



***In vitro* anti-inflammatory activity of 5 Cambodian *Stephania* species: from crude extracts to bioactive alkaloids**

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(Accepted October 27, 2024)

ABSTRACT

Context: *Stephania* species are widely used in Cambodian traditional medicine for joint pains and edema. **Objectives:** The aim of this study was to analyze the *in vitro* anti-inflammatory activity of different extracts of 5 *Stephania* species in relation with their alkaloid composition to contribute to validating their medicinal applications. **Methods:** The anti-inflammatory activity of crude extracts and seven alkaloids (cepharanthine, crebanine, palmatine, roemerine, stephanine, tetrahydropalmatine, and xylopinine) from these *Stephania* species was evaluated by investigating their ability to inhibit lipopolysaccharides-induced nitric oxide production in macrophage RAW 264.7 cells. **Results:** For each species evaluated, hydro-ethanolic extract showed the highest anti-inflammatory ratio with the values of half maximal concentration $IC_{50-NO} < 10 \mu\text{g/ml}$. Interestingly, none of the aqueous extracts were active. **Conclusion:** This study demonstrates for the first time that the tubers of 5 different *Stephania* species collectively known in Khmer language as “Komar Pich” have potent anti-inflammatory effects and this may be attributed to the main alkaloids found in each species. These results suggest that these plants would serve as a natural source for the discovery of novel anti-inflammatory agents.

Keywords: Anti-inflammatory, Cambodia, isobologram, LPS, pharmacognosy, *Stephania*

INTRODUCTION

Macrophages play an important role in defending the host during inflammation (Marzocco et al., 2015). They detect pathogenic substances through pattern-recognition receptors and subsequently initiate and regulate inflammatory responses by using a wide range of soluble pro-inflammatory mediators such as nitric oxide (NO), pro-inflammatory enzymes (e.g. cyclooxygenase-2), reactive oxygen species (ROS), and cytokines (Medzhitov and Janeway, 1997). Macrophages and monocytes that have been activated by lipopolysaccharides (LPS) are known to produce inflammatory mediators (Grahames et al., 1999, Mehta et al., 2001). During the inflammatory process, the inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2) produce significant amounts of the pro-inflammatory mediators NO and prostaglandin E2 (PGE2). Several studies have suggested that the overexpression of COX-2 or iNOS is closely link with the pathogenesis of inflammation, cancer, multiple sclerosis, Parkinson's syndrome, Alzheimer's disease. Consequently, the development of enzyme inhibitors or repressors of enzyme synthesis that selectively target the inducible forms of these enzymes has been the subject of numerous studies. Inhibiting the production of NO and PGE2 becomes an important therapeutic target in the development of anti-inflammatory agents (Yang et al., 2012).

The species belonging to the genus *Stephania* (Menispermaceae) are herbaceous or woody vines frequently with a tuberous rootstock. This genus is characterized by unicarpellous ovaries and peltate synandria and leaves (Hul et al., 2017). They are mainly distributed in Asia, and have long been used in traditional medicine for the treatment of various pathologies (Zhu et al., 1983). A wide number of chemical and pharmacological studies have been conducted on the tuber of this genus. Over 150 alkaloids together with coumarins, flavonoids, lignans, steroids, and terpenoids have been isolated and characterized in the genus, and many of these have been evaluated for biological activities such as antimalarial, anticancer, anti-inflammatory and analgesic activities (Semwal et al., 2010).

Seven species of *Stephania* were recorded in Cambodia: *S. cambodica* Gagnep., *S. japonica* (Thunb.) Miers var. *discolor* (Blume) Forman and var. *timoriensis* (DC.) Forman, *S. oblata* Craib, *S. pierrei* Diels, *S. rotunda* Lour., *S. suberosa* Forman and *S. venosa* (Blume) Spreng. (Dary, 2016). They are collectively known in Khmer language as "Doeum Komar Pich" because of their similar shape of leaves, tuber and twinning habit. "Doeum Komar Pich" is traditionally used for treatment of a number of ailments and symptoms with special attention on malaria, anxiety, wounds, joint pains and inflammation (MoH, 2013). To our knowledge, despite the well-established use of "Komar Pich" tuber in Cambodian traditional medicine for inflammation related illness, their anti-inflammatory activity has not yet been investigated.

