The toxic effects of bisphenol S showing immunomodulation in fish macrophages

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SI Materials and Methods

Phagocytic activities of primary macrophages. The phagocytic activities of primary macrophages against the pathogenic gram-positive *Staphylococcus aureus* (CGMCC 1.363), gram-negative *Vibrio parahaemolyticus* (CGMCC 1.1615), and green florescent protein (GFP)-expressing *Escherichia coli* (CGMCC 1.2389) were measured following modified protocols described in previous studies[1, 2]. The GFP-expressing *E. coli* were generated prior to the experiment. A GFP-expressing plasmid was constructed using a pET-28a expression vector (Novagen). After confirming by DNA sequencing, the pET-28a/GFP plasmid was used to transform *E. coli* BL21(DE3)pLysS cells. A single colony of *E. coli* BL21(DE3)pLysS harboring the pET28/GFP plasmid was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to LB medium at 37°C. The culture was incubated with shaking at 180 rpm and 28°C for 3 h until the OD_{600} reached 0.2–0.3. *S. aureus* and *V. parahaemolyticus* were cultured to the logarithmic phase and counted using the OD value.

Detached primary macrophages were separated on a 34%/51% (v/v) Percoll density gradient (GE, USA) by centrifugation. After exposure to bisphenol S (BPS)-supplemented culture medium for 6 h, the macrophages were washed three times and resuspended in 0.85 mL of antibiotic-free culture medium. Fifty milliliters of freshly thawed ice-cold normal serum and 0.1 mL of bacteria were added to each well ensuring that the proportion of macrophages to bacteria was 1:10. After cultivation on a Labquake shaker at 8 rpm for 90 min at 26°C, the cells were washed four times to remove the bacteria that had not been engulfed. Final suspensions of macrophages were resuspended in 200 μL of PBS and applied to slides using a cytocentrifuge. The phagocytic activity of macrophages against GFP-expressing *E. coli* was evaluated under a fluorescence microscope (LSM 780 NLO, ZEISS) or directly in a fluorescence microplate (Infinite® M1000 Pro, Tecan). The phagocytic activity of macrophages against *S. aureus* and *V.*

parahaemolyticus was analyzed at ×100 under an oil-immersion microscope (Axio Observer A1, ZEISS) after staining using a Diff-Quik Kit (Nanjing Jian Cheng Bioengineering Institute, China). The phagocytosis of macrophages was evaluated using a phagocytic index, which as determined by using the following formula: phagocytic index = (percentage of macrophages containing at least one bacterium) × (mean number of bacteria per positive cell).

REFERENCES

1. Qiu, W. H.; Liu, S.; Chen, J. S.; Hu, L.; Wu, M. H.; Yang, M. The primary culture of carp (*Cyprinus carpio*) macrophages and the verification of its phagocytosis activity. *In Vitro Cell Dev-An.* **2016,** *52* (1), 10-19.
2. Campbell, P. A.; Canono, B. P.; Drevets, D. A. Measurement of bacterial ingestion and killing by macrophages. *Curr. Protoc. Immunol.* **2001,** *Chapter 14*, Unit 14 6.

