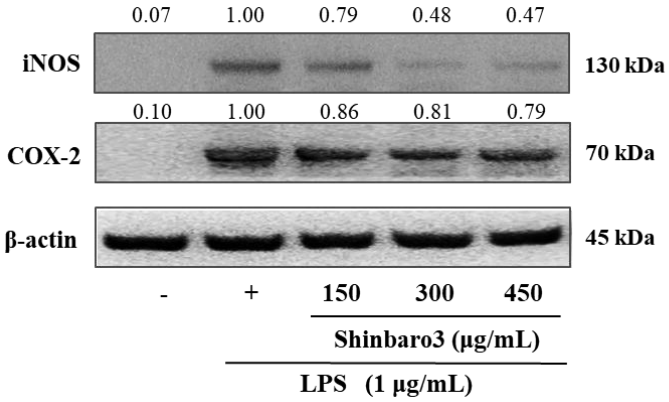
Supplementary materials

Supplementary 1

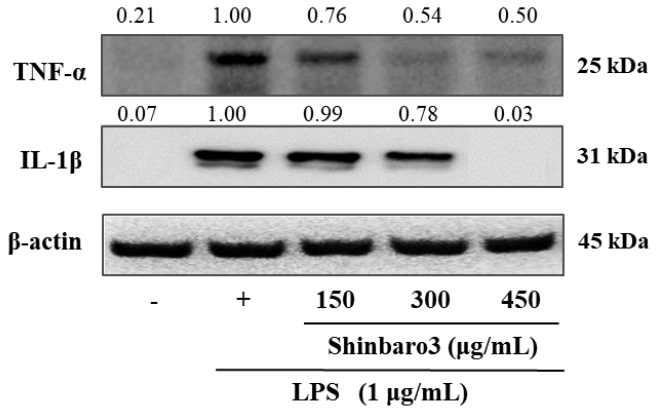
Supplemental Figure 1

: All western blots were assessed of their quantified density.

Figure 3 (c)



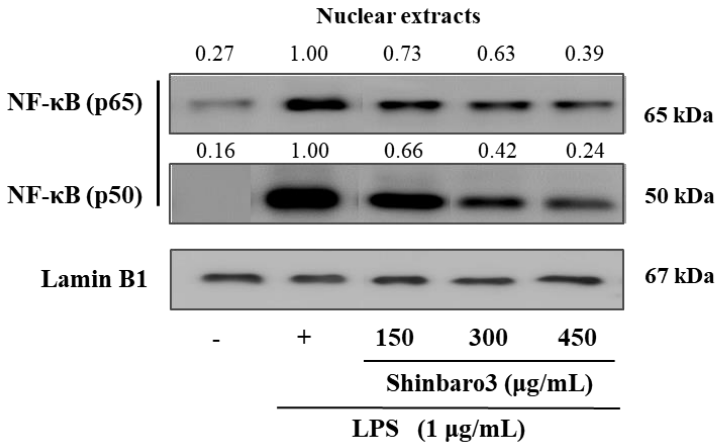
	iNOS	COX-2	beta-actin		**iNOS**	**COX-2**
-	870.0	3165.3	22493.9		**0.07**	**0.10**
+	12194.4	32023.1	22605.4		**1.00**	**1.00**
150	9089.0	25998.4	21264.0		**0.79**	**0.86**
300	5485.8	23954.9	21005.4		**0.48**	**0.81**
450	5416.6	23810.8	21264.0		**0.47**	**0.79**



	TNF-a	IL-1b	beta-actin		**TNF-a**	**IL-1b**
-	5350.2	1906.7	33278.6		**0.21**	**0.07**
+	26409.9	30548.7	35221.4		**1.00**	**1.00**
150	19103.1	28814.4	33477.6		**0.76**	**0.99**
300	13552.5	22405.3	33247.9		**0.54**	**0.78**
450	12262.9	940.0	32477.6		**0.50**	**0.03**