Here, we assess the *in vitro* anti-inflammatory potential of the crude tuber extracts of 5 species of *Stephania*: *S. cambodica*, *S. pierrei*, *S. rotunda*, *S. suberosa*, and *S. venosa* and identify their bioactive compounds *via* inhibition of NO production stimulated by *E. coli*'s LPS. In a second step, the 2 most active alkaloids are selected for isobologram analysis.

METHODS

Plant material: The tubers of 5 *Stephania* species: *S. cambodica*, *S. pierrei*, *S. rotunda*, *S. suberosa*, and *S. venosa* were collected in different provinces in Cambodia. The plants were botanically authenticated by Prof. Hul Sovanmoly and the vouchers were deposited at The Paris Herbarium, France (*Stephania pierrei* Hul et al. 5001-5005, P02440215; *S. cambodica* Dary 18, P00750367; *S. venosa* Dary & Kim 7, P00688173). Tubers were cut into pieces, allowed to air dry at room temperature for two weeks, and then ground into a fine powder.

Chemicals: Acetonitrile, ethanol and formic acid of HPLC Ultra-Gradient grade alongside with dichloromethane, and ammonia of analytical grade were purchased from Carlo Erba (Val de Reuil, France). Potassium phosphate monobasic was obtained from Fluka (Saint Quentin Fallavier, France). Cepharanthine (#89599) and tetrahydropalmatine (#89807) were purchased from Phytolab (Vestenbergsgreuth, Germany). Palmatine (#361615) was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Roemerine (#Amb4417140) and xylopinine (#Amb8470359) were purchased from Ambinter (Orléans, France).

Preparation of crude extracts: Aqueous and hydro-ethanolic extracts were prepared in order to mimic as closely as possible the traditional modes of preparation of the 5 species in Cambodia. As *Stephania* species are well-known for alkaloid rich composition, percolation using dichloromethane was also carried out. For aqueous extract, 10 g of dried and powdered tuber were extracted with 150 ml of boiling distilled water for 30 min and filtered. The obtained aqueous extracts were then freeze-dried. As for hydro-ethanolic extract, 150 ml of 50% (v/v) ethanol was macerated with 10 g of dried and powdered plant material for seven days at room temperature and free from light. The extract was then filtered, evaporated under low pressure, and the resulting aqueous solution was freeze-dried. Another crude extract was dichloromethane extract in which 15 ml of a 3% (v/v) ammonia solution was used to moisten 10 g of dried and powdered plant material for 4 hours at room temperature. Then the mixture was percolated with 100 ml of dichloromethane (CH₂Cl₂). Dichloromethane extract was evaporated *in vacuo* at 40°C. All dried extracts were stocked at room temperature until time use.

HPLC analyses of dichloromethane extracts: High performance liquid chromatography was performed using the previously described method with minor modification of sample preparation in order to obtain a chemical HPLC fingerprint of dichloromethane extracts of *Stephania* species (Bory et al., 2010). The retention times and UV spectra of the samples and standard alkaloids were compared in order to identify the plant compounds of interest in the extracts.

Alkaloid isolation and characterization from each Stephania species: *S. cambodica:* the isolation of nine alkaloids: angkorwatine, asimilobine-β-D-glucopyranoside, isocorydine, jatrorrhizine, oblongine, palmatine, roemerine, stepharine and tetrahydropalmatine, from the hydro-ethanolic extract of *S. cambodica* tuber by preparative HPLC has been described in our previous study (Dary et al., 2017b). *S. pierrei:* the identification of cepharantine in dichloromethane extract

