Supplementary materials

Supplementary 1

Supplemental Figure 1

All western blots were assessed of their quantified density.

Figure 3 (c)

| Shinbaro3 (µg/mL) | LPS (1 µg/mL) |
|-------------------|---------------|
| -                 | +             |
| 0.07              | 0.10          |
| 1.00              | 1.00          |
| 0.79              | 0.86          |
| 0.48              | 0.81          |
| 0.47              | 0.79          |

| 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   |

| Shinbaro3 (µg/mL) | LPS (1 µg/mL) |
|-------------------|---------------|
| -                 | +             |
| 0.21              | 0.76          |
| 0.76              | 0.54          |
| 0.54              | 0.50          |
| 0.07              | 0.99          |
| 0.99              | 0.78          |
| 0.78              | 0.03          |
| 0.03              |               |

| iNOS  | COX-2  | beta-actin | iNOS  | COX-2  |
|-------|--------|------------|-------|--------|
| -     | 25 kDa | 31 kDa     | 45 kDa |       |       |
| +     |        |            |       |       |       |
| 150   | 21264.0 | 23810.8    | 21264.0 | 0.47   | 0.79   |
| 300   | 21005.4 | 23954.9    | 21264.0 | 0.48   | 0.81   |
| 450   | 22493.9 | 3165.3     | 22605.4 | 1.00   | 1.00   |

Figure 1
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.

|     | 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 4 (a)
### Figure 4 (b)

**Cytoplasm extracts**

| Shinbaro3 (μg/mL) | LPS (1 μg/mL) |
|-------------------|---------------|
| -                 | +             |
| 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)

**Cytoplasm extracts**

| Shinbaro3 (μg/mL) | LPS (1 μg/mL) |
|-------------------|---------------|
| -                 | +             |
| 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

| Shinbaro3 (μg/mL) | 0.79 | 1.00 | 0.82 | 0.63 | 0.60 |
|------------------|------|------|------|------|------|
| 150              | 44 kDa | 42 kDa | 44 kDa | 42 kDa | 45 kDa |
| 300              | - | + | 150 | 300 | 450 |
| 450              | + | + | 150 | 300 | 450 |

| LPS (1 μg/mL) | 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 |
**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)**

| Shinbaro3 (μg/mL) | p-IRF3 | β-actin | IRF3 | p-IRF3 | IRF3 |
|-------------------|--------|---------|------|--------|------|
| 0.58              | 1.00   | 0.49    | 0.33 | 0.31   |      |
| 0.99              | 1.00   | 0.87    | 0.79 | 0.70   |      |

| LPS (1 μg/mL) | 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 |

| Shinbaro3 (μg/mL) | p-STAT1 | β-actin | STAT1 | p-STAT1 |
|-------------------|---------|---------|-------|---------|
| 0.95              | 1.00    | 0.96    | 0.76  | 0.17    |
| 0.37              | 1.00    | 0.81    | 0.70  | 0.65    |

| LPS (1 μg/mL) | p-STAT1 | STAT1 | β-actin |
|---------------|---------|-------|---------|
| -             | 91 KDa  |       | 91 KDa  |
| +             |         |       |         |
| 150           |         |       |         |
| 300           |         |       |         |
| 450           |         |       |         |
### Table 1

| Shinbaro3 (μg/mL) | 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  |

### Table 2

| Shinbaro3 (μg/mL) | 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)

- **p-STAT1**
- **STAT1**
- **beta-actin**

- **p-JAK1**
- **JAK1**
- **beta-actin**

- **INF-β**
- **β-actin**

- **LPS (1 μg/mL)**
Figure 6 (d)

|           | 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    |

|           | 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       |
|-----------|---------|------------|------------|---------|------------|
| -         | 0.02    | 1.03       | 0.76       | 0.53    | 0.88       |
| +         | 1.00    | 1.00       | 0.94       | 0.89    | 0.88       |
| 150       |         |            |            |         |            |
| 300       |         |            |            |         |            |
| 450       |         |            |            |         |            |
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.

|        | 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 7 (a)
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)**

![MTT assay graph showing cell viability](image)
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)

![Graph showing NO production and cell viability](image-url)
(b) MTT assay (harpagoside, harpagide, cinnamic acid)