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A new protoberberine alkaloid from *Meconopsis simplicifolia* (D. Don) Walpers with potent antimalarial activity against a multidrug resistant *Plasmodium falciparum* strain

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A new protoberberine alkaloid from *Meconopsis simplicifolia* (D. Don) Walpers with potent antimalarial activity against a multidrug resistant *Plasmodium falciparum* strain

Abstract

Ethnopharmacological relevance The aerial components of *Meconopsis simplicifolia* (D. Don) Walpers are indicated in Bhutanese traditional medicine for treating malaria, coughs and colds, and the infections of the liver, lung and blood. This study is to validate the ethnopharmacological uses of this plant and also identify potent antimalarial drug leads through bioassays of its crude extracts and phytochemical constituents.

Materials and methods *Meconopsis simplicifolia* (D. Don) Walpers was collected from Bhutan and its crude MeOH extract was subjected to acid-base fractionation. Through repeated extractions, separations and spectroscopic analysis, the alkaloids obtained were identified and tested for their antimalarial and cytotoxicity activities.

Results Phytochemical studies resulted in the isolation of one new protoberberine type alkaloid which we named as simplicifolianine and five known alkaloids: protopine, norsanguinarine, dihydrosanguinarine, 6-methoxydihydrosanguinarine and oxysanguinarine. Among the five of the alkaloids tested, simplicifolianine showed the most potent antiplasmodial activities against the *Plasmodium falciparum* strains, TM4/8.2 (chloroquine–antifolate sensitive strain) and K1CB1 (multidrug resistant strain) with IC₅₀ values of 0.78 µg/mL and 1.29 µg/mL, respectively. The compounds tested did not show any significant cytotoxicity activities against human oral carcinoma KB cells and normal Vero cells of African kidney epithelial cells.

Conclusions This study validated the traditional uses of the plant for the treatment of malaria and identified a new alkaloid, simplicifolianine as a potential antimalarial drug lead.

Keywords

potent, walpers, don, simplicifolia, meconopsis, alkaloid, protoberberine, plasmodium, resistant, multidrug, against, strain, activity, falciparum, antimalarial, CMMB

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A new protoberberine alkaloid from *Meconopsis simplicifolia* (D. Don) Walpers with potent antimalarial activity against a multidrug resistant *Plasmodium falciparum* strain

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ABSTRACT

Ethnopharmacological relevance: The aerial components of *M. simplicifolia* are indicated in Bhutanese traditional medicine for treating malaria, coughs and colds, and the infections of the liver, lung and blood.

Aim of the study: To validate the ethnopharmacological uses of this plant and also identify potent antimalarial drug leads through bioassays of its crude extracts and phytochemical constituents.

Materials and methods: *M. simplicifolia* was collected from Bhutan and its crude MeOH extract was subjected to acid-base fractionation. Through repeated extractions, separations and spectroscopic analysis, the alkaloids obtained were identified and tested for their antimalarial and cytotoxicity activities.

Results: Phytochemical studies resulted in the isolation of one new protoberberine type alkaloid which we named as simplicifolianine and five known alkaloids: protopine, norsanguinarine, dihydrosanguinarine, 6-methoxydihydrosanguinarine and oxysanguinarine. Among the five of the alkaloids tested, simplicifolianine showed the most potent antiplasmodial activities against the *Plasmodium falciparum* strains, TM4/8.2 (chloroquine-antifolate sensitive strain) and K1CB1 (multidrug resistant strain) with IC₅₀ values of 0.78 µg/ml and 1.29 µg/ml, respectively. The compounds tested did not show any significant cytotoxicity activities against human oral carcinoma KB cells and normal Vero cells of African kidney epithelial cells.

Conclusions: This study validated the traditional uses of the plant for the treatment of malaria and identified a new alkaloid, simplicifolianine as a potential antimalarial drug lead.

Keywords: *Meconopsis simplicifolia*, simplicifolianine, antimalarial, anticancer, medicinal plant, Bhutanese traditional medicine.

