Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Inhibition of Oxidative Stress and Autophagy by Arbutin in Lipopolysaccharide-Induced Myocardial Injury

Beibei Zhang[1,2], Mengnan Zeng[1,2], Benke Li[1,2], Yangyang Wang[1,2], Yuxuan Kan[1,2], Shengchao Wang[1,2], Yijia Meng[1,2], Jiji Gao[1,2], Weisheng Feng[1,2], Xiaoke Zheng[1,2]

[1]College of Pharmacy, Henan University of Chinese Medicine, [2]Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment and Chinese Medicine Development of Henan Province, Zhengzhou, China

Submitted: 29-10-2018 Revised: 27-11-2018 Published: 16-05-2019

ABSTRACT

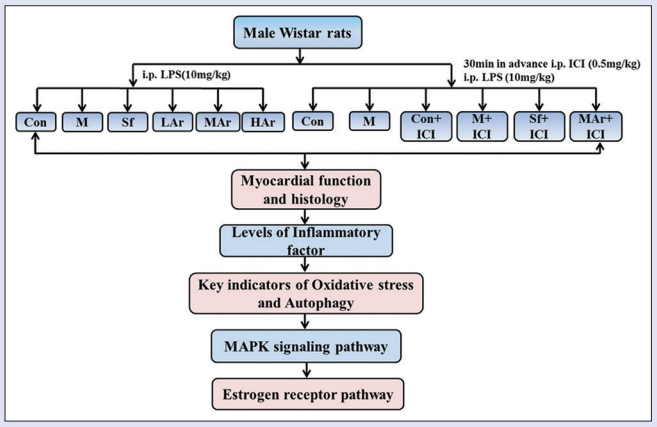
Background: Sepsis is a syndrome characterized by a systemic inflammatory response. Arbutin (Ar) is an active natural product known for its bactericidal and anti-inflammatory effects. Many studies have reported the diverse pharmacological actions of Ar, but there is no relevant research on the effect of Ar on lipopolysaccharide (LPS)-induced myocardial injury. **Objective:** The purpose of this study was to investigate the effect of Ar on LPS-induced myocardial injury and its underlying mechanisms. **Materials and Methods:** The levels of tumor necrosis factor alpha, interleukin 6, cardiac troponin-I, and procalcitonin were detected by ELISA. The levels of phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated extracellular regulated protein kinase (p-ERK), and phosphorylated p38 (p-p38) proteins were detected by flow cytometry using Cytometric Bead Array. Western blot was used to detect the expression of autophagy-related and estrogen receptor (ER)-associated proteins. Levels of the oxidative stress-related markers were detected by the cuvette assay. **Results:** The levels of the inflammatory factors, LC3B, malondialdehyde, p-JNK, and p-p38 were increased in LPS-treated rats, while the ERK, total superoxide dismutase, glutathione peroxidase, p62, and ER-associated proteins were decreased. These effects could be effectively reversed by Ar, which could be blocked by ER antagonist ICI182780. Our previous study found Ar to possess an estrogen-like activity. **Conclusion:** Ar inhibits the oxidative stress and autophagy and offers protection from the LPS-induced myocardial injury via the ER pathway.

Key words: Arbutin, autophagy, estrogen receptor, lipopolysaccharide, oxidative stress, sepsis

SUMMARY

- The purpose of this study was to investigate the effect of arbutin (Ar) on the lipopolysaccharide-induced cardiomyopathy and its underlying mechanisms. It was found that Ar exhibited a protective effect in the rats with septic cardiomyopathy by inhibiting the oxidative stress and autophagy via estrogen receptor pathway.

Abbreviations used: Ar: Arbutin; LPS: lipopolysaccharide; Sf: Soybean isoflavone; TNFα: Tumor necrosis factor α; IL-6: Interleukin 6; CTnI: Cardiac troponin-I; PCT: Procalcitonin; p-JNK: Phosphorylated c-Jun N-terminal kinase; p-ERK: Phosphorylated exeracellular regulated protein kinase; p-p38: Phosphorylated p38; ER: Estrogen receptor; MDA: Malondialdehyde; T-SOD: Total superoxide dismutase; GSH-Px: Glutathione peroxidase; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular shortening rate; ROS: Reactive oxygen species; Con: Control; M: LPS (10 mg/kg); Sf: Sf + LPS (Sf 30 mg/kg, LPS 10 mg/kg); LAr: Ar low + LPS (Ar 25 mg/kg, LPS 10 mg/kg); MAr: Ar medium + LPS (Ar 50 mg/kg, LPS 10 mg/kg); HAr: Ar high + LPS (Ar 100 mg/kg, LPS 10 mg/kg); Con + ICI: control + ICI182780 (0.5 mg/kg); Sf + ICI: Sf + LPS + ICI; MAr + ICI: MAr + LPS + ICI; CBA: Cytometric Bead Array; MAPK: Mitogen-activated protein kinase.



Correspondence:

Prof. Xiaoke Zheng,
Henan University of Chinese Medicine, Zhengzhou, China.
Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment and Chinese Medicine Development of Henan Province, Zhengzhou, China.
E-mail: zhengxk.2006@163.com
DOI: 10.4103/pm.pm_552_18

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Sepsis is a clinical syndrome characterized by a severe systemic inflammatory response. Severe sepsis induces multiple organ dysfunctions and has a very high mortality rate.[1-3] Every year, more than 19 million people develop sepsis, which is defined as a life-threatening acute organ dysfunction that is secondary to an infection.[4] The underlying pathogenesis of sepsis is unclear and involves complex systemic inflammatory network effects, genetic polymorphisms, immune dysfunction, coagulopathy, tissue damage, and abnormal response to different infectious pathogenic microorganisms.[5-7] Sepsis and related complications have a high morbidity and mortality, with mortality rates ranging from 30% to 50%.[8] More and more research is on sepsis because of its high morbidity and mortality. The cardiovascular system

Cite this article as: Zhang B, Zeng M, Li B, Wang Y, Kan Y, Wang S, *et al*. Inhibition of oxidative stress and autophagy by arbutin in lipopolysaccharide-induced myocardial injury. Phcog Mag 2019;15:507-13.