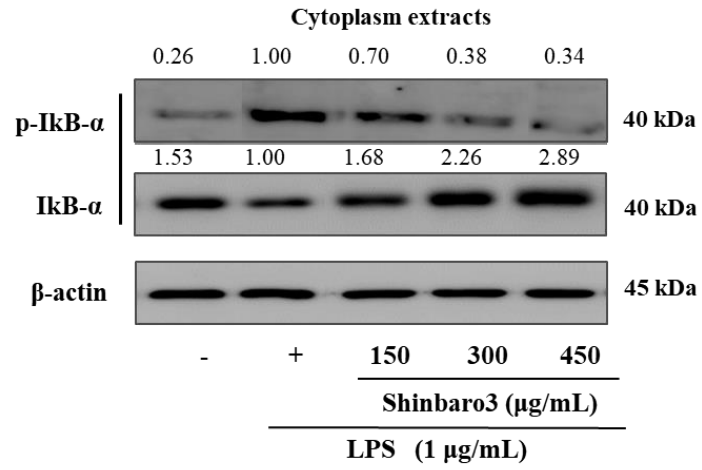
Figure 3 (c): Effects of Shinbaro3 on the expression of inflammatory mediators in LPS-stimulated RAW 264.7 macrophage cells. (Figure 3c) RAW 264.7 cells were treated with LPS (1 μg/mL) and Shinbaro3 (150, 300, and 450 μg/mL) for 18 h to investigate protein expression. iNOS, COX-2, TNF-α and IL-1β mRNA and protein expression levels were then analysed in Western blot assays. β-actin was used as an internal control. The data are representative of three separate experiments.

Figure 4 (a)



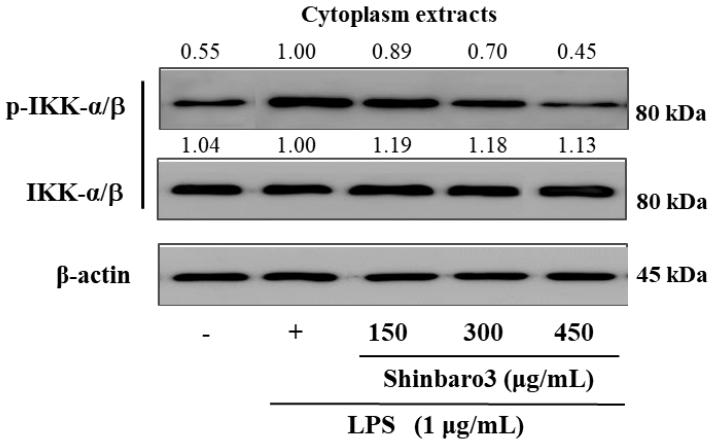
	p65	p50	beta-actin		**p65**	**p50**
-	9196.0	6789.9	25192.2		**0.27**	**0.16**
+	27881.4	34791.6	20777.9		**1.00**	**1.00**
150	22012.7	24970.5	22566.6		**0.73**	**0.66**
300	18011.3	14786.2	21224.4		**0.63**	**0.42**
450	13658.2	10579.4	26105.7		**0.39**	**0.24**

Figure 4 (b)



	p-IkB	IkB	beta-actin		**p-IkB**	**IkB**
-	8217.9	19862.3	30644.2		**0.26**	**1.53**
+	31103.5	12815.2	30306.8		**1.00**	**1.00**
150	19830.7	19439.8	27441.6		**0.70**	**1.68**
300	10417.9	25388.5	26582.4		**0.38**	**2.26**
450	9376.5	32768.6	26808.4		**0.34**	**2.89**