was carried out with the comparison of the retention times, and UV spectra between the sample and standard compound. *S. rotunda*: the dried powder of *S. rotunda* tuber (0.5 g) was extracted with 10 ml of ethanol 50% (v/v) for 15 min in a microwave (CEM Corporation Matthews, NC, USA). This extractive protocol was developed in our previous study (Dary et al., 2017a). The dried extract (1 mg) was dissolved in 1 ml of methanol before UHPLC analysis. Chromatographic separation was performed on a Zorbax Eclipse Plus RRHD-C18 column (1.8 μ m, 50 \times 2.1 mm, Agilent), operated at 30°C. The mobile phase consisted of a gradient elution of formic acid 0.1% (v/v) (solvent A) and ethanol (solvent B). The gradient program was: 0–1 min at 5% of B, 1–7 min from 5 to 42% of B with 3 min of post-time at a flow rate of 0.35 ml/min. The injected volume was 2 μ l. The identification of three alkaloids (palmatine, roemerine, tetrahydropalmatine) in the extract was based on the retention times, and UV spectra in samples and standard solutions. *S. suberosa*: three alkaloids (cepharantine, tetrahydropalmatine, and xylopinine) were identified in the dichloromethane extract by comparing the UV spectra and retention times of the samples with those of standard alkaloids. *S. venosa*: the dried powder of *S. venosa* tuber (0.5 g) was extracted with 10 ml of ethanol 50% (v/v) for 10 min in a microwave (CEM Corporation Matthews, NC, USA). This extractive protocol was developed in our previous study (Dary et al., 2017a). The 10 mg of dried extract was dissolved in 2.5 ml of methanol (70:30, v/v) and 0.1% (v/v) formic acid mixture. A Gilson PLC 2020@ preparative chromatograph with a DAD detector (LT350026, Gilson inc., USA) was used to isolate the compounds. The components were separated and purified using the Luna C18 column (5 μ m, 150 \times 21.2 mm, Phenomenex). The mobile phase in gradient mode was a solvent system with methanol (B) and formic acid 0.1% (v/v) (A). A linear gradient from 35% to 100% B was used to optimize the eluting program (15–40 min). Monitoring was done at 272 nm, and the flow rate was 12 ml/min. A total of 33 fractions was collected and controlled by HPLC analysis to obtain 5 pure compounds: tetrahydropalmatine (0.7 mg); palmatine (1.2 mg); roemerine (0.4 mg); crebanine (3.1 mg) and stephanine (4.0 mg). The structural elucidation of isolated compounds was based on spectroscopic experiments: 1D and 2D NMR, ESIMS/HRESIMS and by comparison of the spectral and chemical data with literature (Blanchfield et al., 2003, Liu et al., 2014, Ma et al., 2014).

NO production assay: Organic extracts and alkaloids (cepharantine, crebanine, palmatine, roemerine, stephanine, tetrahydropalmatine and xylopinine) used in NO production assay were dissolved in DMSO while aqueous extracts were dissolved in sterile water. Murine macrophages from the RAW 264.7 cell line are activated using *E. coli* LPS and then treated with the test material for 24 hours. After incubation, nitric oxide production is assessed by indirectly measuring nitrite/nitrate accumulation, which are the stable end products of nitric oxide oxidation, in the culture medium using a spectrophotometric technique based on the Griess reaction. Cells were seeded into 48-well tissue culture plates at the concentration of 1.105 cells/ml (200 μ l/well) in DMEM medium with stable L-glutamine, supplemented with penicillin 100 IU/ml - streptomycin 100 μ g/ml and 10% of inactivated calf serum. They were then incubated for 24 h at 37°C (5% CO₂). At the end of the incubation period the culture medium was replaced by 200 μ l of medium containing the appropriate concentrations of the test material (C1, C2, C3), and cells were incubated at 37°C (5% CO₂) during 1 h. At the end of the incubation period, pro-inflammatory

LPS from *E. coli* were added to cell cultures (1 µg/ml), and cells were incubated at 37°C (5% CO₂) during 24 h. 100 µl of the supernatants were transferred into the wells of a 96-well tissue culture plate, and 100 µl of the Griess reagent were added in each well. The Optical Density of each well was read at 540 nm by a fluorescence-luminescence reader Infinite M200 Pro (TECAN) following a 15-minute incubation period at room temperature. The test material-treated well results were compared to the 100% viability, untreated control wells (DMSO, 100% viability), and the results were converted to percentage values. All assays were performed in triplicate wells and a positive control with various concentrations of dexamethasone was included. Concentrations that result in a 50% decrease of NO release (IC_{50-NO}) were determined by non-linear regression analysis, using software Tablecurve Version 2.0.