SI Figures

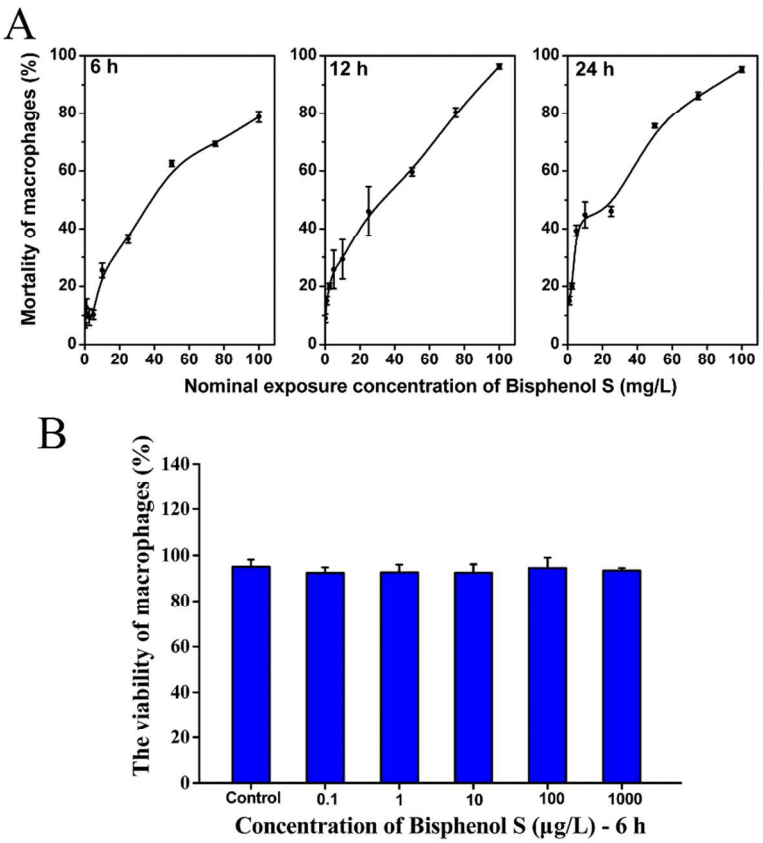

Figure. S1. Acute toxicity (A) and sublethal toxicity (B) of bisphenol S to the primary macrophages of red carp. Mortality was observed at 6, 12, and 24 h after exposure. Values are the means and standard error of the means (n = 6) relative to the control.

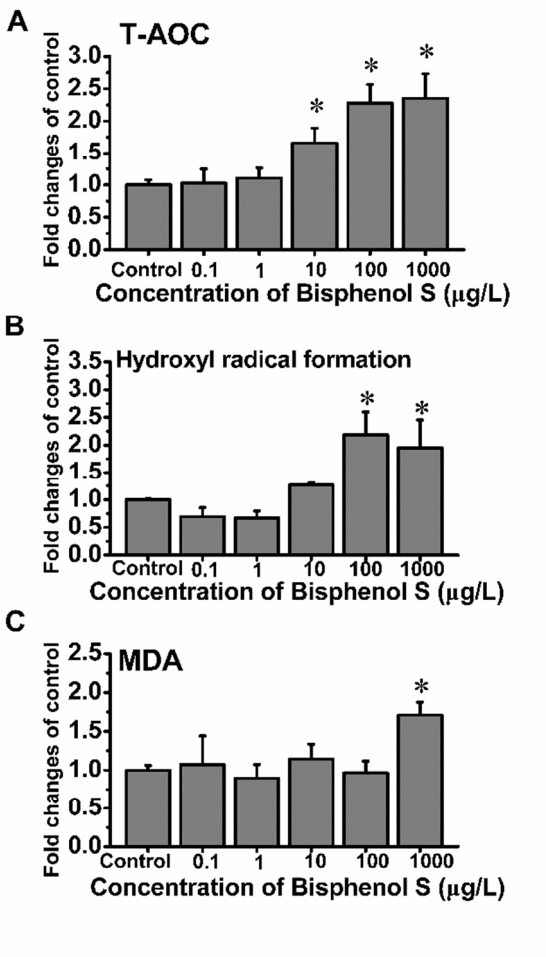

Figure. S2. Induction of total antioxidant capacity (T-AOC, A), hydroxyl radical formation level (B) and malonaldehyde (MDA) content (C) in primary macrophages following a 6-h exposure to bisphenol S (BPS). The level of MDA represents the lipid peroxidation level. Values are the means ± standard deviation (n = 3). An asterisk (*) indicates an significant difference versus the control at $p < 0.05$ (ANOVA, Tukey's test).