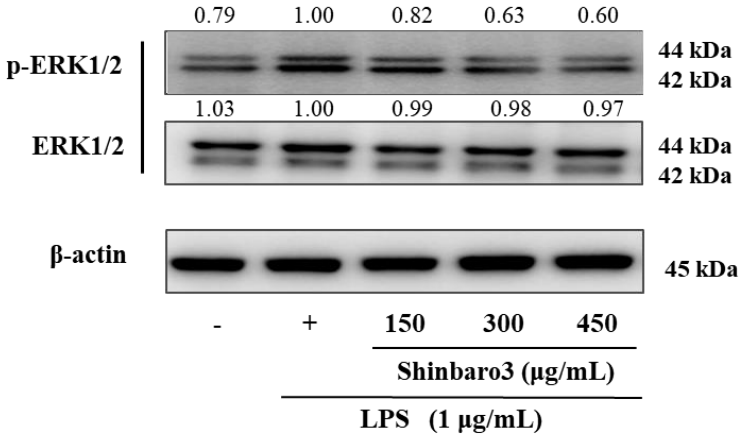
Figure 4 (c)



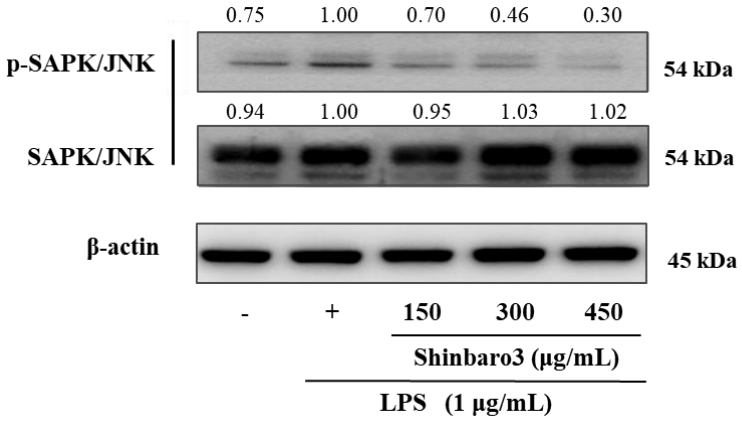
	p-IKK	IKK	beta-actin		**p-IKK**	**IKK**
-	20344.7	29100.9	30644.2		**0.55**	**1.04**
+	36741.6	27701.1	30306.8		**1.00**	**1.00**
150	29723.1	29823.2	27441.6		**0.89**	**1.19**
300	22710.6	28694.5	26582.4		**0.70**	**1.18**
450	14476.0	27680.1	26808.4		**0.45**	**1.13**

Figure 4: Effects of Shinbaro3 on NF-κB activation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/mL) and Shinbaro3 (150, 300, and 450 μg/mL) for 2 h to investigate protein expression. Nuclear and cytoplasm extracts were analysed via Western blotting. (Figure 4a, 4b, 4c) The expression levels of NF-κB (p65 and p50 subunits) (nuclear fraction), p-IκB-α, IκB-α, p-IKK-α, and IKK-α (cytoplasmic fraction) were measured using specific antibodies. Lamin B1 (nuclear fraction) and β-actin (cytoplasmic fraction) were used as an internal control. The data are representative of three separate experiments.

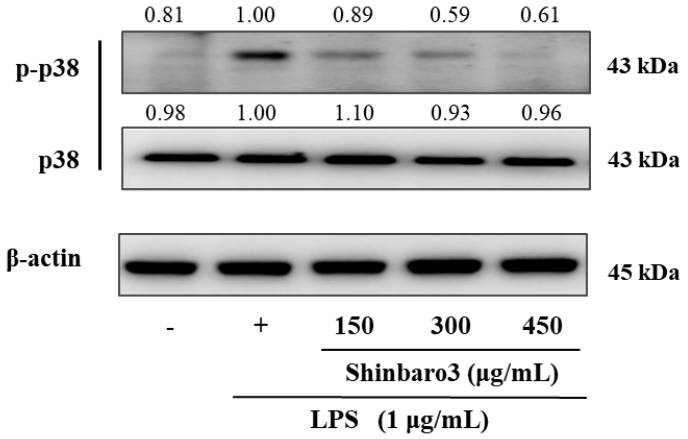
Figure 5



	p-ERK	ERK	beta-actin		**p-ERK**	**ERK**
-	22718.3	271426.7	26719.3		**0.79**	**1.03**
+	32302.7	296485.4	29976.4		**1.00**	**1.00**
150	25646.6	285785.6	29145.9		**0.82**	**0.99**
300	22937.5	324473.6	33525.6		**0.63**	**0.98**
450	20769.0	307585.9	31918.0		**0.60**	**0.97**



