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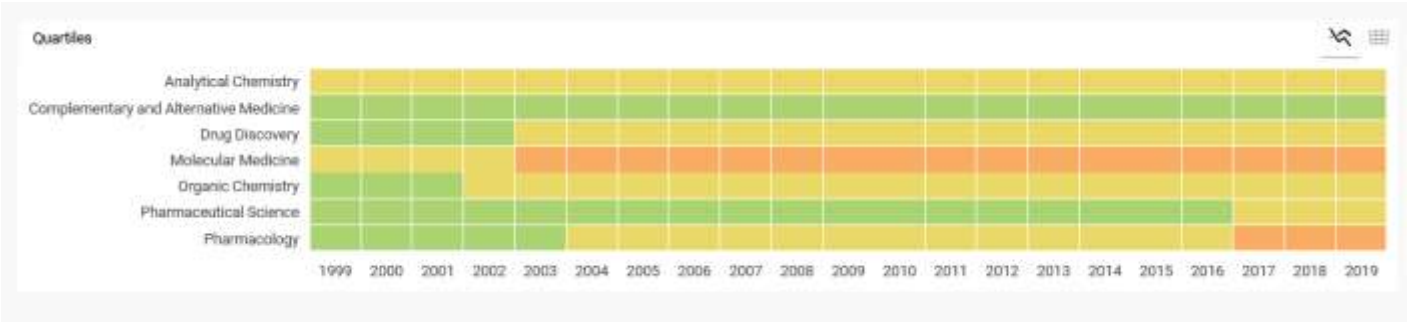
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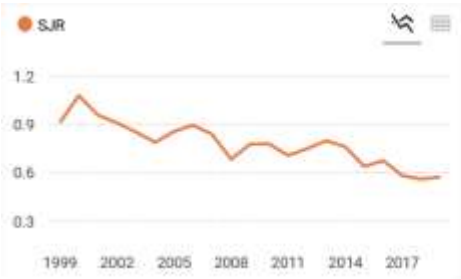
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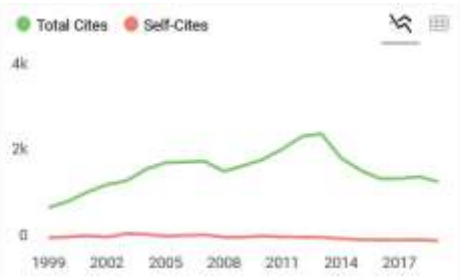
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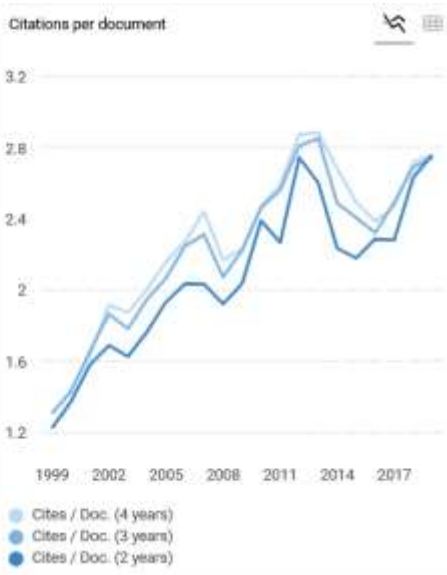
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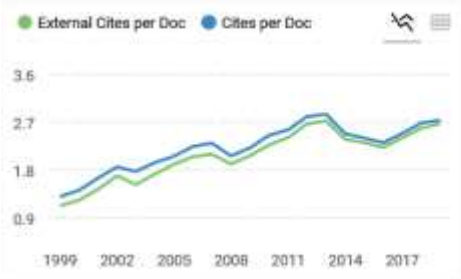
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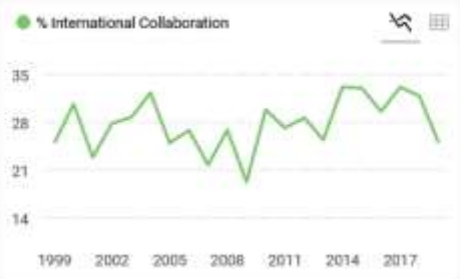
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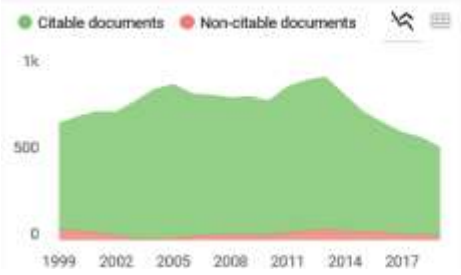
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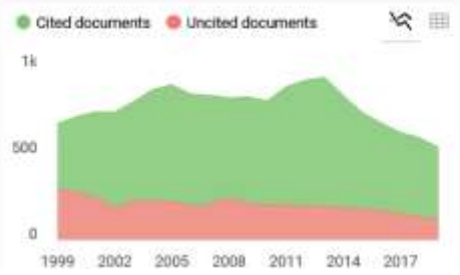
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Inhibitors of Nitric Oxide Production from *Stemona javanica*

