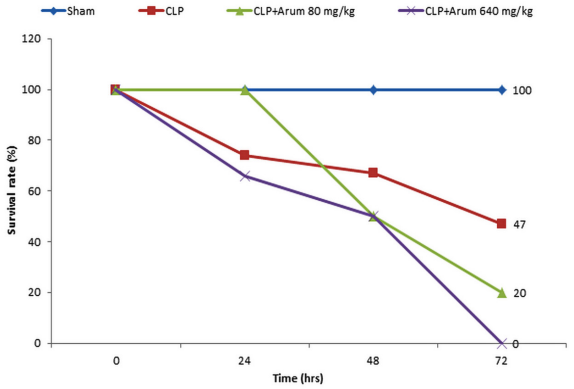

Figure 5. The effect of hydroalcoholic extract of *A. orientale* on survival rate after 72 h. (n=10). CLP: Cecal ligation and puncture; Arum: *A. orientale*

of 80 and 640 mg/Kg body weight decreased survival rate to 20% and 0 in comparison with CLP group respectively (Figure 5)

Discussion

In the present study, we showed that hydroalcoholic extract of *A. orientale* improves hemodynamic parameters and shows antibacterial activity. In addition, the extract unexpectedly increased the inflammatory response and mortality rate in rats with polymicrobial sepsis. To ensure, we repeated the study twice and obtained the same results on mortality rate. Besides, preliminary phytochemical screenings showed that hydroalcoholic extract of *A. orientale* contains alkaloids, polyphenols, flavonoids, tannins, and saponins. Compounds including anthraquinones, coumarins and cardiac glycosides were not found in the extract.

Our results showed that hydroalcoholic extract *of A. orientale* has antibacterial activity against *E. coli* and *S. aureus*. As these bacteria are predominant cause of sepsis,[3] this edible flower can have beneficial effects against sepsis. In line with our results, other studies have reported antibacterial effects of other arum species.[13-15] The antibacterial activity of *A. orientale* might be related to its phytochemical contents including alkaloids, flavonoids, phenolic acids, saponins, and tannins because the antibacterial activities of these compounds have been reported by several studies.[30-32]

As mentioned, sepsis causes cardiac and endothelial dysfunction. Hemodynamic monitoring in this study showed attenuation of hemodynamic parameters in 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, hemodynamic stability is essential in the management of sepsis.[5] Administration of hydroalcoholic extract of *A. orientale* to septic rats increased mean arterial and mean blood pressure. Therefore, administration of medications-extracted from this plant might be useful for treatment of sepsis.

MPO, the major enzyme in azurophilic granules of neutrophils,[33] is a marker of inflammation initiation in plasma.[34] Thus, increased MPO activity indicates the onset of inflammatory response and neutrophil infiltration due to induction of microbial sepsis. Our results showed that MPO activity increased in the serum of animals with CLP-induced poly-microbial sepsis in comparison to normal animals, which is similar to previous studies.[26,27,35] Reports regarding *A. orientale* uses for remedial purposes are contradictory. In addition to the therapeutic effects, toxic effects of this plant have also been reported.[9] In the present study, administration of *A. orientale* extract increased MPO activity which shows pro-inflammatory effect of this extract. Evidences related to pro-inflammatory activities of *Arum* species is poor. Alencar *et al.*[36] revealed that an isolated lectin from *A. maculatum* enhanced neutrophil migration both *in vivo* and *in vitro*. Plant lectins have immunomodulatory and pro-inflammatory activities[37] that can cause inflammatory disorders via activation of NLRP3 inflammasome.[38] In addition to lectins, the toxicity and pro-inflammatory effects of *Arum* spp. can be attributed to the presence of raphides, alkaloids and cyanogenic glycosides.[9,39] The present study, in agreement with previous studies demonstrated a reduction in survival rate in CLP-induced sepsis in rats.[26,27,35] The reason of high mortality rates in sepsis is excessive release of cytokines which results in hyper-inflammatory state.[40]

Considering all this, unexpectedly administration of *A. orientale* decreased survival rate. To ensure, we repeated the study twice and obtained the same results on mortality rate. We believe that it could be due to some toxic substances of this plant that needs further studies. *Arum* plants contain calcium oxalate, cyanoglycosides, alkaloids and saponins which are toxicant.[10,41] Phytochemical analysis in this study indicated that *A. orientale* extract possesses alkaloids and saponins. Studies have reported toxic effects of some *Arum* species in liver,[42-44] which confirmed that *Arum* genus can be poisonous.

Conclusion

Our results for the first time showed that *A. orientale*, as a herbal plant, is a double edge sword in the treatment of CLP-induced sepsis. This plant improved hemodynamic parameters and showed antibacterial effect. *A. orientale*, on the other hand, increases MPO activity and reduces survival rate. Therefore, more caution is needed in the use of traditional medicines. Considering all aspects, we believe that this plant deserves more attention.

Acknowledgements

The present study was supported by a grant from the Research Vice Chancellors of Urmia University of Medical Sciences, Urmia, Iran.

Ethical Issues

This study was approved by the Ethics Committee of Urmia University of Medical sciences (code: IR.UMSU.REC.1395.394).

Author Contributions

SS: Carried out animal grouping, handling and sepsis experiments. MK: Contributed in data acquisition and drafting the work. SH and NM: Performed plant extraction and phytochemical screening. FG: Carried out microbial tests. HS: Supervising and directing the project, carried out the data analysis and interpretations and prepared the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

Dr. Sanaz Hamedeyazdan is the associate editor of Pharmaceutical Sciences. She did not involve in peer review process of the submission. The other authors declare no conflict of interest.

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