	p-JNK	JNK	beta-actin		**p-JNK**	**JNK**
-	19955.8	24196.3	26719.3		**0.75**	**0.94**
+	29983.6	28837.4	29976.4		**1.00**	**1.00**
150	20463.8	26685.5	29145.9		**0.70**	**0.95**
300	15373.8	33137.2	33525.6		**0.46**	**1.03**
450	9689.2	31453.2	31918.0		**0.30**	**1.02**

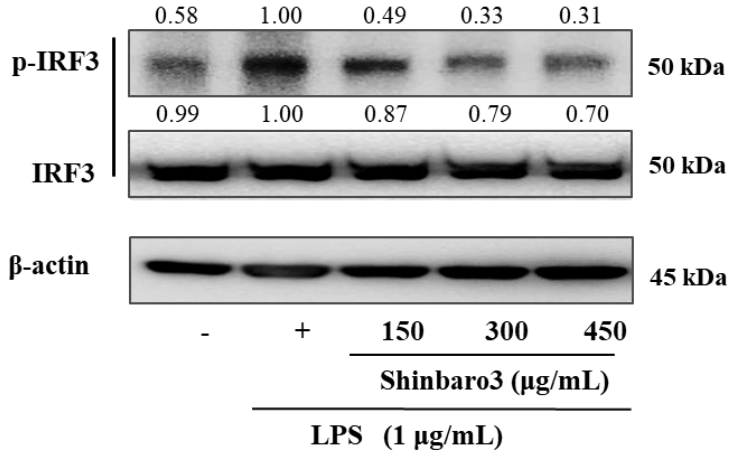


	p-p38	p38	beta-actin		**p-p38**	**p38**
-	18867.1	27028.4	26719.3		**0.81**	**0.98**
+	26113.8	30970.2	29976.4		**1.00**	**1.00**
150	22651.9	33125.2	29145.9		**0.89**	**1.10**
300	17183.4	32096.8	33525.6		**0.59**	**0.93**
450	16853.9	31696.8	31918.0		**0.61**	**0.96**

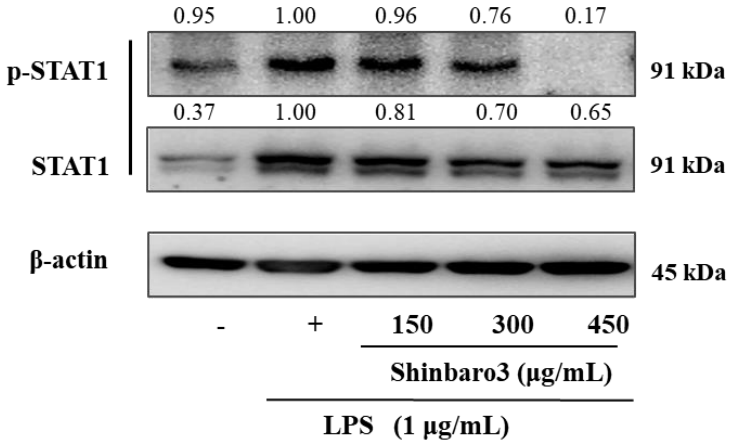
Figure 5: Effects of Shinbaro3 on MAPK phosphorylation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/mL) and Shinbaro3 (150, 300, and 450 μg/mL) for 2 h. Total cell lysates were analysed via Western blotting using anti-phospho-

ERK1/2, anti-phospho-SAPK/JNK, and anti-phospho-p38 antibodies. β-actin was used as an internal control. The data are representative of three separate experiments.