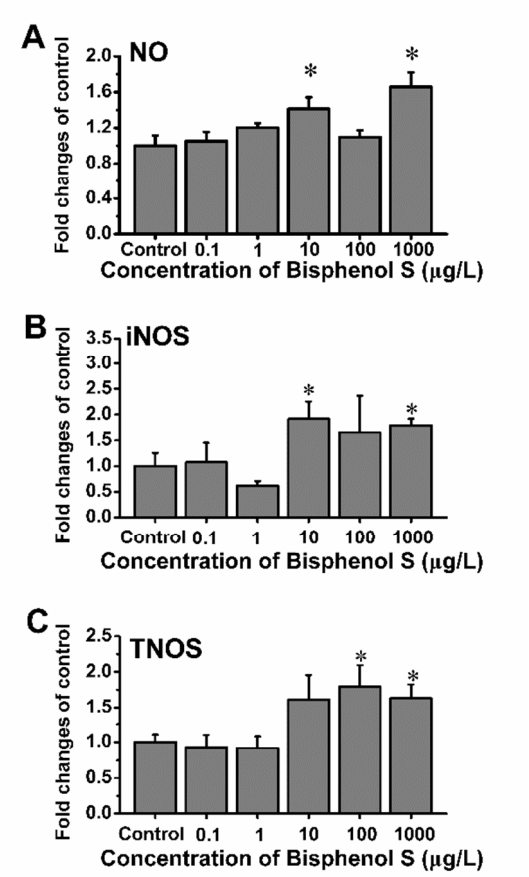

Figure S3. Induction of nitric oxide production (NO, A), and induced nitric oxide synthase (iNOS, B) and total nitric oxide synthase (TNOS, C) activities in primary macrophages following a 6-h exposure to bisphenol S (BPS). Values are the means ± standard deviation (n = 3). An asterisk (*) indicates a significant difference versus the control at $p < 0.05$ (ANOVA, Tukey's test).

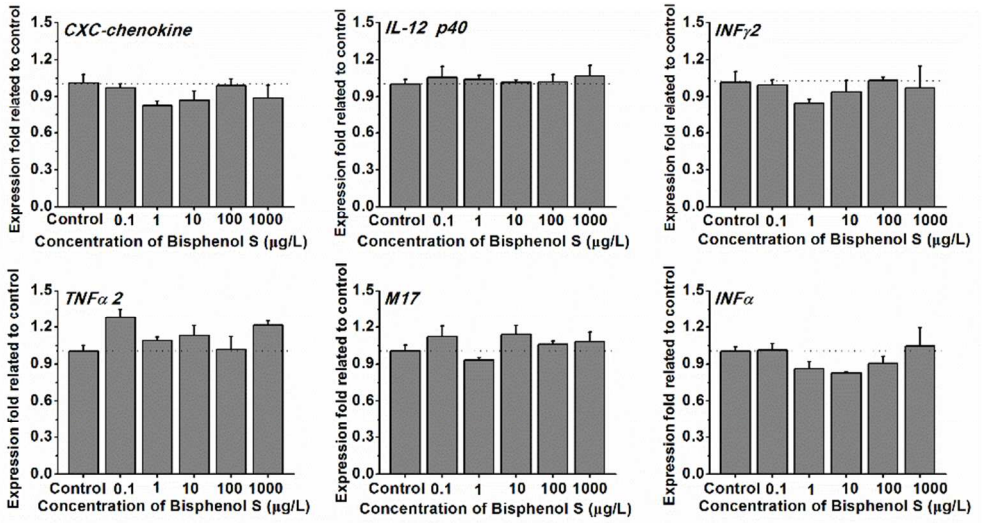

Figure S4. The expression of immune-related genes in primary macrophages following a 6-h exposure to bisphenol S (BPS). Values are the means ± standard deviation (n = 3). An asterisk (*) indicates a significant difference versus the control at $p < 0.05$ (ANOVA, Tukey's test).