Cell viability assay: To validate the NO production assay, cell viability was concurrently assessed. Each well received 100 µl of WST-1 reagent (1/10 dilution) after the culture medium was decanted. The Optical Density in each well was measured at 450 nm using a fluorescence-luminescence reader Infinite M200 Pro (TECAN) following a 30-min incubation period at 37°C (5% CO₂). The test material-treated well results were compared to the 100% viability, DMSO-treated control wells, and the results were converted to percentage values. Using the program Tablecurve Version 2.0, concentrations that result in a 50% reduction in cell viability (IC_{50-cytotox}) were determined through non-linear regression analysis. The anti-inflammatory ratio (AI) was obtained from the ratio between the toxicity and the anti-inflammatory activity. It was expressed as follows: Anti-inflammatory ratio = IC_{50-cytotox} / IC_{50-NO}. This ratio is used to quantify the selectivity of *in vitro* anti-inflammatory agents.

Isobologram analysis: Following pharmacological test of the main compounds of the 5 *Stephania* species, synergism between the 2 most anti-inflammatory compounds was investigated *in vitro* using standard isobolar analysis.

RESULTS AND DISCUSSION

The anti-inflammatory effects of each *Stephania* species (Table 1, Figure 1) are presented in Table 2. For each species evaluated, hydro-ethanolic extract showed the highest anti-inflammatory ratio compared with other types of extracts with IC_{50-NO} < 10 µg/ml. Hydro-ethanolic extracts of tubers of *Stephania suberosa* and *S. venosa* were more efficacious against NO production with anti-inflammatory ratio of AI>181.82 and AI>163.93, respectively. The dichloromethane extracts of studied *Stephania* species exhibited moderate activities except for that of *S. suberosa* which demonstrated potent anti-inflammatory activity with appreciable anti-inflammatory ratio (AI=42.63). Despite not having a significant inhibitory effect on NO production, the aqueous extracts were not cytotoxic to RAW 264.7 cells at the concentration of 100 µg/ml. These findings indicate potential safety of aqueous extracts used in traditional medicine. Furthermore, they constitute the first report about NO production inhibitory effects of these 5 Cambodian species collectively known in Khmer language as “Komar Pich” tubers. According to these results, *S. suberosa* has the highest anti-inflammatory potential among the 5 *Stephania* species tested.

Table 1. Voucher information of 5 *Stephania* species.

Species (Codes)	Localities District, Province	Date of collection	Voucher number	Global Sites GPS	Positional
<i>Stephania cambodica</i> (Dary 18)	Choam Ksan, Preah Vihear	March 2014	P00750367	14.223143 104.980178 E	N—
<i>Stephania pierrei</i> (Hul et al. 5001-5005)	Samrong Tong, Kampong Speu	July 2014	P02440215	11.23902 N-104.29132 E	
<i>Stephania rotunda</i> (Dary 14)	Kulen mountain, Siem Reap	September 2013	To be digitalized	13.39910 N-104.03012 E	
<i>Stephania suberosa</i> (Hul et al. 5013)	Banan, Battambang	December 2014	To be digitalized	13.115298 N-103.32571 E	
<i>Stephania venosa</i> (Dary & Kim 7)	Rabbit island, Kep	July 2013	P00688173	10.26237 N-104.19262 E	