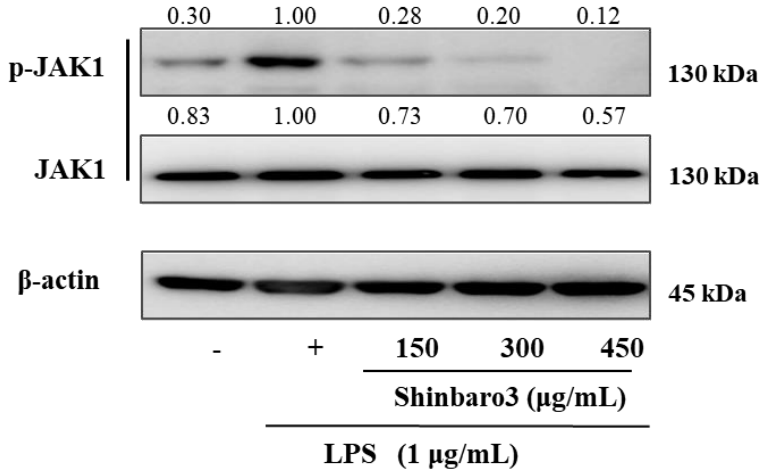
Figure 6 (a)



	p-IRF3	IRF3	beta-actin		**p-IRF3**	**IRF3**
-	15196.2	30895.9	23346.8		**0.58**	**0.99**
+	27062.7	32466.5	24294.2		**1.00**	**1.00**
150	15060.0	32232.9	27657.0		**0.49**	**0.87**
300	10943.1	30998.1	29494.5		**0.33**	**0.79**
450	10612.6	28391.4	30402.1		**0.31**	**0.70**

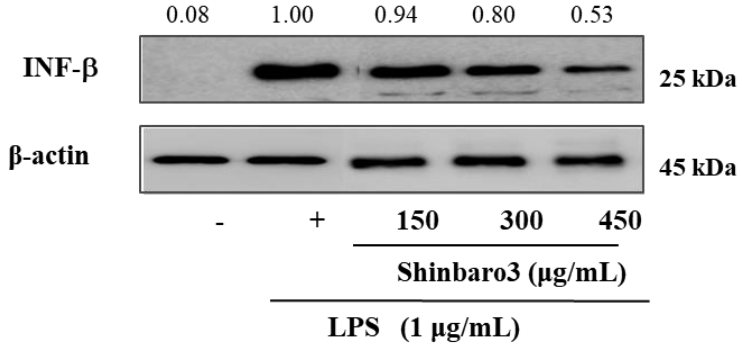


	p-STAT1	STAT1	beta-actin		**p-STAT1**	**STAT1**
-	23289.3	12242.0	23346.8		**0.95**	**0.37**
+	25496.2	34478.0	24294.2		**1.00**	**1.00**
150	27835.5	31865.5	27657.0		**0.96**	**0.81**
300	23523.5	29254.8	29494.5		**0.76**	**0.70**
450	5375.7	28110.3	30402.1		**0.17**	**0.65**



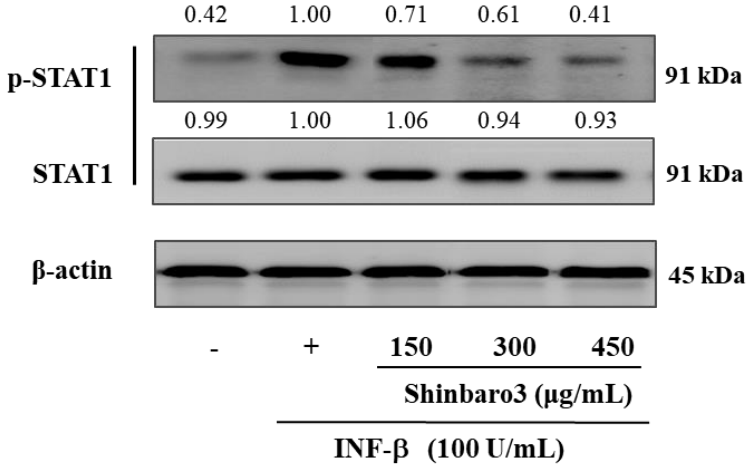
	p-JAK1	JAK1	beta-actin		**p-JAK1**	**JAK1**
-	8203.8	30045.2	23346.8		**0.30**	**0.83**
+	28300.9	37758.2	24294.2		**1.00**	**1.00**
150	8873.5	31392.7	27657.0		**0.28**	**0.73**
300	6728.6	32296.4	29494.5		**0.20**	**0.70**
450	4418.8	26883.8	30402.1		**0.12**	**0.57**