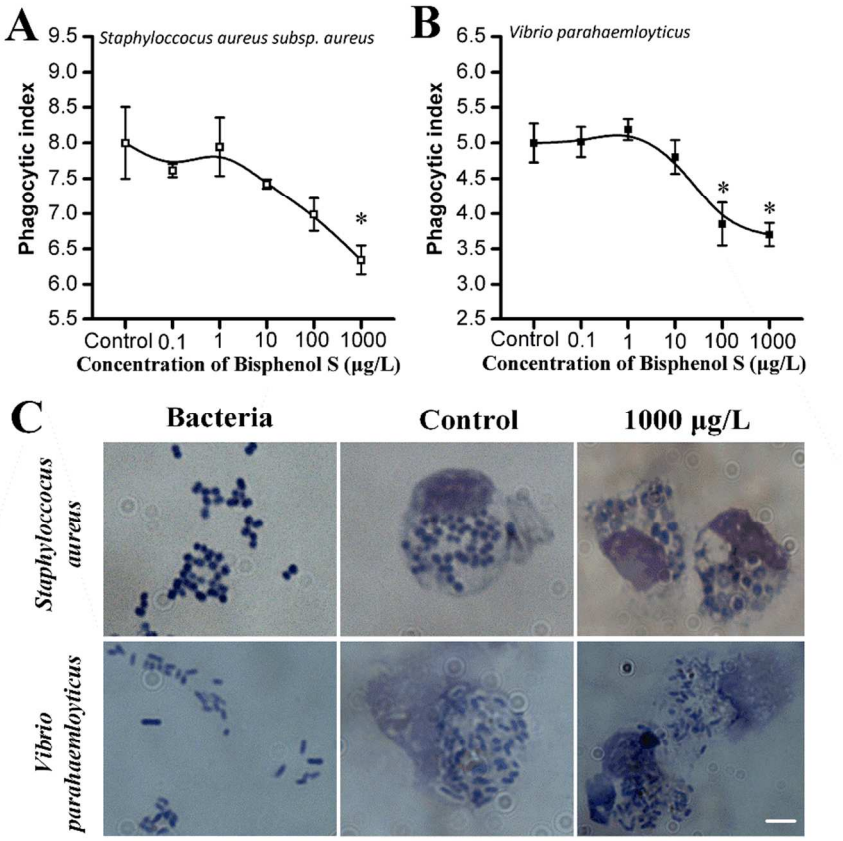

Figure S5. Changes in the phagocytic index of primary macrophages after a 6-h exposure to bisphenol S (BPS). The phagocytic index was measured by counting the intracellular *Staphylococcus aureus* (n = 6, A) and the intracellular *Vibrio parahaemolyticus* (n = 6, B) (C) Representative images comparing the phagocytic activities of the control group and the 1000 μg/L BPS group (scale bar = 5 μm). Values are the means ± standard deviation. An asterisk (*) indicates a significant difference versus the control at $p < 0.05$ (ANOVA, Tukey's test).

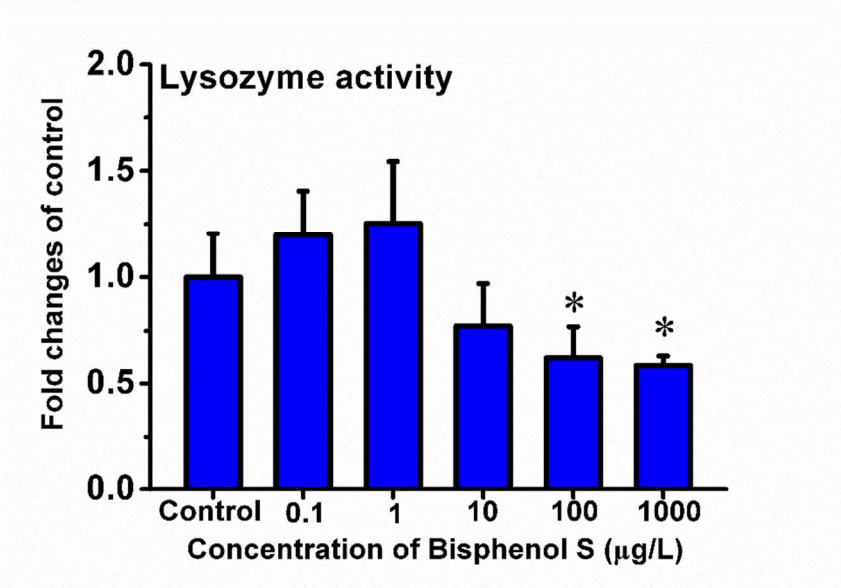

Figure S6. Changes in lysozyme activity of primary macrophages after a 6-h exposure to bisphenol S (BPS). Values are the means ± standard deviation (n = 3). An asterisk (*) indicates a significant difference versus the control at $*p < 0.05$ (ANOVA, Tukey's test).