is one of the important target organs for sepsis-induced multiple organ dysfunction.[9] Myocardial dysfunction and impaired cardiac function, being the key features of sepsis, have a mortality rate of up to 70% in sepsis patients with myocardial dysfunction.[10,11] Studies have shown that myocardial dysfunction in patients with sepsis is mainly characterized by a left ventricular shortening (LVFS) rate and left ventricular ejection fraction (LVEF).[12] Sepsis is a complex pathophysiological state whose complications are caused due to the host's strong reaction to an infection, leaving the body in an inflammatory state, releasing pro-inflammatory cytokines and oxidative stress to release the active oxygen (ROS).[8,13] There are many mechanisms involved in the pathogenesis of sepsis, such as activation of inflammation process, immune response, and bioenergy response. One of the key factors is increase of the causative substances accompanied with the failure of the antioxidant system, which leads to an irreversible oxidative stress.[14] The normal process of autophagy is very important in the smooth progress of the life activities of an organism, which is the pro-survival pathway during cellular stress of the heart.[8] Recent evidence shows that autophagy has a protective effect in sepsis.[15] Studies have reported that downregulation of autophagy can lead to a systemic inflammation after sepsis and its recovery is a potential treatment strategy for sepsis.[16] However, excessive autophagy occurring *in vivo* can cause a serious damage to myocardial tissue.

Arbutin (Ar) is a naturally occurring phytochemical with anti-irritant, antiseptic, anti-inflammatory, and other pharmacological effects. In our earlier work, Ar was isolated from Yam and known to have estrogenic activity.[17] The present work envisaged to study the protective potential of Ar in sepsis-mediated cardiomyopathy and whether this effect correlates with its estrogenic activity. Soybean isoflavones (Sfs), a class of soy plant estrogens, are known to possess anti-cancer activity, whose effect is believed to be achieved by reducing the synthesis of estrogen and changing genotoxic metabolites toward inactive metabolites.[18] Hence, it was used as a control in our experiment.

MATERIALS AND METHODS

Materials

Ar and lipopolysaccharide (LPS) (Sigma, St Louis, MO, USA), Sf (Zbd Pharmaceutical Co., Ltd. Heilongjiang, China), and ICI182780 (Tocris, Bristol, UK) were procured from their respective suppliers. Microplate reader and semi-dry transfer film (Bio-Rad 680); high-speed cryogenic centrifuge (Thermo Fisher Scientific), flow cytometry (FACSAria β, BD, USA); and ODYSSEY (Clx, Li-COR, USA) were employed in the study.

Animals

The experiment was conducted in accordance with the regulations for the experimental animal management promulgated by the National Science and Technology Commission of the People's Republic of China. Male Wistar rats (200 ± 20 g, n = 90) were purchased and procured from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (SCXK2016-0011) and adapt to the environment for a week with provision of regular food and water every day. According to the principle of random distribution, they were divided into six groups: control (Con), LPS (M, 10 mg/kg), Sf + LPS (Sf, Sf 30 mg/kg, LPS 10 mg/kg), Ar low (LAr) + LPS (LAr, Ar 25 mg/kg, LPS 10 mg/kg), Ar medium (MAr) + LPS (MAr, Ar 50 mg/kg, LPS 10 mg/kg), and Ar high (HAr) + LPS (HAr, Ar 100 mg/kg, LPS 10 mg/kg). All rats were injected intraperitoneally with LPS, except for the normal group, which were injected intraperitoneally with normal saline. Three hours later, the Sf, LAr, Mar, and HAr groups were intragastrically administered with Sf solution, low, medium, and high concentrations of Ar solution, respectively; Con and M were administered distilled water at the same time by gavage, twice a day for 3 days. Twenty-four hours after the last administration, chloral hydrate was administered intraperitoneally for anesthesia. The cardiac function was measured, and organs were carefully immediately removed, weighed, and stored in liquid nitrogen. The blood obtained from the abdominal aorta was centrifuged at 3000 rpm for 10 min to obtain the supernatant and stored at − 80°C for inspection. The organ index is equal to the wet organ weight divided by body weight times 100%.

In another experiment, rats were divided into groups as follows: control, control + ICI182780 (Con + ICI, 0.5 mg/kg), LPS, LPS + ICI, Sf + LPS + ICI (Sf + ICI), and a group with MAr + LPS + ICI (MAr + ICI).

Western blot analysis

Total protein from heart tissue was extracted according to the instructions of the mammalian protein extraction kit (Beijing ComWin Biotech Co., Ltd., Beijing, China) and quantified by the Bradford protein assay kit (Wuhan Boster Biological Technology., Ltd., Wuhan, China). Protein (40 μg) was loaded per protein sample and separated by SDS-PAGE gel. Then, nonfat milk was used to block the membrane for 1½ h, followed by addition of the primary antibody: estrogen receptor alpha (ERα) (ab75635, Abcam, Massachusetts, US), estrogen receptor beta (ERβ) (ab3576, Abcam, Massachusetts, US), p62 (ab56416, Abcam, Massachusetts, US), LC3B (ab48394, Abcam, Massachusetts, US), and GAPDH (AC033, ABclonal Biotechnology Co., Ltd.) overnight for incubation at 4°C and washed five times with PBST for 5 min each time. Then, secondary antibody (goat anti-rabbit 925-68071, goat anti-mouse 925-32210, Li-COR, MO, USA) was added, incubated for 1 h in the dark, and washed four times with PBST for 5 min each time and finally with PBS. The expression of the proteins was quantified by ODYSSEY (Clx, Li-COR, USA).

Cytometric bead array

The content of phosphorylated c-Jun N-terminal kinase (p-JNK) (7170701, BD), phosphorylated extracellular regulated protein kinase (p-ERK) 1/2 (7205548, BD), phosphorylated p38 (p-p38) (7202572, BD) in rats was detected by the Cytometric Bead Array according to the manufacturer's instructions. Initially, the different concentrations of standard products were formulated according to the respective preparation, and a standard curve was plotted. Subsequently, 50 mg of heart tissue was weighed and subjected to 10 times mixture of prechilled PBS, phosphoprotease inhibitor, and five times denaturation solution (800:1:200). After crushing by a homogenizer and incubation with the lysate treatment reagent (6299995, BD Biosciences, New York, USA) on ice, the mixture was centrifuged and the supernatant was taken to complete the protein denaturation at 95°C for 5 min. Fifty microliters was taken as the sample to be tested, to which 50 μl microspheres were added and incubated at room temperature in the dark for 3 h. Then, 50 μl antibody was added and incubated for 1 h at room temperature in the dark; 1 ml washing solution was added and centrifuged followed by gently discarding the supernatant. The resulting suspension was re-suspended in 500 μl buffer, and finally, protein content was measured by flow cytometry and the results were analyzed using the Cell Quest software (BD).