Figure 6 (b)

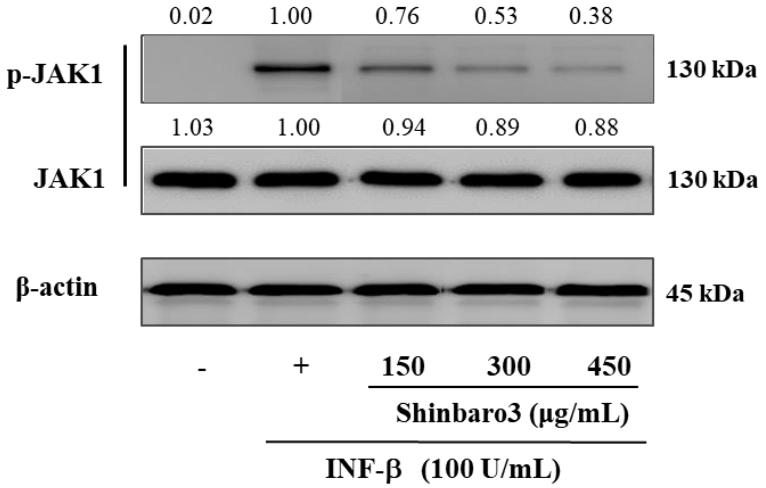


	INF-b		beta-actin		**INF-b**
-	2302.3		25028.3		**0.08**
+	33474.1		28768.0		**1.00**
150	31509.6		28664.0		**0.94**
300	25480.4		27248.0		**0.80**
450	15315.9		24985.9		**0.53**

Figure 6 (d)



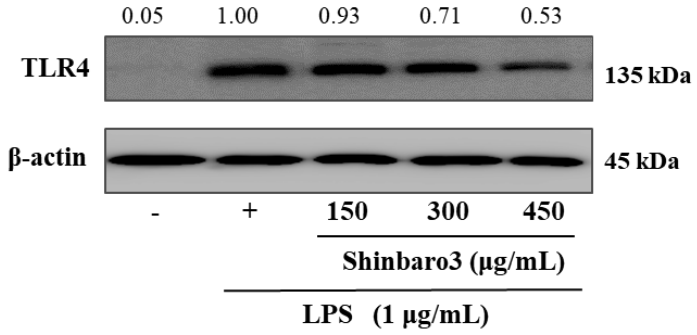
	p-STAT1	STAT1	beta-actin		**p-STAT1**	**STAT1**
-	12757.3	26469.0	32263.4		**0.42**	**0.99**
+	30963.1	27069.0	32527.1		**1.00**	**1.00**
150	21267.7	27669.5	31384.2		**0.71**	**1.06**
300	18860.8	25499.8	32665.1		**0.61**	**0.94**
450	12907.6	25781.5	33377.2		**0.41**	**0.93**

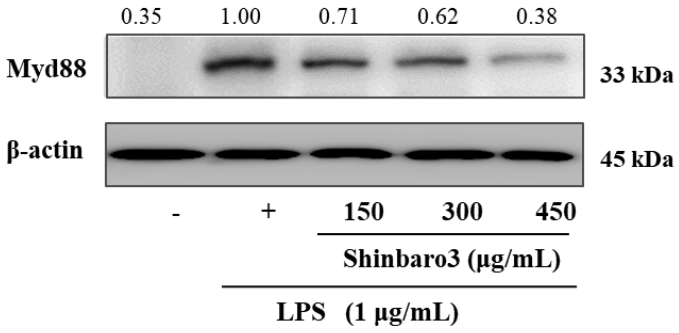


	p-JAK1	JAK1	beta-actin		**p-JAK1**	**JAK1**
-	620.2	32534.3	32263.4		**0.02**	**1.03**
+	31462.5	31994.3	32527.1		**1.00**	**1.00**
150	23101.2	29128.9	31384.2		**0.76**	**0.94**
300	16621.3	28609.0	32665.1		**0.53**	**0.89**
450	12197.9	28819.2	33377.2		**0.38**	**0.88**