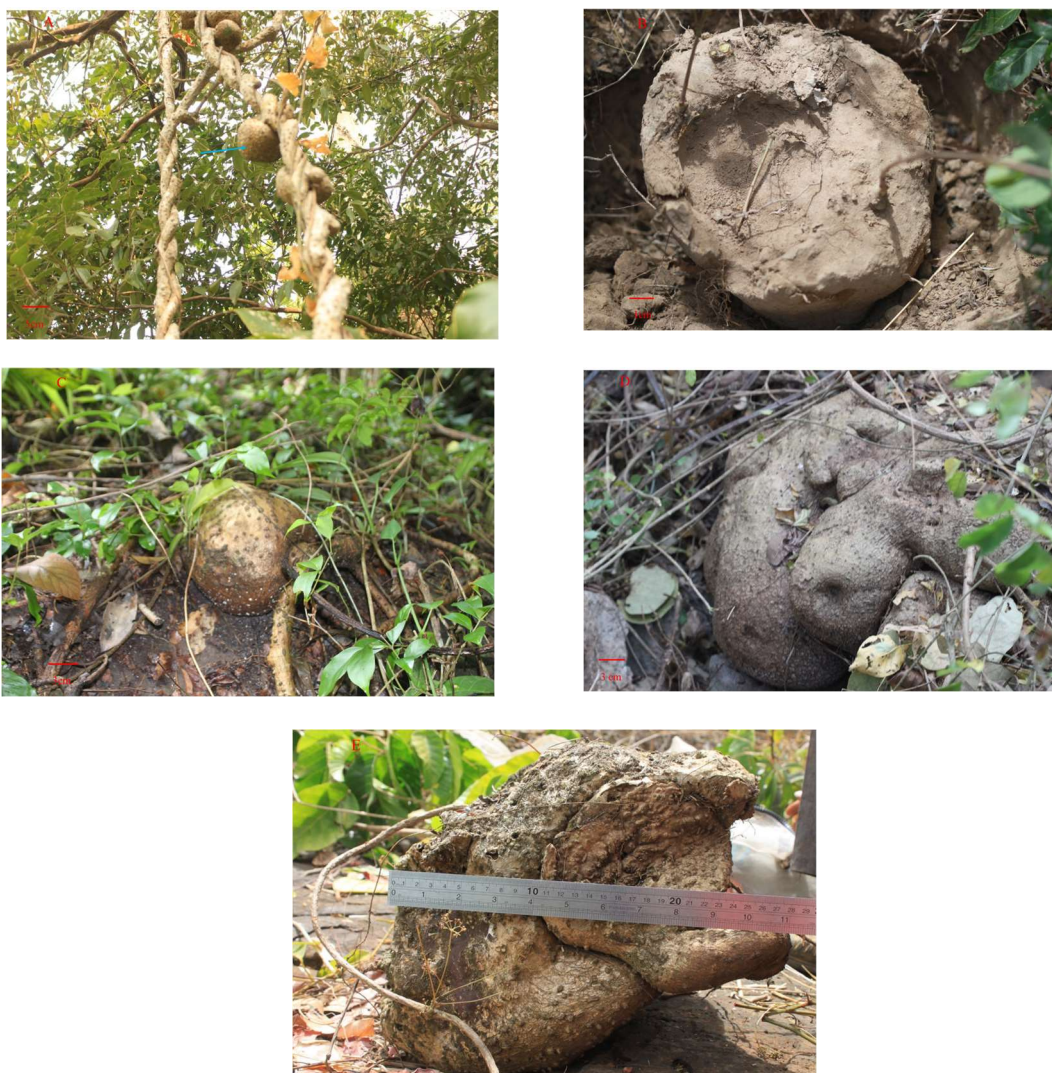


Figure 1: Tubers *in situ* of the five Cambodian *Stephania* species. A: aerial tubers of *S. cambodica*, B: underground tuber of *S. pierrei*, C: tuber of *S. rotunda*, D: tuber of *S. suberosa*, E: tuber of *S. venosa*.

Table 2. Inhibitory NO production and cytotoxic effects of extracts of 5 *Stephania* species.

<i>Stephania</i> species	Aqueous extracts			Hydro-ethanolic extracts			Dichloromethane extracts		
	IC ₅₀ -NO (µg/ml)	IC ₅₀ -cytox (µg/ml)	AI	IC ₅₀ -NO (µg/ml)	IC ₅₀ -cytox (µg/ml)	AI	IC ₅₀ -NO (µg/ml)	IC ₅₀ -cytox (µg/ml)	AI
<i>S. cambodica</i>	64.44	>100	>1.55	7.27	63.40	8.72	18.15	48.76	2.69
<i>S. pierrei</i>	>100	>100	ND	2.36	34.50	14.62	8.12	15.40	1.90
<i>S. rotunda</i>	78.81	>100	>1.27	2.91	>100	>34.36	4.25	68.52	16.12
<i>S. suberosa</i>	>100	>100	ND	0.55	>100	>181.82	1.80	76.73	42.63
<i>S. venosa</i>	>100	>100	ND	0.61	>100	>163.93	19.60	22.50	1.15

AI: anti-inflammatory ratio.