ELISA analysis

Plasma was collected and used to detect the levels of interleukin 6 (IL-6) (R180109-003a, NeoBioscience Co., Ltd., Shenzhen, China), tumor necrosis factor alpha (TNF-α) (R180109-102a, NeoBioscience Co., Ltd., Shenzhen, China), procalcitonin (PCT, CSB-E13419r, CUSABIO BIOTECH Co., Ltd, Wuhan, China), CRP (3007961, EMD Millipore Corporation, Billerica, Massachusetts, USA), and cardiac troponin-I (CTnI, 2HSPA1018, Life Diagnostics, Inc., West Chester, PA, USA) according to the manufacturer's instructions.

Colorimetric method

Fifty milligrams of heart tissue was weighed and nine times precooled physiological saline was added to it. Subsequently, the tissue was cut into pieces and homogenized by the homogenizer. The tissue homogenate was centrifuged and supernatant was used to detect the levels of malondialdehyde (MDA, A003-1, Nanjing Jiancheng Bioengineering Institute, China), glutathione peroxidase (GSH-Px, A005, Nanjing Jiancheng Bioengineering Institute, China), and total superoxide dismutase (T-SOD) (A001-1, Nanjing Jiancheng Bioengineering Institute, China) according to the instructions of the colorimetric method.

Data processing analysis

Analyses were performed using SPSS 20.0 (IBM, New York, NY, USA). Statistical significance of differences between groups was evaluated by one-way ANOVA with LSD's multiple comparison test. P <0.05 was accepted as statistically significant.

RESULTS

Effect of arbutin on cardiac function and organ index in lipopolysaccharide-induced myocardial injury

As shown in Table 1, the results of ultrasound scanner showed that LVEF and LVFS were significantly reduced in model rats. Results of the organ index experiment showed that the heart, kidney, and thymus index were significantly reduced in the LPS-induced cardiomyopathy rats, while the spleen index was increased, which could be relieved by Ar and Sf to varying degrees. The effects of Sf and Ar could be effectively blocked by ICI182780 effectively. The results are shown in Table 2. As shown in Figure 1, there were no significant pathological changes in the normal group; however, muscle gap broadening, a number of red blood cell aggregates, partial myocardial bundle breakage, and even structurally blurring were observed in the model group. These symptoms were effectively alleviated by Ar and Sf. It was observed that the effect of Ar and Sf on myocardial tissue in model rats could be blocked by ICI182780 effectively.

Effect of arbutin on inflammatory factors in lipopolysaccharide-induced myocardial injury

Table 3 indicates the significant increase in the inflammatory factors in the cardiomyopathic rats, which were reduced by Ar and Sf effectively. The effect of Ar on inflammatory cytokines was dose dependent, with middle and high doses of Ar exhibiting better alleviation effects. In addition, we found that the effects of Ar and Sf on CTnI, PCT, TNFα, and IL-6 could be blocked by ICI182780 in different degrees. The results are shown in Table 4.

Effect of arbutin on oxidative stress indices in lipopolysaccharide-induced myocardial injury

As shown in Table 5, the MDA levels in LPS-induced cardiomyopathy rats were significantly increased than the normal control group, while T-SOD and GSH-Px indicated an obvious decrease that could be significantly reversed by Ar and Sf. Moreover, the effects of Ar and Sf were significantly antagonized by ICI182780. The results are shown in Table 6.

Effect of arbutin on the levels of LC3B and p62 in lipopolysaccharide-induced myocardial injury

It was observed that the level of LC3B in model rats significantly increased, while p62 was significantly reduced compared with the normal group, which could be reversed by Ar and Sf. Moreover, the effects of LC3B and p62 could be blocked by ICI182780. The results are shown in Figure 2.

Effect of arbutin on mitogen-activated protein kinase signaling pathway in lipopolysaccharide-induced myocardial injury

The level of p-ERK1/2 was significantly reduced, while the p-JNK and p-p38 were significantly increased in the LPS-induced cardiomyopathy rats, which were notably reversed by Ar and Sf, but for p-ERK 1/2. The results are shown in Tables 7 and 8.

Table 1: Effect of arbutin on cardiac function and organ index in lipopolysaccharide-induced myocardial injury

	Heart	Liver	Kidney	Spleen	Thymus	LVEF	LVFS
Con	0.7238±0.0579*	7.362±0.365	1.802±0.159*	0.7012±0.0509**	0.4547±0.054**	81.14±3.65**	56.86±3.345**
M	0.6613±0.0250	7.413±0.459	1.757±0.134	1.1202±0.1428	0.1722±0.0406	69.01±7.81	40.77±2.06
Sf	0.7303±0.0567*	7.303±0.638	1.826±0.142*	0.8909±0.0723**	0.2357±0.0647**	78.46±7.64**	48.89±5.69**
LAr	0.7025±0.0272	6.956±0.596	1.757±0.142	0.8664±0.0276**	0.1652±0.0505	78.02±7.87**	52.91±2.78**
MAr	0.7142±0.0371	7.709±1.032	1.761±0.138	0.9457±0.1977**	0.1885±0.0221	80.72±4.09**	53.91±3.31**
HAr	0.7309±0.0392*	7.729±1.208	1.828±0.233*	1.0016±0.1579**	0.2021±0.0664*	82.84±4.73**	54.96±3.87**

Mean±SD, n=8 rats per group. *P<0.05, **P<0.01 compared with model group. LVFS: Left ventricular fractional shortening; LVEF: Left ventricular ejection fraction; Con: Control; M: LPS (10 mg/kg); Sf: Sf+LPS (Sf 30 mg/kg, LPS 10 mg/kg); LAr: Ar low+LPS (Ar 25 mg/kg, LPS 10 mg/kg); MAr: Ar medium+LPS (Ar 50 mg/kg, LPS 10 mg/kg); HAr: Ar high + LPS (Ar 100 mg/kg, LPS 10 mg/kg); LPS: Lipopolysaccharide; Ar: Arbutin; Sf: Soybean isoflavone; SD: Standard deviation

Table 2: Effect of arbutin on cardiac function and organ index in lipopolysaccharide-induced myocardial injury with addition of ICI182780