Figure 6: Effects of Shinbaro3 on the IRF3/STAT1 signalling pathway in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/mL) and Shinbaro3 (150, 300, and 450 μg/mL) for 4 h. The expression levels of (a) IRF3, STAT1, JAK1 and their phosphorylated forms were detected with specific antibodies. (b) Protein expression of INF-β was assessed in RAW 264.7 cells under the above condition. (d) RAW 264.7 cells were stimulated with Shinbaro3 (150, 300, and 450 μg/mL) in presence of IFN-β (100 U/mL) for 4 h. Protein expression of JAK1 and STAT1, and their phosphorylated forms were detected. β-actin was used as an internal control. The data are representative of three separate experiments.

Figure 7 (a)





	TLR4	Myd88	beta-actin		**TLR4**	**Myd88**
-	2221.9	13480.9	36984.5		**0.05**	**0.35**
+	35266.5	33485.4	31935.5		**1.00**	**1.00**
150	30754.8	22165.9	29808.6		**0.93**	**0.71**
300	25596.2	21163.5	32687.5		**0.71**	**0.62**
450	14788.3	10016.5	25294.7		**0.53**	**0.38**

Figure 7: Effects of Shinbaro3 on the TLR4/Myd88 signalling pathway in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/mL) and Shinbaro3 (150, 300, and 450 μg/mL) for 6 h. (a) TLR4 and (c) Myd88 protein expression levels were analysed via Western blotting. β-actin was used as an internal control. The data are representative of three separate experiments.