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Abstract

In our screening program for bioactive natural products from our library of tropical plants, the extract prepared from the roots of *Stemona javanica* inhibited NO production in mouse macrophage-like cell line J774.1 stimulated by lipopolysaccharide (LPS). Bioassay-guided fractionation of the extract from *S. javanica* led to the isolation of two active compounds, stemofoline (**1**) and stemanthrene C (**2**). The inhibition mechanism of **1** was proposed to suppress iNOS expression in J774.1 cells stimulated by LPS, whereas that of **2** was due to potent radical scavenging activity resulting in NO inhibitory activity.

Key words
Stemona javanica · Stemonaceae · stemofoline · stemanthrene C · iNOS · DPPH radical scavenge

Abbreviations

DPPH:	diphenylpicrylhydrazine radical
IC_{50}:	inhibitory concentration at 50%
iNOS:	inducible NO synthase
J774.1:	mouse macrophage-like cell line
LPS:	lipopolysaccharide
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NO:	nitric oxide

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Nitric oxide (NO) is an important intracellular and intercellular signaling molecule that acts as a mediator in the cardiovascular, nervous, and immunological systems [1]. NO is involved in biological reactions such as vasorelaxation [2], inhibition of platelet aggregation [3], neurotransmission [4], inflammation [5], and immunoregulation [6]. In mammalian cells, NO is synthesized from L-arginin (L-Arg) by NO synthase (NOS), which is classified into three homologues: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) [7]. iNOS produces large amounts of NO in macrophages stimulated with lipopolysaccharide (LPS) and in proinflammatory cytokines such as tumor necrosis factor (TNF) and interferon-γ (IFN-γ) [8]. Therefore, inhibition of excess NO production by iNOS might be of potential therapeutic value for oxidative stress–induced inflammatory diseases and septic shock [9].

Plants belonging to the genus *Stemona* such as *S. tuberosa, S. japanica*, and *S. sessilifolia* (Stemonaceae) are widely used in China

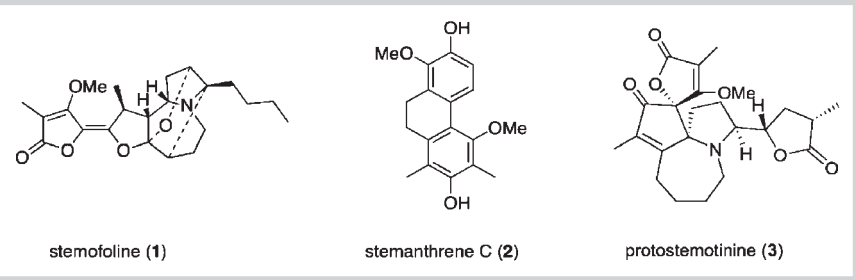

Fig. 1 Structures of stemofoline (**1**) and stemanthrene C (**2**) as inhibitors of NO production.

and Japan for their insecticide and antitussive activities [10]. Most of the previous research related to the chemical constituents in *Stemona* species has been limited to the structures of a class of polycyclic complex alkaloids [11, 12]. In this paper, we describe the isolation of two inhibitors of NO production from *S. javanica* and their inhibitory mechanisms.