	Heart	Liver	Kidney	Spleen	Thymus	LVEF	LVFS
Con	0.7342±0.0549*	7.147±0.641	1.827±0.442*	0.8543±0.0342**	0.4872±0.0561**	84.59±3.65**	62.41±4.12**
Con + ICI	0.7188±0.0678*	7.275±0.432	1.812±0.359*	0.8621±0.0601**	0.4492±0.064**	85.48±3.65**	61.62±6.03**
M	0.6778±0.0641	7.412±0.608	1.765±0.451	1.1201±0.1427	0.2031±0.0564	73.92±6.89	45.28±3.02
M + ICI	0.6504±0.02370	7.428±0.901	1.743±0.634	1.1292±0.1471	0.1822±0.0626	74.61±8.29	47.30±3.41
Sf + ICI	0.6613±0.0614	7.603±0.468	1.782±0.341	1.0891±0.0729	0.2057±0.0647	76.41±3.71	47.17±5.78
MAr + ICI	0.6908±0.0391	7.502±0.935	1.769±0.398	1.0721±0.3177	0.1817±0.0351	77.41±3.77	48.54±6.36

Mean±SD, n=8 rats per group. *P<0.05, **P<0.01 compared with model group. Con: Control; M: LPS (10 mg/kg); Sf: Sf + LPS (Sf 30 mg/kg, LPS 10 mg/kg); MAr: Ar medium + LPS (Ar 50 mg/kg, LPS 10 mg/kg); Con + ICI: control + ICI182780 (0.5 mg/kg); LPS: Lipopolysaccharide; Ar: Arbutin; Sf: Soybean isoflavone; SD: Standard deviation

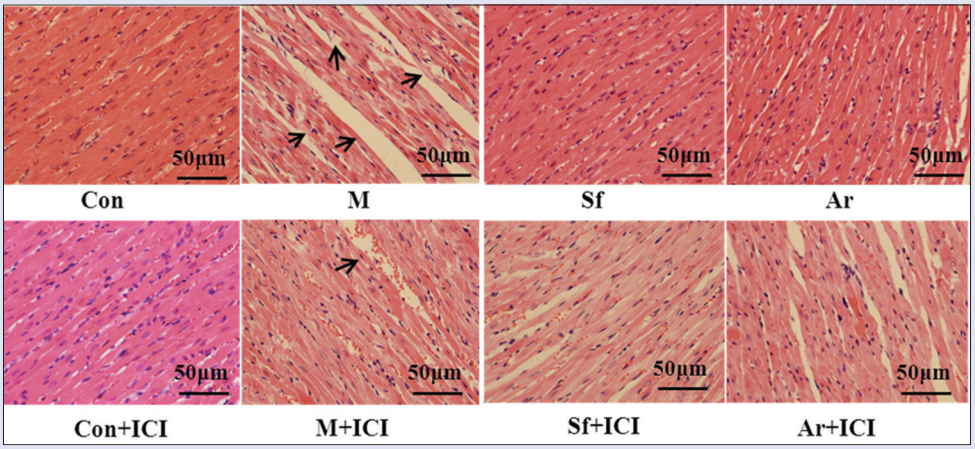


Figure 1: Representative histomorphometric images of formalin-fixed myocardial tissues from each group (H and E, ×400). Con: Control; M: Lipopolysaccharide (10 mg/kg); Sf: Soybean isoflavone + lipopolysaccharide (soybean isoflavone 30 mg/kg, lipopolysaccharide10 mg/kg), Con + ICI: control + ICI182780 (0.5 mg/kg); Sf + ICI: Soybean isoflavone + lipopolysaccharide + ICI182780

Table 3: Effects of arbutin on inflammatory factors in lipopolysaccharide-induced myocardial injury

	TNFα (pg/ml)	IL-6 (pg/mL)	CTnI (ng/ml)	PCT (ng/ml)
Con	351.54±51.21**	381.23±59.53**	23.01±2.13**	746.12±81.43**
M	479.55±26.18	593.12±69.57	31.06±2.04	1200.13±94.14
Sf	379.46±43.39**	498.37±47.39*	26.52±1.66*	837.14±84.59**
LAr	427.19±40.76	552.31±76.21	29.12±1.69*	1160.45±107.76
MAr	401.25±33.34*	513.64±47.83*	26.21±1.66*	960.1±84.31*
HAr	364.25±43.28**	473.12±54.21**	24.23±1.65**	850.9±94.37**

Mean±SD, n=8 rats per group. *P<0.05, **P<0.01 compared with model group. TNFα: Tumor necrosis factor α; IL: Interleukin; CTnI: Cardiac troponin-I; PCT: Procalcitonin; Con: Control; M: LPS (10 mg/kg); Sf: Sf + LPS (Sf 30 mg/kg, LPS 10 mg/kg); LAr: Ar low + LPS (Ar 25 mg/kg, LPS 10 mg/kg); MAr: Ar medium + LPS (Ar 50 mg/kg, LPS 10 mg/kg); HAr: Ar high + LPS (Ar 100 mg/kg, LPS 10 mg/kg); LPS: Lipopolysaccharide; Ar: Arbutin; Sf: Soybean isoflavone; SD: Standard deviation

Table 4: Effect of arbutin on inflammatory factors in lipopolysaccharide-induced myocardial injury with addition of ICI182780

	TNFα (pg/ml)	IL-6 (pg/mL)	CTnI (ng/ml)	PCT (ng/ml)
Con	348.49±69.69**	389.22±59.53**	22.32±5.58**	712.39±68.55**
Con + ICI	351.46±70.29**	381.23±52.25**	22.64±5.66**	729.89±98.42**
M	479.55±95.91	587.35±56.87	30.02±7.51	1106.1±50.93
M + ICI	481.34±96.26	598.81±38.21	30.34±7.58	1154.3±64.32
Sf + ICI	391.12±75.35*	486.17±74.31*	28.12±6.78	1037.3±64.32
MAr + ICI	386.42±77.28*	497.26±71.31*	28.86±6.96	1009.6±59.81

Mean±SD, n=8 rats per group. *P<0.05, **P<0.01 compared with model group. TNFα: Tumor necrosis factor α; IL: Interleukin; CTnI: Cardiac troponin-I; PCT: Procalcitonin; Con: Control; M: LPS (10 mg/kg); Sf: Sf + LPS (Sf 30 mg/kg, LPS 10 mg/kg); MAr: Ar medium + LPS (Ar 50 mg/kg, LPS 10 mg/kg); Con + ICI: control + ICI182780 (0.5 mg/kg); LPS: Lipopolysaccharide; Ar: Arbutin; Sf: Soybean isoflavone; SD: Standard deviation