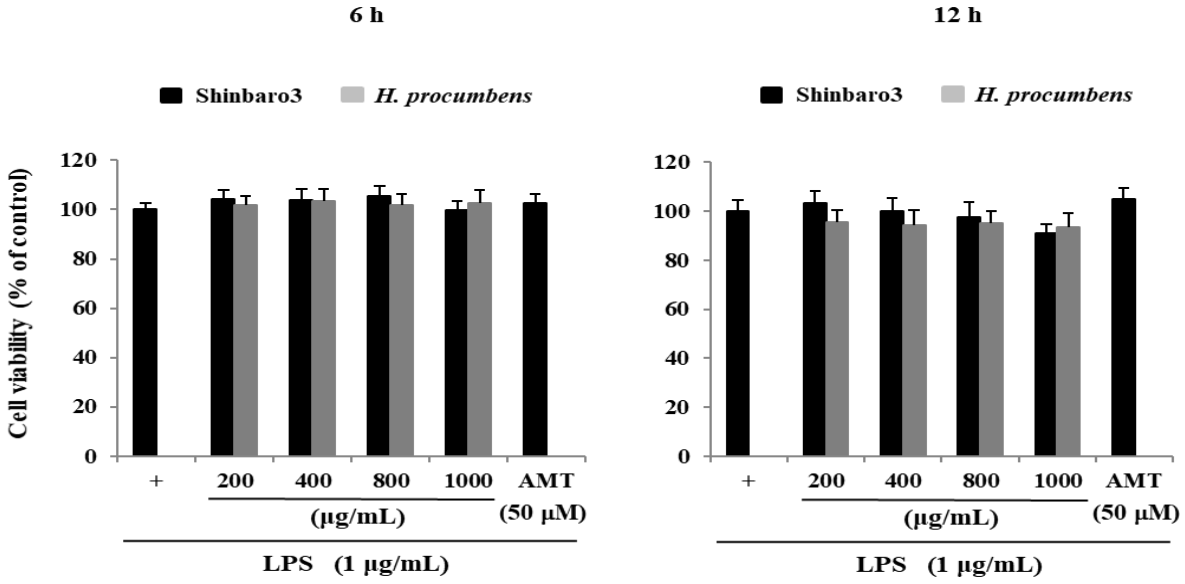
Supplementary materials

Supplementary 2

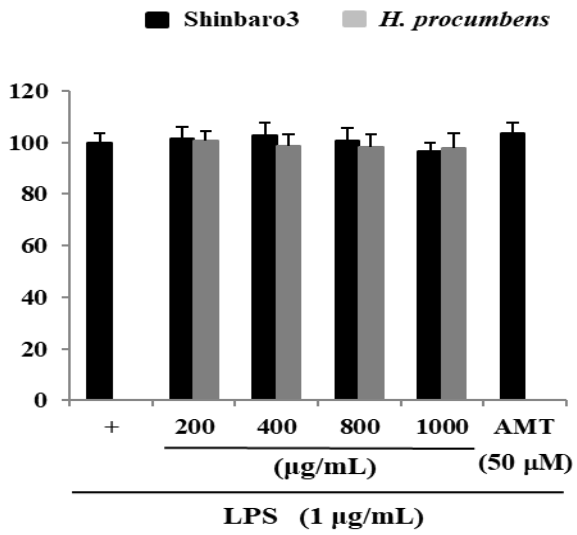
Supplemental Figure 2

: Shinbaro3 did not show any significant cytotoxicity in RAW 264.7 cells at 6, 12, and 24 h exposure. Cell viability upon Shinbaro3 or *H. procumbens* treatment for 6, 12, and 24 h were evaluated using the MTT assay, as described in the Materials and Methods. Data are presented as the mean ± SD (n=3).

MTT assay (6, 12, 24 h)



24 h


Shinbaro3
H. procumbens
120
100
80
60
40
20
0
+
200
400
800
1000
AMT
(μg/mL)
(50 μM)
LPS (1 μg/mL)

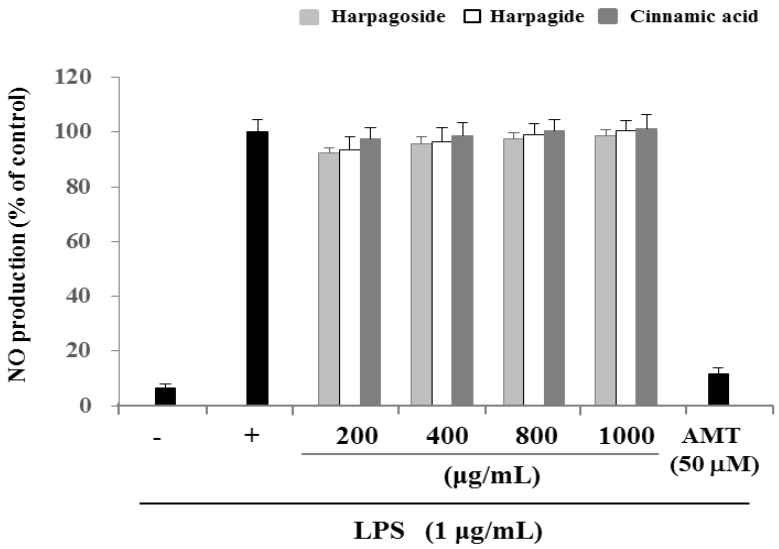
Supplementary materials

Supplementary 3

Supplemental Figure 3

: NO generation and cell viability has been found to be superior to equivalent amount of harpagoside, harpagide, or cinnamic acid in LPS-stimulated RAW 264.7 macrophage cells. (a) RAW 264.7 cells were stimulated with LPS (1 μg/mL) for 20 h in the absence or presence of harpagoside, harpagide, or cinnamic acid (200, 400, 800 or 1000 μg/mL). The nitrite concentration in the supernatant was detected via the Griess reaction. (b) Cell viability upon harpagoside, harpagide, or cinnamic acid treatment for 20 h was evaluated using the MTT assay, as described in the Materials and Methods. Data are presented as the mean ± SD (n=3).

(a) NO production (harpagoside, harpagide, cinnamic acid)



(b) MTT assay **(harpagoside, harpagide, cinnamic acid)**