Screening of NO production inhibitory activity for our tropical plant extract library collected in Malaysia and Indonesia was carried out at 50, 25, and 12.5 µg/mL of each MeOH extract. The methanol extract from the root of *Stemona javanica* collected at Alas Purwo, Banyuwangi, Indonesia, exhibited NO production inhibitory activity in J774.1 cells stimulated by LPS. The inhibition ratios at 100, 50, 25, and 12.5 µg/mL were 76%, 63%, 49%, and 19%, respectively, although cell viability was above 95% at each concentration. The methanol extract of *S. javanica* was partitioned between H_2O and hexane, chloroform, and then ethyl acetate. The *n*-hexane– and chloroform-soluble fractions showed NO production inhibitory activity of 60% and 40%, respectively, at 50 µg/mL. We used bioguided fractionation of the chloroform-soluble fraction to identify stemofoline (**1**) [13] and stemanthrene C (**2**) [14] (**Fig. 1**) as inhibitors of NO production by comparing their spectral data with that of the nonactive compound protostemotinine (**3**) [15]. Stemofoline (**1**) was one of the major *Stemona* alkaloids, and stemanthrene C (**2**) was classified as a stilbenoid.

Stemofoline (**1**) and stemanthrene C (**2**) inhibited NO production in a dose-dependent manner, and they showed little effect on cell viability (**Fig. 2A, B**). The IC_{50} values in this assay were 16.4 and 18.3 µg/mL, respectively. To evaluate the compounds' effect on the expression of iNOS protein in J774.1 cells stimulated by LPS, Western blotting analysis was performed. When the cells were stimulated by LPS, iNOS protein in the cells was overexpressed due to overproduction of NO. Stemofoline (**1**) decreased iNOS protein expression dose-dependently, although stemanthrene C (**2**) did not (**Fig. 3**).

LPS stimulation in J774.1 cells induces iNOS overexpression, subsequent to NO synthesis, resulting in physiological reactions such as inflammation and mutagenesis [1]. We focused on DPPH radical scavenging activity to answer the question of whether the isolated compounds were active as radical scavengers. Stemanthrene C (**2**) scavenged the DPPH radical dose-dependently, with an IC_{50} value of 27.5 µg/mL. On the other hand, stemofoline (**1**) had no activity against the DPPH radical even at 100 µg/mL (**Table 1**).

Stemona alkaloids from Stemonaceae species have been classified into eight groups [16]. Due to the interest in complex chemical structures, there have been many reports on their structure elucidation, total synthesis, and biological activities [11]. Stemofoline (**1**), possessing a rigid pentacyclic core and a pendant conjugated butenolide, has been isolated from stems and leaves of the *Stemona* species, and its insecticidal and antifeedant activities have been reported [17].