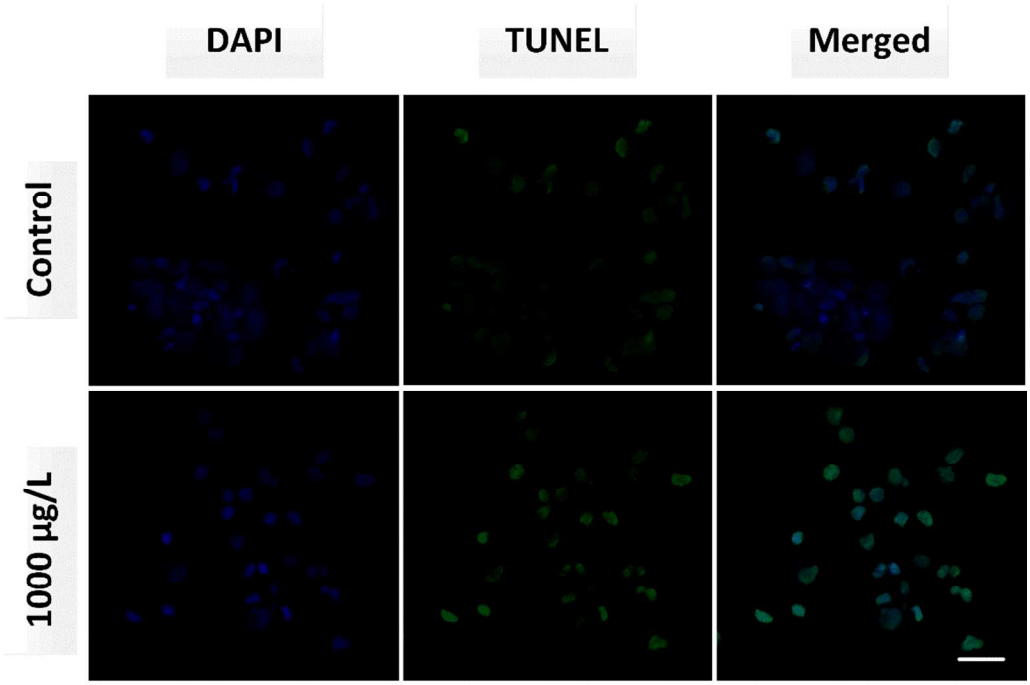

Figure S7. Representative fluorescent images showing a significant increase in TUNEL-positive nuclei (green) in 1,000 µg/L bisphenol S (BPS)-treated samples are compared with the control (Scale bar = 20 µm).