Table 5: Effects of arbutin on oxidative stress indices in lipopolysaccharide-induced myocardial injury

	MDA (nmol/mgprot)	T-SOD (U/mgprot)	GSH-Px (U/mgprot)
Con	2.379±0.4392**	86.73±4.793**	250.59±61.04**
M	4.293±0.5756	61.03±4.311	157.01±13.56
Sf	2.262±0.4192**	80.88±2.618**	247.51±28.80**
LAr	2.155±0.6043**	71.71±3.110**	201.79±27.33**
MAr	2.115±0.3776**	75.11±5.294**	245.34±11.26**
HAr	2.024±0.4672**	91.06±6.645**	257.64±34.00**

Mean±SD, n=8 rats per group. *P<0.05, **P<0.01 compared with model group. MDA: Malondialdehyde; T-SOD: Total superoxide dismutase; GSH-Px: Glutathione peroxidase; Con: Control; M: LPS (10 mg/kg); Sf: Sf+LPS (Sf 30 mg/kg, LPS 10 mg/kg); LAr: Ar low + LPS (Ar 25 mg/kg, LPS 10 mg/kg); MAr: Ar medium + LPS (Ar 50 mg/kg, LPS 10 mg/kg); HAr: Ar high + LPS (Ar 100 mg/kg, LPS 10 mg/kg); LPS: Lipopolysaccharide; Ar: Arbutin; Sf: Soybean isoflavone; SD: Standard deviation

Table 6: Effect of arbutin on oxidative stress indices in lipopolysaccharide-induced myocardial injury with addition of ICI182780

	MDA (nmol/mgprot)	T-SOD (U/mgprot)	GSH-Px (U/mgprot)
Con	2.178±0.4535**	88.64±6.161**	243.12±29.06**
Con + ICI	2.833±0.4804**	85.67±2.378**	239.82±21.04**
M	4.916±0.7966	66.94±1.584	175.15±15.35
M + ICI	4.722±0.8439	64.07±1.714	156.95±20.58
Sf + ICI	4.219±0.9549	62.63±4.986	131.66±31.46
MAr + ICI	4.446±0.5841	61.86±2.719	144.50±10.23

Mean±SD, n=8 rats per group. *P<0.05, **P<0.01 compared with model group. MDA: Malondialdehyde; T-SOD: Total superoxide dismutase; GSH-Px: Glutathione peroxidase; Con: Control; M: LPS (10 mg/kg); Sf: Sf + LPS (Sf 30 mg/kg, LPS 10 mg/kg); MAr: Ar medium + LPS (Ar 50 mg/kg, LPS 10 mg/kg); Con + ICI: control+ICI182780 (0.5 mg/kg); LPS: Lipopolysaccharide; Ar: Arbutin; Sf: Soybean isoflavone; SD: Standard deviation

Effect of arbutin on the levels of estrogen receptor alpha and estrogen receptor beta in lipopolysaccharide-induced myocardial injury

To further explore the mechanism of Ar and Sf in the LPS-induced cardiomyopathy rats, we detected the expression of ERα and ERβ by Western blot analysis. Results are shown in Figure 1. The levels of ERα and ERβ were reduced in the LPS-induced cardiomyopathy rats significantly but were higher in the Ar and Sf-treated cases. Furthermore, these effects of Ar and Sf could be effectively blocked by ICI182780 [Figure 3].

DISCUSSION

Sepsis is a systemic infection syndrome of the inflammatory response, leading to an organ dysfunction.[8] In this experiment, the results of the organ index showed that the heart, spleen, and kidney of LPS-challenged rats were severely damaged, which were relieved by

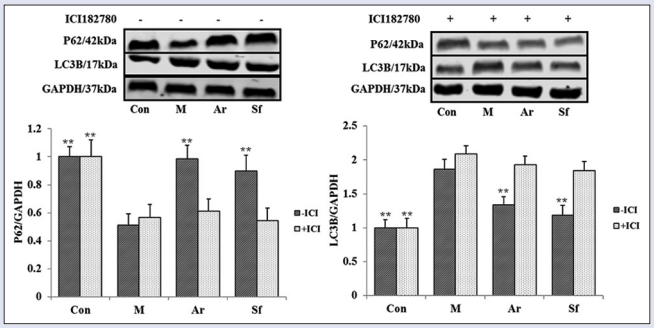


Figure 2: Effect of arbutin on the levels of LC3B and p62 in lipopolysaccharide-induced myocardial injury. Mean ± standard deviation, n = 8 rats per group, *P < 0.05, **P < 0.01 compared with model group. Con: Control; M: Lipopolysaccharide (10 mg/kg); Sf: Soybean isoflavone + lipopolysaccharide (soybean isoflavone 30 mg/kg, lipopolysaccharide10 mg/kg); Ar: Arbutin

Table 7: Effect of arbutin on protein content in mitogen-activated protein kinase pathway in lipopolysaccharide-induced myocardial injury

	p-ERK1/2 (Unit/ml)	p-JNK (Unit/ml)	p-p38 (Unit/ml)
Con	8.09±1.04**	46.36±4.53**	140.83±6.13**
M	3.36±0.51	92.13±10.02	318.02±78.54
Sf	5.64±1.01**	69.84±7.36**	242.11±81.06**
LAr	3.06±0.43	68.04±5.12**	239.78±64.19**
MAr	3.18±0.56	61.08±6.48**	234.69±56.99**
HAr	3.20±0.67	59.62±5.21**	229.78±75.16**

Mean±SD, n=8 rats per group. *P<0.05, **P<0.01 compared with model group. Con: Control; M: LPS (10 mg/kg); Sf: Sf + LPS (Sf 30 mg/kg, LPS 10 mg/kg); LAr: Ar low + LPS (Ar 25 mg/kg, LPS 10 mg/kg); MAr: Ar medium + LPS (Ar 50 mg/kg, LPS 10 mg/kg); HAr: Ar high + LPS (Ar 100 mg/kg, LPS 10 mg/kg); LPS: Lipopolysaccharide; Ar: Arbutin; Sf: Soybean isoflavone; SD: Standard deviation