1. Introduction

Meconopsis (Papaveraceae) comprises about 43-49 species with the majority of these restricted to the Himalaya and only one species, *M. cambrica*, being endemic to Europe (Debnath and Nayar, 1986; Zhou et al., 2009). The plants of this genus are prized for their ornamental and medicinal qualities. Many *Meconopsis* species, such as *M. integrifolia*, *M. torquata*, *M. horridula*, *M. racemosa* and *M. quintuplinervia* have long been used in Tibetan folk remedies for treating various disorders (Luo et al., 1984; Zhou et al., 2009; Yue et al., 2010). Out of 13 *Meconopsis* species reported from Bhutan (Grierson and Long, 1984), seven of them (*M. horridula*, *M. paniculata*, *M. napaulensis*, *M. superba*, *M. primulina*, *M. discigera* and *M. simplicifolia*) were said to have medicinal properties. However, only two species (*M. horridula* and *M. simplicifolia*) are currently used in Bhutanese traditional medicine (BTM) for various formulations (Tenzin, 2007; Wangchuk et al., 2008).

M. simplicifolia is locally known as *ud-pel-sngon-po* and grows to 30-70 cm tall with narrowly oblongated seed capsules, blue flowers, and hairy stems and leaves (Anonymous, 2008). Its aerial parts (stems, leaves, flowers and fruit), in combination with other ingredients, are used for preparing more than eight important BTM multi-ingredient formulations. As an individual plant, it is indicated for treating coughs and colds, fever and infections in the liver, lung and blood which show correlations to the symptoms of cancer, microbial infections and malaria (Wangchuk et al., 2008, 2011). Malaria causes 2-3 million deaths each year (Marti et al., 2010). In Bhutan, there were 436 microscopy-confirmed indigenous cases with 140 (32%) cases being due to *Plasmodium falciparum* (Yangzom et al., 2010). As the artemisinin-based combination therapies (ACTs), including artesunate monotherapy, have shown signs of lower efficacy (Dondorp et al., 2009) against the resistant *Plasmodium falciparum* strains, the battle against malaria is seriously impaired. Therefore, the need for new antimalarial agents has

become imperative. History tells us that the plant-based drugs including traditional medicinal plants and compounds derived from them are the important sources of antimalarial drugs. The BTM, which depends upon the country's rich biodiversity, may offer alternative treatment regimens by promising a source of antimalarial extracts and new drug lead compounds as revealed by previous studies on Bhutanese medicinal plants (Wangchuk et al., 2008, 2010, 2011, 2012a, 2012b). Our recent biological activity studies of different solvent extracts of *M. simplicifolia* showed remarkable antiplasmodial activity against a multidrug resistant strain (K1CB1) and a chloroquine and antifolate sensitive wild type strain (TM4/8.2) of *Plasmodium falciparum* with IC₅₀ values of 6.39 µg/ml and 0.40 µg/ml, respectively (Wangchuk et al., 2011). Based on this earlier finding we have further investigated this plant for its phytochemicals and their biological activities and consequently discovered a new protoberberine type alkaloid along with five known protopine and benzophenanthridine type alkaloids (**1-6**) (Fig. 1) with potent antimalarial activities and cytotoxicities. The findings are described in this paper.

2. Materials and methods

2.1. Plant material

The aerial components of wild type *M. simplicifolia* were collected from near the Lingzhi Makhang (Altitude: 4183 m; Latitude: 27° 50' 29.9"; Longitude: 89° 25' 41.5"; global positioning system (GPS) point number: 138; Site number: P138; Slope: 25°; Aspect: North-East), under Lingshi region of Bhutan in July 2009. The herbarium voucher specimen number 2 was authenticated by Mr. Samten and deposited at the herbarium of the Manjong Sorig Pharmaceuticals, Thimphu, Bhutan. The plant has a low average population density of 0.6 plants/m² and usually inhabits damp ground, rocky alpine hillsides, and screes at the margins

of shrubby mountain vegetation (Anonymous, 2008) growing in association with *Rubus* and *Rhodiola* species, *Rhododendron anthopogon* and *Bistorta macrophylla*. It is distributed in Lingzhi, Bumthang and Gasa districts of Bhutan.