SI Tables

Table S1. Primers used for real-time PCR

Gene name	GenBank accession number	Sequence of the primer (5'-3')
40S ribosomal protein S11	AB012087.1	F: GTTCTCGCTGTTGAAGGAAGTGG
		R: TTGCGGATGTAATGCAAGTAGTC
Interleukin-1β	AJ245635.1	F: AAGGAGGCCAGTGGCTCTGT
		R: CCTGAAGAAGAGGAGGCTGTCA
Tumor necrosis factor-α1	AJ311800.2	F: CACGCTCAACAAGTCTCAGAACAA
		R: CGAAGTAAATGCCGTCAGTAGGA
Interleukin-10	AB110780.1	F: AGCGGGATATGCGGAAATGTAGG
		R: TGCCAAATACTGCTCGATGTACTTAA
CXCL8	AB470924.1	F: TCACTTCACTGGTGTTGCTC
		R: GGAATTGCTGGCTCTGAATG
γ-Interferon-1	AM261214.1	F: TGCACTTGTCAGTCTCTGCT
		R: TGTACTTGTCCCTCAGTATTT
Interleukin-12 p35	AJ580354.1	F: TGCTTCTCTGTCTCTGTGATGGA
		R: CACAGCTGCAGTCGTTCTTGA
Interleukin-6	KC858889.1	F: GGCGTATGAAGGAGCGAAGA
		R: ATCTGACCGATAGAGGAGCG
Interleukin-11	AJ632159.1	F: GCTGTCACGTCATGAACGAGAT
		R: CCCGCTTGAGATCCTGAAATAT
CXC chemokine	AB082985.1	F: GTGTGAACATGGTTCCTCCA
		R: GGATTGAAGCATTTCTGCTCT
Interleukin-12 p40	AJ621425.1	F: GAGCGCATCAACCTGACCAT
		R: AGGATCGTGGATAGTGACCTCTAC
γ-Interferon-2	AM168523.1	F: GAGGAACCTGAGCAGAATCT
		R: CCTTGATCGCCCATAGTGTT
Tumor necrosis factor-α2	AJ311801.2	F: AGAACAATCAGGAAGGTGGAAATG
		R: CTGCCGTAGGACTCAGAGTAGCG
Interleukin-6 subfamily-like cytokine M17	AY102632.1	F: AGCTAAATTCAGAATGATCCTCGCTAT
		R: GCAGAAACTCCTTCAGGTGGGTG
α-Interferon	AB376667.1	F: TGCATATGGCTCGGCCAATA
		R: GTCAAGACAAGAAACCTCACC
Estrogen receptor α	AB334722.1	F: CACAGCCGCCCATACACCGAGAT
		R: GGAAGCCTGGTACTTTCTTAGCC
Estrogen receptor β1	AB334723.1	F: GCCGTGCTCCTCTTTGTTGGTAGC
		R: ACCTCGGCCATGACTTCACCACTC
Estrogen receptor β2	AB334724.1	F: TTCCCTTCAGGGGACAGAGCTGAG
		R: AGTCCAGCAGCCTCAGAACCTTCC

Table S2. The lethal concentrations of bisphenol S against fish primary macrophages

Exposure time (h)	LC_{50} [a] (mg/L)	LC_5 [b] (mg/L)
6	39.1 (34.2–44.9)	1.52 (1.21–1.99)
12	29.7 (25.4–35.7)	0.25 (0.07–0.57)
24	16.8 (13.3–21.5)	0.12 (0.04–0.26)

a. Lethal concentration for 50% of the tested populations calculated using the trimmed Spearman–Karber (TS–K) method. The values in parentheses represent 95% confidence limits.

b. Lethal concentration for 5% of the tested populations calculated using the Probit analysis method. The values in parentheses represent 95% confidence limits.

Table S3. Correlation coefficients between all evaluated parameters in primary macrophages after a 6-h exposure to bisphenol S