Table 8: Effect of arbutin on protein content in mitogen-activated protein kinase pathway in lipopolysaccharide-induced myocardial injury with addition of ICI182780

	p-ERK1/2 (Unit/ml)	p-JNK (Unit/ml)	p-p38 (Unit/ml)
Con	14.99±1.81**	39.81±6.72**	136.5±28.4**
Con + ICI	13.29±1.23**	42.74±3.14**	145.1±20.3**
M	4.58±1.03	62.56±2.46	359.1±77.6
M + ICI	4.89±0.46	61.74±4.12	367.1±84.5
Sf + ICI	7.77±1.70	60.27±4.91	372.5±70.4
MAr + ICI	5.19±1.42	61.81±3.25	357.2±71.6

Mean±SD, n=8 rats per group. *P<0.05, **P<0.01 compared with model group. Con: Control; M: LPS (10 mg/kg); Sf: Sf + LPS (Sf 30 mg/kg, LPS 10 mg/kg); MAr: Ar medium + LPS (Ar 50 mg/kg, LPS 10 mg/kg); Con + ICI: control + ICI182780 (0.5 mg/kg); LPS: Lipopolysaccharide; Ar: Arbutin; Sf: Soybean isoflavone; SD: Standard deviation

Ar and Sf in varying degrees. Studies have shown that the cardiac dysfunction is an important mechanism for the pathogenesis of sepsis in rats.[19] Therefore, promoting the cardiac function plays a key role in improving the survival rate of sepsis rats. We found that the decrease of LVEF and LVFS in LPS-induced cardiomyopathy rats could be significantly reversed by Ar and Sf, which in turn protects the heart function and other organs in the LPS-challenged rats. By observing the pathological sections, we could clearly confirm the severity of the lesions and the site of myocardial injury induced by LPS. The results showed that severe histopathological changes occurred in the myocardial tissue of rats induced by LPS, which could be alleviated by Ar and Sf.

However, the inflammatory response is an important factor, leading to heart dysfunction. Studies have shown that acute heart failure caused by the inflammatory reactions is one of the main causes of death in patients with sepsis.[20] TNFα is a key factor that triggers the initiation of many cytokines and even systemic inflammatory response, which could directly induce the release of IL-6 and numerous other inflammatory factors, triggering an inflammatory cascade. PCT reflects the degree of systemic inflammatory response. Increased PCT levels occur in severe shock, systemic inflammatory response syndrome, and multiple organ dysfunction syndromes. When the body's myocardial tissue is damaged, CTnI is released into the blood circulation. Our results showed that the production of TNFα, IL-6, PCT, and CTnI in the myocardial tissue of rats injected by LPS was significantly reduced by Ar and Sf. The levels of inflammatory cytokines in LPS-challenged rats' serum were significantly higher than those in normal group, which were significantly inhibited by Ar and Sf, which means that the LPS-induced myocardial injury rats produced a severe inflammatory response and that Ar and Sf exhibited significant anti-inflammatory effects. Thus, we speculate Ar and Sf to play a protective role in the heart function and other organs in LPS-induced cardiomyopathy rats, which may be attributed to its anti-inflammatory effect.

There are many factors that cause the increase of inflammatory parameters; however, a number of studies have shown that the inflammatory response is accompanied by different degrees of oxidative stress reaction in the body.[21-23] The production of inflammatory factors could be reduced by reducing the oxidative stress response.[24] The early stages of sepsis are characterized by production of large amounts of reactive oxygen species.[25] Oxidative stress reaction is a kind of response when the body is stimulated by external factors. MDA is cytotoxic and is one of the most important products of membrane lipid peroxidation, which reflects the degree of membrane lipid peroxidation. SOD is an important antioxidant enzyme in organisms and the primary substance in the body to scavenge free radicals. GSH-Px is an important peroxide-degrading enzyme widely found in the body. The levels of SOD and GSH-Px reflect the ability of the body to resist the oxidation levels. Interestingly, we found that the level of MDA was significantly increased in the LPS-induced rats' myocardial tissue, while SOD and GSH-Px were significantly reduced, which were effectively reversed by Ar and Sf. This indicated that excessive oxidative stress occurred in model rats could be inhibited by Ar and Sf. Therefore, we could conclude that a number of oxidative stress products are produced in LPS-induced myocardial injury rats, which might be closely related to inflammatory response.

Studies in the recent years have shown that autophagy and inflammation are inextricably linked[26,27] and the occurrence of autophagy could inhibit the inflammatory response under normal circumstances.[28] However, neurogenic inflammation might cause autophagic damage.[29] There is evidence showing that the autophagic activity in cardiomyocytes changes during sepsis, but the results were unclear.[30] Autophagy is a dynamic process, in which the damaged organelles and long-lived proteins are delivered to the lysosome for degradation and recycling.[31] During this dynamic process, the outer membrane of the autophagosomes (a double-membrane vesicle) fuses with the lysosomal membrane forming an autolysosome. LC3 is a main mediator in autophagy and functions at an early stage of phagophore expansion. The level of LC3 increases as the number of autophagosome increases. There are three types of LC3 in mammals, including LC3A, LC3B, and LC3C. LC3B is an autophagy-specific cargo that is a notable marker of the autophagosomes.[32] When the autophagosomes bind to the lysosomes to form autophagosomes, p62 is an essential reaction substrate and the level of p62 decreases as the number of autophagosomes increases. In our study, the level of p62 decreased and LC3B increased in the model