2.2. Phytochemical investigation and sample preparation methods

2.2.1 General instrumentations

The crude methanol extract, fractions and pure isolates of *M. simplicifolia* were stored at 5 °C until required for further purification or testing. The extracts and fractions were concentrated using a rotary evaporator under reduced pressure at 35-50 °C. Separation and purification of alkaloids were achieved using flash column chromatography (CC) packed with Merck Kieselgel 60 PF₂₅₄ and an aluminium-backing silica plates (0.2 mm silica thickness, Merck). Separated bands or spots on TLC plates were visualized under UV light (short wavelength of 254 nm, long wavelength of 366 nm) and by staining with Dragendorff's reagent which was prepared using the methods described by Svendsen and Verpoorte (1983). LR-ESI-MS, LR-EI-MS and the HR-ESI-MS were obtained using a Micromass Waters Platform LCZ (single quadrupole, MeOH as solvent), Shimadzu GCMS-QP-5050 (direct insertion technique at 70 eV) and the Micromass Waters Q-ToF Ultima (quadrupole time-of-flight) mass spectrometer, respectively. The ¹H-NMR, gCOSY, ¹³C-NMR, APT, gHMBC, gHSQC, and gNOESY spectral data of the relevant compounds (dissolved in deuterated solvents CD₃OD or CDCl₃) were generated using either a 500 MHz Varian Unity Inova or 500 MHz Varian Premium Shield (VNMRS PS 54) or 300 MHz Varian Mercury NMR spectrometer. A hot-stage apparatus was used for determining the melting points.

2.2.2. Separation/isolation of alkaloids

The air-dried plant material (2 kg) was chopped into small pieces and was repeatedly extracted with analytical grade or HPLC grade methanol (5 x 3 L over 48 h). The extract was filtered and then concentrated using a rotary evaporator to afford the crude methanol extract (43.1 g). This extract was acidified with 5% HCl and then extracted with CH₂Cl₂ (5 x 60 mL) which upon evaporation of the solvent yielded the CH₂Cl₂ extract (9.8 g). The remaining acidified aqueous solution was basified (pH 9-12) with NH₄OH solution and then extracted with CHCl₃ (5 x 60 mL) to obtain the crude alkaloid CHCl₃ extract (446 mg). Repeated fractional crystallizations and separations by CC on silica gel (mobile phase: gradient eluant of increasing solvent polarities mainly using MeOH and CHCl₃) and preparative thin layer chromatography (PTLC) yielded six alkaloids (**1–6**) (Fig. 1).

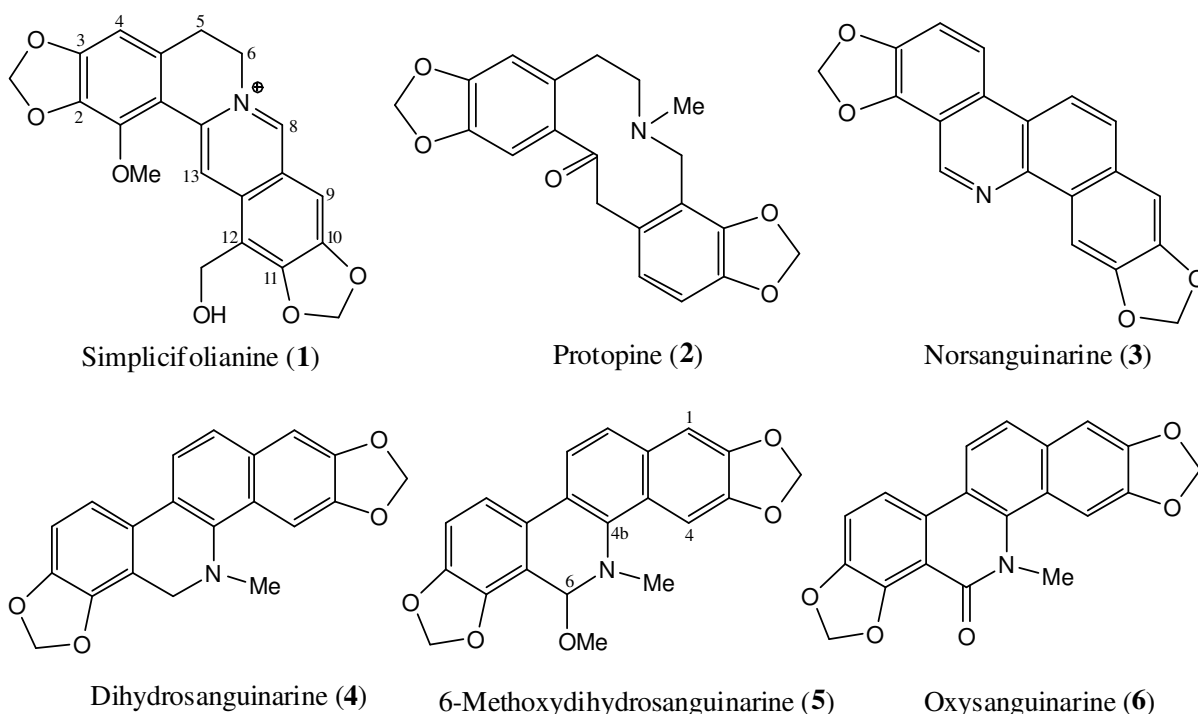


Fig. 1. The structures of the six isolated compounds from *M. simplicifolia*.