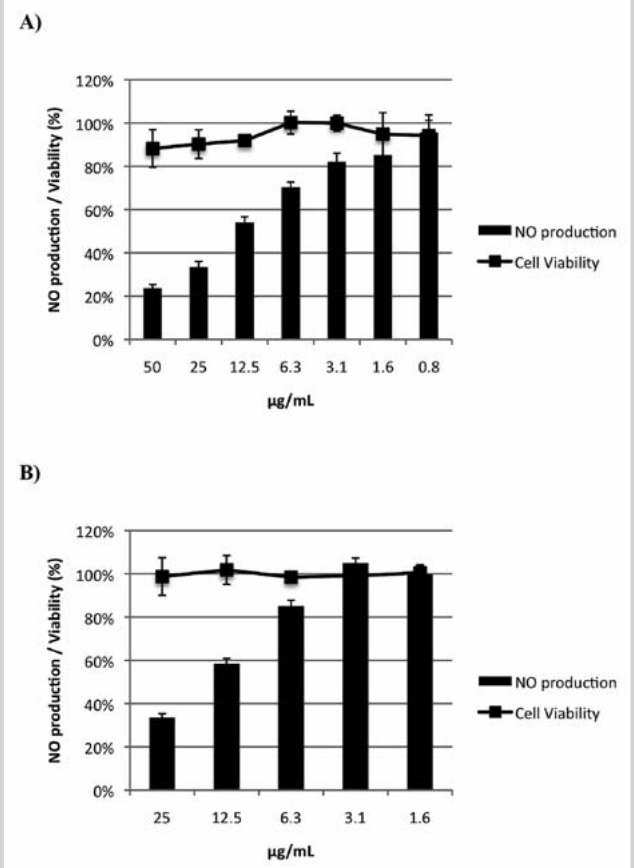

Fig. 2 NO production ratio in J774.1 cells stimulated with LPS. Panel **A** stemofoline; panel **B** stemanthrene C. The assays were performed in triplicate. Error bars represent SD.

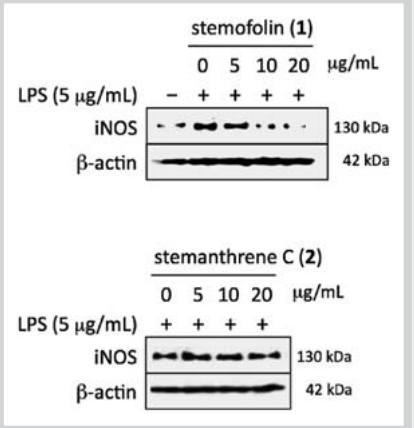

Fig. 3 iNOS protein expression levels in J774.1 cells treated with **1** or **2** and with/without LPS.

Table 1 PPH radical scavenging activity of **1** and **2** at different concentrations.

Compounds (μg/mL)	Radical scavenging ratio (%)	
	Stemanthrene C (2)	Stemofoline (1)
100	80	15
50	70	12
25	55	10
12.5	30	8

From *Stemona* species, stilbenoids such as pinosylvin, stilbostemins, and stemofurans, which show biological activities such as antifungal activity [14] and inhibition of leukotriene biosynthesis [18], have been isolated together with *Stemona* alkaloids. However, no activity of stemanthrenes A–C, which possess a dihydrophenanthrene structure, has been reported. Resveratrol, one of the major stilbenoids, showed NO production inhibition by suppression of iNOS expression [19], but stemanthrene C (**2**) showed NO production inhibitory activity by scavenging radicals. This is the first report of the NO production inhibitory activity of **1** and **2**.

In summary, from a bioguided fractionation assay on NO production inhibition, two active compounds were isolated from the roots of *S. javanica*. Stemofoline (**1**), one of the major *Stemona* alkaloids, suppressed iNOS expression stimulated by LPS, resulting in the inhibition of NO production, but it was not able to scavenge radicals. On the other hand, stemanthrene C (**2**), a stilbenoid from *Stemona*, decreased NO production in J774.1 cells stimulated by LPS, but the mode of action of **2** was not to suppress iNOS expression but to scavenge radicals.

Materials and Methods

The roots of *Stemona javanica* were collected at Alas Purwo, Banyuwangi, Indonesia, in 2006. The botanical identification was made by Ms. Sri Wuryanti, Purwodadi Botanical Garden, Indonesia. A voucher specimen (no. AP070912) has been deposited in the herbarium at Purwodadi Botanical Garden, Pasuruan, Indonesia.

Supporting information

Detailed protocols for extraction and isolation, for the NO production assay using the J774.1 cell line, for cell viability measurement, for Western blotting, and for the DPPH radical assay are available as Supporting Information.

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