ND: not determined

The results of anti-inflammatory activity were investigated with the alkaloidal composition of the studied extracts. The HPLC analysis of dichloromethane extracts of each *Stephania* species showed similarities between *S. cambodica* and *S. rotunda* with common peaks while *S. pierrei* was characterized by a main peak. Using chromatographic methods, the main alkaloids of each species were isolated and identified (Table 3). Palmatine, roemerine, and tetrahydropalmatine were the main alkaloids in the tubers of *S. cambodica* and *S. rotunda* while crebanine, palmatine, stephanine were isolated from the tuber of *S. venosa*. Cepharanthine was identified in *S. pierrei* and *S. suberosa* tubers as xylopinine was only presented in that of *S. suberosa*. Tetrahydropalmatine was commonly found in four *Stephania* species except *S. pierrei*. The anti-inflammatory effects of these alkaloids are summarized in Table 4. Among the seven tested alkaloids, cepharanthine, crebanine, roemerine, and stephanine displayed the most important anti-inflammatory activity while only crebanine and stephanine exhibited high anti-inflammatory ratio. The presence of cepharanthine in *S. suberosa* and crebanine and stephanine in *S. venosa* tubers, respectively, may contribute to the potent anti-inflammatory effects of those extracts. Roemerine (IC₅₀-NO = 2.01 µM) and cepharanthine (IC₅₀-NO = 2.39 µM) exhibited remarkable effects but also high cytotoxicity while crebanine (IC₅₀-NO = 2.29 µM) and stephanine (IC₅₀-NO = 1.32 µM) demonstrated potent inhibitory NO production effects with satisfactory anti-inflammatory ratio of AI=66.56 and AI=111.39, respectively.

Interestingly, palmatine and tetrahydropalmatine exhibited moderate anti-inflammatory effects with no cytotoxicity at concentration more than 280 µM. The *in vitro* anti-inflammatory activities by inhibiting NO production in RAW 264.7 cells of cepharanthine, crebanine, and palmatine were consistent with previous studies (Aota et al., 2018, Intayoung et al., 2016, Ishikawa et al., 2016, Paudel et al., 2016).

Table 3. Chemical structures of the main isolated alkaloids in each *Stephania* species

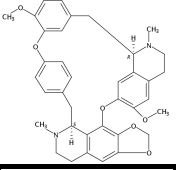
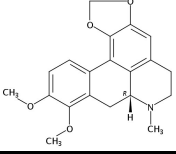
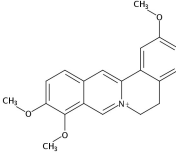
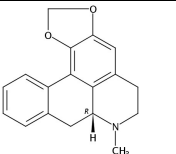
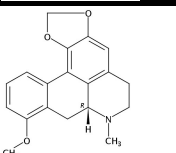
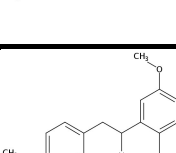
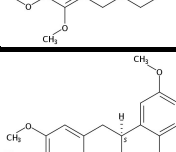
Alkaloids (group)	Chemical structures	Species (References)
Cepharanthine (bisbenzylisoquinoline)		<i>S. pierrei</i> <i>S. suberosa</i> (Patra et al., 1986)
Crebanine (aporphine)		<i>S. venosa</i> (Le et al., 2017)
Palmatine (protoberberine)		<i>S. cambodica</i> (Dary et al., 2017b) <i>S. rotunda</i> (Thuy et al., 2005) <i>S. venosa</i>
Roemerine (aporphine)		<i>S. cambodica</i> (Dary et al., 2017b) <i>S. rotunda</i> (Thuy et al., 2005) <i>S. venosa</i>
Stephanine (aporphine)		<i>S. venosa</i> (Le et al., 2017)
Tetrahydropalmatine (tetrahydroprotoberberine)		<i>S. cambodica</i> (Dary et al., 2017b) <i>S. rotunda</i> (Thuy et al., 2005) <i>S. suberosa</i> <i>S. venosa</i> (Le et al., 2017)
Xylopinine (tetrahydroprotoberberine)		<i>S. suberosa</i> (Semwal et al., 2010)

Table 4. Inhibitory NO production and cytotoxic effects of main alkaloids found in 5 *Stephania* species.

Compounds	IC ₅₀ -NO (µg/ml)	IC ₅₀ -cytox (µg/ml)	AI ^(a)
Cepharanthine ^(b)	2.39	30.41	12.72
Crebanine ^(c)	2.29	152.42	66.56
Palmatine ^(b)	17.91	283.77	15.84
Roemerine ^(b)	2.01	3.04	1.51
Stephanine ^(c)	1.32	147.04	111.39
Tetrahydropalmatine ^(b)	22.96	281.35	12.25
Xylopinine ^(b)	99.77	281.35	2.82
Dexamethasone	4.31	163.22	37.87