The CHCl_3 extract upon initial flash CC over silica gel (120 g, 200-300 mesh), eluting with a gradient solvent system of MeOH-CHCl_3 (in a v/v% ratio of 0:100, 2:98, 4:96, 6:94, 8:92, 10:90, 20:80, 30:70, 50:50), yielded fractions $\text{CHCl}_3\text{-F1}$ to $\text{CHCl}_3\text{-F8}$. Fraction $\text{CHCl}_3\text{-F1}$ upon final separation using PTLC and CHCl_3 as the mobile phase, yielded compound **3** (3 mg). Fractions $\text{CHCl}_3\text{-F5}$ and $\text{CHCl}_3\text{-F6}$ were combined and separated using CC (gradient eluant, MeOH-CHCl_3) followed by PTLC (mobile phase, MeOH-CHCl_3 (4:96, 100 mL)) which yielded compound **2** (69.8 mg, major alkaloid of a plant). Fractions $\text{CHCl}_3\text{-F7}$ and $\text{CHCl}_3\text{-F8}$ were combined and separated by CC with a gradient solvent system of MeOH-CHCl_3 (200 mL, v/v% ratio of 20:80, 30:70) to obtain sub-fractions $\text{CHCl}_3\text{-F78.1}$ to $\text{CHCl}_3\text{-F78.5}$. Final separation of sub-fraction $\text{CHCl}_3\text{-F78.4}$ using PTLC and the mobile phase system of MeOH-CHCl_3 (v/v ratio of 15:85) yielded a new compound **1** (3 mg). The CH_2Cl_2 extract upon initial flash CC on silica gel eluting with a gradient solvent system of $\text{MeOH-CH}_2\text{Cl}_2$ (in a v/v% ratio of 0:100, 0.5:99.5, 1.5:98.5, 2.5:97.5, 3.5:96.5, 5:95, 10:90, 20:80, 50:50) yielded fractions $\text{CH}_2\text{Cl}_2\text{-F1}$ to $\text{CH}_2\text{Cl}_2\text{-F18}$. Fraction $\text{CH}_2\text{Cl}_2\text{-F4}$ upon crystallization from $\text{CHCl}_3\text{-MeOH}$ (1:1) gave crystals of compound **4** (11.6 mg). Its mother liquor was purified by CC eluting with CHCl_3 (100%) to obtain five sub-fractions $\text{CH}_2\text{Cl}_2\text{-F4.1}$ to $\text{CH}_2\text{Cl}_2\text{-F4.5}$. The sub-fractions $\text{CH}_2\text{Cl}_2\text{-F4.4}$ and $\text{CH}_2\text{Cl}_2\text{-F4.5}$ were combined and repeatedly separated. Final separation of fraction $\text{CH}_2\text{Cl}_2\text{-F4.45.3.1}$ by PTLC ($\text{CHCl}_3\text{-ethyl acetate-MeOH}$ in v/v ratio of 90:8:2), yielded compound **6** (0.9 mg). Fraction $\text{CH}_2\text{Cl}_2\text{-F10}$ upon separation using CC with a gradient solvent system of $\text{MeOH-CH}_2\text{Cl}_2$ (in a v/v ratio of 0:100, 0.5:99.5, 1:99, 2:98, 5:95, 10:90, 100:0) gave sub-fractions $\text{CH}_2\text{Cl}_2\text{-F10.1}$ to $\text{CH}_2\text{Cl}_2\text{-F10.4}$. Crystallization of the sub-fraction $\text{CH}_2\text{Cl}_2\text{-F10.1}$ from $\text{CHCl}_3\text{-MeOH}$ (97:3) furnished compound **5** (3.3 mg).