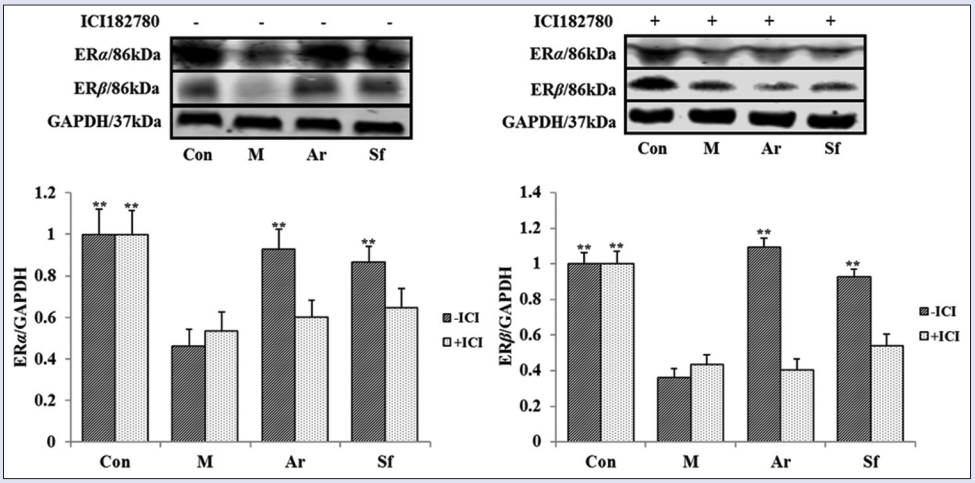


Figure 3: Effect of arbutin on estrogen receptor related proteins content in lipopolysaccharide-induced myocardial injury. Mean ± standard deviation, n = 8 rats per group, *P < 0.05, **P < 0.01 compared with model group. Con: Control; M: Lipopolysaccharide (10 mg/kg); Sf: Soybean isoflavone + lipopolysaccharide (soybean isoflavone 30 mg/kg, lipopolysaccharide10 mg/kg); Ar: Arbutin, Ar was isolated from Yam and known to have estrogenic activity[17]

rats, which means that the expression of autophagy increased and the autophagy process was complete in LPS-induced cardiomyopathy rats. Our results indicate that Ar and Sf could reduce the level of LC3B protein in the myocardial tissue and blocked the formation of barrier membrane. However, the level of p62 protein in rat myocardial tissue treated with Ar and Sf was significantly increased when compared with the model group, which indicates that the production of autophagosomes is reduced, indicating that Ar and Sf could block the occurrence of autophagy. This may be an interesting finding that Ar and Sf could prevent autophagy from different stages.

Mitogen-activated protein kinase (MAPK) signaling pathway is one of the important signal transduction systems *in vivo*. The p38 and JNK are the two critical pathways in the MAPK, both of which play an important role in different stress responses. The activation of JNK and p38 on the signaling pathway promotes the inflammatory response. Previous studies have confirmed that MAPK signaling pathway has a profound impact on the development of the inflammation-related cancers.[33] Studies have shown that β-amyloid-induced inflammatory response could be inhibited by the MAPK signaling pathway.[34] We found that p-JNK and p-p38 were significantly elevated in LPS-induced cardiomyopathy rats when compared with normal group and this effect was significantly reversed by Ar and Sf. However, it is strange that we detected no significant changes in the level of p-ERK1/2 in LPS-induced cardiomyopathy rats. To clearly understand the mechanism of action of Ar and Sf, further studies are needed.

It is well known that Sf is a fine phytoestrogen, and it was found that Ar has a significant estrogenic activity in our previous study. In this experiment, Ar and Sf showed protection against LPS-induced cardiomyopathy rats through anti-inflammatory effects. Studies have shown that phytoestrogens to possess a variety of pharmacological activities. Similarly, substances with estrogen-like activity might also have phytoestrogen-like pharmacological activities. Compounds with estrogenic potency will bind to the ER *in vivo* and show estrogen-like activity. When Ar binds to the ER, it is possible to show significant pharmacological changes and the effect on sepsis might be exerted through ER. Therefore, we hypothesize that the anti-inflammatory effect of Ar has a certain relationship with estrogenic activity. Moreover, the anti-inflammatory activity of Ar might be exerted via the ER pathway, and it is hence necessary to detect the levels of ERα and ERβ in the LPS-treated rats' heart. Interestingly, we found that the levels of ERα and ERβ were significantly decreased in the LPS-induced cardiomyopathy rats. Ar and Sf significantly increased the expression of ERα and ERβ. Downregulation of ERα expression in the MCF-7 cells could induce autophagy and produce large amounts of ROS. Autophagy is considered to be the main driver of the anti-estrogen resistance.[35] Studies have shown that the ER-specific antagonists could block the activation of MAPK.[36] The ER-specific antagonist ICI182780 reduced the ERα expression to induce excessive autophagy and oxidative stress and enhanced the inflammation *in vivo* to induce myocardial injury in rats. To further confirm the effects of Ar on LPS-induced cardiomyopathy rats through ER pathway, we used ICI182780 to block the effects of Ar. The results showed that Ar and Sf inhibited the excessive autophagy and oxidative stress and their actions could be blocked by ICI182780 effectively.

CONCLUSION

We could conclude that Ar could protect the rats from LPS-induced myocardial injury via ER pathway. Our study reflects that Ar could be used as a potential drug for the treatment of sepsis cardiomyopathy.

Acknowledgements

Our work was supported by the Central Government Guide Local Science and Technology Development Funds (14104349). We thank Xiaoke Zheng and Mengnan Zeng for the experimental design, manuscript drafting, and statistical analysis. We are thankful to Benke Li, Yangyang Wang, Yuxuan Kan, ShengchaoWang, Yijia Meng, and Jiji Gao for helping with experiments and Weisheng Feng for supervising the project.