	ROS	*TAOC*	*OH ·*	*MDA*	*NO*	*INOS*	*TNOS*	*IL1ß*	*TNFα1*	*IL10*	*CXCl8*	*INFγ1*	*IL-12 p35*	*IL-6*	*IL11*	*BA (S.)*	*BA (E.)*	*BA (V.)*	*LYSO*	*Caspase 3*	*Tunel*	*ERα*	*ERß1*
TAOC	0.564**																						
OH ·	0.511**	0.794**																					
MDA	-0.206	-.0353	0.150																				
NO	0.092	0.132	0.111	0.122																			
INOS	0.240	0.281	0.554**	0.364*	0.473**																		
TNOS	0.072	0.212	0.436**	0.130	0.299	0.804**																	
IL1ß	0.662**	0.762**	0.749**	-0.23	0.318	0.450**	0.265																
TNFα1	0.530**	0.623**	0.558**	-0.341*	0.108	0.234	0.157	0.746**															
IL10	0.263	0.250	0.273	-0.020	-0.059	0.164	0.074	0.373*	0.484**														
CXCl8	0.620**	0.460**	0.352*	-0.512**	0.118	0.123	0.063	0.637**	0.575**	0.362*													
INFγ1	0.519**	0.608**	0.603**	-0.069	0.346*	0.306	0.088	0.699**	0.542**	0.136	0.452**												
IL-12 p35	0.459**	0.512**	0.607**	-0.272	0.102	0.311	0.268	0.652**	0.496**	0.186	0.508**	0.539**											
IL-6	0.511**	0.686**	0.768**	-0.134	0.105	0.390*	0.263	0.823**	0.700**	0.394*	0.452**	0.590**	0.825**										
IL11	0.391*	0.503**	0.553**	-0.248	-0.105	0.202	0.274	0.543**	0.716**	0.485**	0.489**	0.355*	0.344*	0.518**									
BA (S.)	-0.218	-0.054	-0.222	0.168	-0.044	-0.152	-0.091	-0.309	-0.333*	-0.235	-0.255	-0.186	-0.292	-0.230	-0.236								
BA (E.)	-0.362*	-0.318	-0.332*	0.118	0.104	-0.137	-0.164	-0.321	-0.402*	-0.046	-0.163	-0.260	0.041	-0.074	-0.475**	0.545**							
BA (V.)	-0.191	-0.090	-0.319	0.116	-0.050	-0.193	-0.135	-0.320	-0.308	-0.204	-0.194	-0.195	-0.334*	-0.283	-0.245	0.978**	0.534**						
LYSO	-0.409*	-0.599**	-.639**	0.168	-0.029	-0.207	-0.185	-0.587**	-0.487**	-0.179	-0.363*	-.582**	-0.506**	-0.595**	-0.353*	0.109	0.180	0.190					
Caspase 3	0.830**	0.522**	0.479**	-0.433**	0.013	0.138	0.059	0.681**	0.502**	0.287	0.682**	0.400*	0.540**	0.555**	0.463**	-0.114	-0.229	-0.118	-0.444**				
Tunel	0.388*	0.625**	0.559**	-0.280	0.101	0.261	0.227	0.700**	0.720**	0.547**	0.630**	0.338*	0.668**	0.772**	0.563**	-0.086	0.112	-0.080	-0.426**	0.508**			
ERα	0.718**	0.563**	0.559**	-0.297	0.038	0.204	0.091	0.687**	0.568**	0.384*	0.634**	0.563**	0.531**	0.652**	0.513**	-0.221	-0.215	-0.214	-0.449**	0.710**	0.552**		
ERß1	0.068	0.210	0.245	0.433**	0.267	0.364*	0.160	0.177	-0.089	0.134	-0.072	0.206	0.108	0.147	-0.152	0.009	0.233	0.009	-0.144	-0.011	0.074	0.016	
ERß2	0.599**	0.470**	0.454**	-0.305	0.264	0.317	0.082	0.662**	0.552**	0.404*	0.606**	0.578**	0.445**	0.495**	0.414*	-0.327	-0.174	-0.284	-0.314	0.565**	0.527**	0.727**	0.246

$p < 0.05$ and *$p < 0.01$* based on Spearman's test.

ROS = reactive oxygen species; TAOC = total antioxidant capacity; OH = hydroxyl radical formation; MDA = lipid peroxidation level; NO = nitric oxide; iNOS = induced nitric oxide synthase; TNOS = total nitric oxide synthase; *IL-1β = interleukin-1β; TNFα1= tumor necrosis factor α1; IL-10 = interleukin-10; CXCl8 = Cxc chemokine-8; INFγ1 = γ-interferon-1; IL-12 p35 = interleukin-12 p35; IL-6 = interleukin-6; IL-10 = interleukin-11;* BA (*S.*) = bactericidal activity against *Staphylococcus aureus* subsp*. aureus*; BA (*E.*) = bactericidal activity against *Escherichia coli*; BA (*V.*) = bactericidal activity against *Vibrio parahaemolyticus*; LYSO = lysozyme; Caspase 3 = caspase 3 activity; *ERα = estrogen receptor α; ERβ = estrogen receptor β*