2.2.3. Structure elucidation and identification of alkaloids

Compound **1** was determined as a new protoberberine type alkaloid, which we named as simplicifolianine (**1**) after the species name of this plant. It was isolated as a faintly brown amorphous solid which melted at 170.8-171.3 °C. The LR-ESI-MS spectrum indicated the $[M]^+$ ion peak at m/z 380. LR-EI-MS (m/z): 380 $[M]^+$, 364, 350, 336, 308, 280, 228, 207, 191, 167, 149, 129, 111. Its even molecular ion suggested either zero or an even number of nitrogen or an iminium species. The HR-ESI-MS spectrum supported the molecular formulae of $C_{21}H_{18}NO_6$ with an actual m/z 380.1130 $[M]^+$ and a calculated mass of 380.1134.

The structure of this new compound (**1**) has been established using 1D and 2D-NMR spectral data. Its 1H and ^{13}C -NMR spectra, and gCOSY correlation are presented in Table 1. The long range gHMBC correlation established the $C \rightarrow H$ and $H \rightarrow C$ cross-correlations among the H and C-atoms (Fig. 2a) and the NOESY correlations (Fig. 2b) further confirmed the structure **1**. This compound is structurally related to alborine ([Hai-feng et al., 2011](#)).

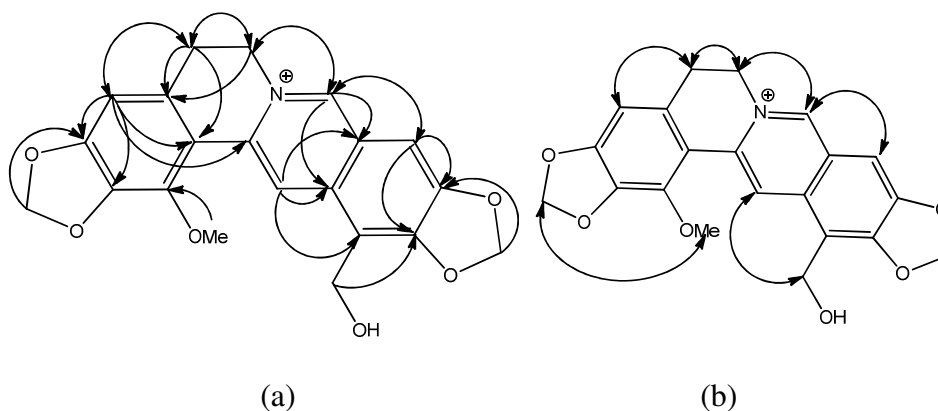


Fig. 2. Key spectral correlations of **1**; (a) gHMBC and (b) NOESY.

Table 1

¹H NMR (500 MHz, methanol-*d*₄), ¹³C NMR (125 MHz, methanol-*d*₄), gCOSY spectroscopic data of simplicifolianine (**1**).

Carbon position	δ C in ppm	δ H in ppm (multiplicity, <i>J</i> in Hz)	gCOSY (¹ H→ ¹ H)
1	143.7		
2	138.3		
3	152.5		
4	104.3	6.71 (1H, s)	3.15
4a	134.2		
5	29.3	3.15 (2H, t, 5.75)	4.70
6	56.4	4.70 (2H, t, 5.75)	3.15
8	146.2	9.31 (1H, s)	7.48
8a	115.6		
9	104.3	7.48 (1H, s)	9.31
10	155.6		
11	139.6		
12	138.4		
12a	125.8		
13	121.9	9.26 (1H, s)	
13a	153.4		
13b	114.2		
OMe	60.9	4.17 (3H, s)	
2,3-OCH ₂ O	103.7	6.09 (2H, s)	
10,11-OCH ₂ O	105.3	6.34 (2H, s)	
C (12)-CH ₂ OH	55.2	5.02 (2H, s)	

The other five known alkaloids were identified as protopine (**2**), norsanguinarine (**3**), dihydrosanguinarine (**4**), 6-methoxydihydrosanguinarine (**5**) and oxysanguinarine (**6**) (Fig.1) through MS library matching techniques, and MS and NMR spectral data comparisons with the pertinent literature. The MS ion fragmentation pattern of compound **2** with a mass of *m/z* 353 [*M*⁺•] matched that of protopine reported in the MS library (NIST08s, Entry # 26245, CAS: 130-86-9, RetIndex: 2943) and further its ¹H and ¹³C-NMR spectroscopic data agreed with those reported (Takahashi et al., 1985; Seger et al., 2004; Wangchuk et al., 2010). It was