^(a)AI=anti-inflammatory ratio. ^(b)Standard alkaloid tested. ^(c)Isolated alkaloid tested

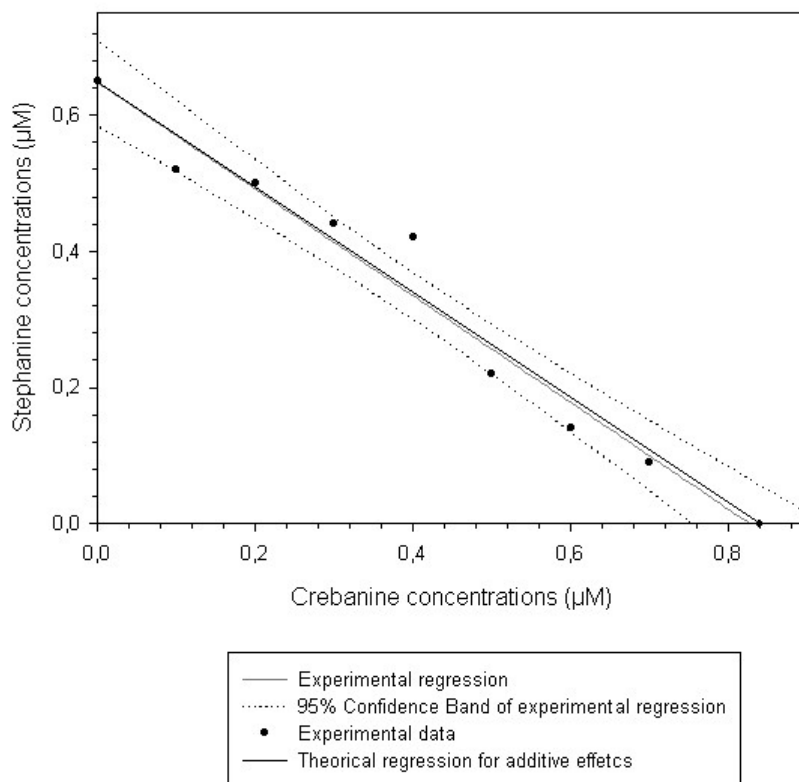


Figure 2: Isobologram showing additive activities of crebanine and stephanine

These findings constitute the first report on anti-inflammatory effects of roemerine and stephanine. Moderate cytotoxicity of cepharanthine could be explained by its high concentration tested in this study as in a previous study this bisclaurine alkaloid did not show cytotoxicity on RAW 264.7 cells at concentration of 10 μ M (Paudel et al., 2016). The potent cytotoxicity of roemerine on blood cells was previously reported (Baghdikian et al., 2013). As crebanine and stephanine isolated from *Stephania venosa* demonstrated promising anti-inflammatory effects with highest values of anti-inflammatory ratio, the anti-inflammatory synergism of both alkaloids was then investigated (Figure 2). The curvilinear curve indicated additive effects of the two alkaloids. These results showed that both alkaloids could be promising anti-inflammatory agents.

CONCLUSION

Taken together, these results revealed that “Komar Pich” tubers corresponding to 5 different *Stephania* species have potent anti-inflammatory effects and this may be attributed to the main alkaloids found in each species. The ethnomedicinal use of 5 Cambodian *Stephania* species in syndromes and diseases associated with inflammation is justified, for the first time, by the *in vitro* anti-inflammatory activity of hydro-ethanolic extracts. The findings that aqueous and hydro-ethanolic extracts are not cytotoxic become an important element in the safety profile of *Stephania* species as these extracts were prepared similarly to the traditional preparation modes. This study also suggests that hydro-ethanolic extracts obtained by tuber maceration should be used as a primary usage form in the management of inflammation related diseases in place of the tuber decoction which is the most common mode of preparation in traditional Cambodian medicine.

DECLARATION OF CONFLICT OF INTEREST

No conflict of interest to declare.

DECLARATION OF HONOUR

We declare in our honor that our results are not fake and made up.

ACKNOWLEDGMENTS

The work has been successfully carried out thanks to scholarship from the French Government (bourse du gouvernement Français). The authors gratefully acknowledge support and encouragement from Professor Cheng Sun Kaing, Professor Tep Rainsy, Doctor Bun Hot, Professor Tea Sok Eng, Doctor Bernard Fabre and Mr. Philippe Bessioud.

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