Financial support and sponsorship

Our work was supported by the Central Government Guide Local Science and Technology Development Funds (14104349).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Buoro S, Manenti B, Seghezzi M, Dominoni P, Barbui T, Ghirardi A, *et al.* Innovative haematological parameters for early diagnosis of sepsis in adult patients admitted in intensive care unit. J Clin Pathol 2018;71:330-5.
2. Wang Y, Yin X, Yang F. Comprehensive analysis of gene expression profiles of sepsis-induced multiorgan failure identified its valuable biomarkers. DNA Cell Biol 2018;37:90-8.
3. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF, *et al.* Neutrophils in development of multiple organ failure in sepsis. Lancet 2006;368:157-69.
4. Prescott HC, Angus DC. Enhancing recovery from sepsis: A review. JAMA 2018;319:62-75.
5. Xie Z, Guo Z, Liu J. Whey acidic protein/Four-disulfide core domain 21 regulate sepsis pathogenesis in a mouse model and a macrophage cell line via the stat3/Toll-like receptor 4 (TLR4) signaling pathway. Med Sci Monit 2018;24:4054-63.
6. Kumar V. T cells and their immunometabolism: A novel way to understanding sepsis immunopathogenesis and future therapeutics. Eur J Cell Biol 2018;97:379-92.
7. Leligdowicz A, Richard-Greenblatt M, Wright J, Crowley VM, Kain KC. Endothelial activation: The Ang/Tie axis in sepsis. Front Immunol 2018;9:838.
8. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, *et al.* The third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA 2016;315:801-10.
9. Lou Y, Lin Z. Pathogenesis of sepsis-induced myocardial dysfunction. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue 2018;30:374-6.
10. Romero-Bermejo FJ, Ruiz-Bailen M, Gil-Cebrian J, Huertos-Ranchal MJ. Sepsis-induced cardiomyopathy. Curr Cardiol Rev 2011;7:163-83.
11. Hsieh CH, Pai PY, Hsueh HW, Yuan SS, Hsieh YC. Complete induction of autophagy is essential for cardioprotection in sepsis. Ann Surg 2011;253:1190-200.
12. Martin L, Derwall M, Thiemermann C, Schürholz T. Heart in sepsis: Molecular mechanisms, diagnosis and therapy of septic cardiomyopathy. Anaesthesist 2017;66:479-90.
13. Guo RF, Ward PA. Role of oxidants in lung injury during sepsis. Antioxid Redox Signal 2007;9:1991-2002.
14. Duran-Bedolla J, Montes de Oca-Sandoval MA, Saldaña-Navor V, Villalobos-Silva JA, Rodriguez MC, Rivas-Arancibia S, *et al.* Sepsis, mitochondrial failure and multiple organ dysfunction. Clin Invest Med 2014;37:E58-69.
15. Zhang L, Ai Y, Tsung A. Clinical application: Restoration of immune homeostasis by autophagy as a potential therapeutic target in sepsis. Exp Ther Med 2016;11:1159-67.
16. Yen YT, Yang HR, Lo HC, Hsieh YC, Tsai SC, Hong CW, *et al.* Enhancing autophagy with activated protein C and rapamycin protects against sepsis-induced acute lung injury. Surgery 2013;153:689-98.
17. Zeng M, Zhang L, Li M, Zhang B, Zhou N, Ke Y, *et al.* Estrogenic effects of the extracts from the Chinese Yam (*Dioscorea* opposite thunb.) and its effective compounds *in vitro* and *in vivo*. Molecules 2018;23. pii: E11.
18. Xu X, Duncan AM, Merz BE, Kurzer MS. Effects of soy isoflavones on estrogen and phytoestrogen metabolism in premenopausal women. Cancer Epidemiol Biomarkers Prev 1998;7:1101-8.
19. Li C, Hua F, Ha T, Singh K, Lu C, Kalbfleisch J, *et al.* Activation of myocardial phosphoinositide-3-kinase p110α ameliorates cardiac dysfunction and improves survival in polymicrobial sepsis. PLoS One 2012;7:e44712.
20. Li Y, Ge S, Peng Y, Chen X. Inflammation and cardiac dysfunction during sepsis, muscular dystrophy, and myocarditis. Burns Trauma 2013;1:109-21.
21. Palanisamy K, Krishnaswamy R, Paramasivan P, Chih-Yang H, Vishwanadha VP. Eicosapentaenoic acid prevents TCDD-induced oxidative stress and inflammatory response by modulating MAP kinases and redox-sensitive transcription factors. Br J Pharmacol 2015;172:4726-40.
22. Bekyarova G, Tancheva S, Hristova M. Protective effect of melatonin against oxidative hepatic injury after experimental thermal trauma. Methods Find Exp Clin Pharmacol 2009;31:11-4.
23. Edirisinghe I, Yang SR, Yao H, Rajendrasozhan S, Caito S, Adenuga D, *et al.* VEGFR-2 inhibition augments cigarette smoke-induced oxidative stress and inflammatory responses leading to endothelial dysfunction. FASEB J 2008;22:2297-310.
24. Nandedkar SD, Weihrauch D, Xu H, Shi Y, Feroah T, Hutchins W, *et al.* D-4F, an apoA-1 mimetic, decreases airway hyperresponsiveness, inflammation, and oxidative stress in a murine model of asthma. J Lipid Res 2011;52:499-508.
25. Yamaguchi J, Nagase M, Yamamoto Y, Sakurai A, Kubo A, Mitsuhashi H, *et al.* Increased oxidative stress and renal injury in patients with sepsis. J Clin Biochem Nutr 2018;63:137-43.
26. Jones SA, Mills KH, Harris J. Autophagy and inflammatory diseases. Immunol Cell Biol 2013;91:250-8.
27. Arnaud F, Faraj T, Thierry J, Agnès RB, Marc P, Guylène P. Involvement of interleukin-1β in the autophagic process of microglia: relevance to Alzheimer's disease. J Neuroinflammation 2013;10:151.
28. Lapaquette P, Guzzo J, Bretillon L, Bringer MA. Cellular and molecular connections between autophagy and inflammation. Mediators Inflamm 2015;2015:398483.
29. Zheng HF, Yang YP, Hu LF, Wang MX, Wang F, Cao LD, *et al.* Autophagic impairment contributes to systemic inflammation-induced dopaminergic neuron loss in the midbrain. PLoS One 2013;8:e70472.
30. Zhang J, Zhao P, Quan N, Wang L, Chen X, Cates C, *et al.* The endotoxemia cardiac dysfunction is attenuated by AMPK/mTOR signaling pathway regulating autophagy. Biochem Biophys Res Commun 2017;492:520-7.
31. Shintani T, Klionsky DJ. Autophagy in health and disease: A double-edged sword. Science 2004;306:990-5.
32. Youn J, Lee SB, Lee HS, Yang HO, Park J, Kim JS, *et al.* Cerebrospinal fluid levels of autophagy-related proteins represent potentially novel biomarkers of early-stage Parkinson's disease. Sci Rep 2018;8:16866.
33. Huang P, Han J, Hui L. MAPK signaling in inflammation-associated cancer development. Protein Cell 2010;1:218-26.
34. Zhao H, Wang SL, Qian L, Jin JL, Li H, Xu Y, *et al.* Diammonium glycyrrhizinate attenuates aβ(1-42) -induced neuroinflammation and regulates MAPK and NF-κB pathways *in vitro* and *in vivo*. CNS Neurosci Ther 2013;19:117-24.
35. Cook KL, Wärri A, Soto-Pantoja DR, Clarke PA, Cruz MI, Zwart A, *et al.* Hydroxychloroquine inhibits autophagy to potentiate antiestrogen responsiveness in ER+breast cancer. Clin Cancer Res 2014;20:3222-32.
36. Arroba AI, Frago LM, Argente J, Chowen JA. Oestrogen requires the insulin-like growth factor-I receptor for stimulation of prolactin synthesis via mitogen-activated protein kinase. J Neuroendocrinol 2005;17:97-104.