the major component of the plant. Compounds **3-6** are benzophenanthridine type alkaloids. While compound **3** was reported from the genus *Meconopsis* (*M. quintuplinervia*) (Shang et al., 2003), compounds **4-6** were isolated from this genus for the first time. The MS, ^1H and ^{13}C -NMR spectroscopic data of compounds **3** (Tousek et al., 2004), **4** (Williams and Ellis, 1993; Choi et al., 2010; Miao et al., 2011; Yao et al., 2011), **5** (Zhang et al., 1995; Dostal et al., 1998; Choi et al., 2010; Miao et al., 2011) and **6** (Williams and Ellis, 1993; Miao et al., 2011) agreed with those from their respective literature. Crystals of compound **4** and **5** were grown using chloroform/methanol and their single crystal X-ray crystallographic structures are presented here for the first time (Fig. 3a-b).

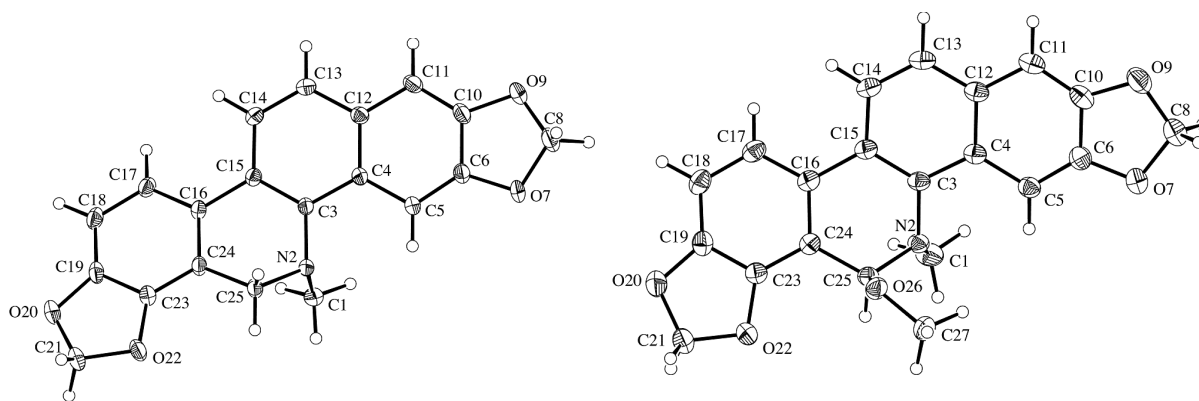


Fig.3. Single crystal X-ray structures of (a) dihydrosanguinarine (**4**), and (b) 6-methoxydihydrosanguinarine (**5**).

X-ray diffraction images were measured on a Nonius KappaCCD diffractometer (Mo $K\alpha$ radiation, graphite monochromator, $\lambda=0.71073 \text{ \AA}$) and data extracted using the DENZO package (Otwinowski and Minor, 1997). Structure solution was by direct methods (SUPERFLIP, SIR92) (Altomare et al, 1994; Palatinus and Chapuis, 2007). The structures were refined using the CRYSTALS program package (Betteridge et al., 2003). Atomic coordinates, bond lengths and angles and displacement parameters have been deposited at the

Cambridge Crystallographic Data Centre (CCDC no. 929725, 929726). These data can be obtained free-of-charge via www.ccdc.cam.ac.uk/data_request/cif, by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033. Crystal data for compound **4**: C₂₀H₁₅NO₄, M_r 333.34, $T = 200\text{K}$, triclinic, $P\bar{1}$, $a = 9.3269$ (2) Å, $b = 10.2708$ (2) Å, $c = 17.5397$ (3) Å, $\alpha = 103.6455$ (9)°, $\beta = 100.3950$ (12)°, $\gamma = 103.6436$ (12)°, $V = 1536.33$ (5) Å³, $Z = 4$, $F(000) = 696$, $D_x = 1.441$ g cm⁻³, $\mu = 0.10$ mm⁻¹, specimen = $0.47 \times 0.34 \times 0.26$ mm (colorless block). 40000 reflections were measured to $2\theta_{\text{max}} = 60^\circ$ and merged to 8969 unique data. Final $R = 0.066$ [for 7763 reflections with $F^2 > 2\sigma(F^2)$], $wR = 0.194$ (all data), $S = 1.02$. Crystal data for compound **5**: C₂₁H₁₇NO₅, M_r 363.37, $T = 200\text{K}$, monoclinic, $P2_1/n$, $a = 12.3894$ (3) Å, $b = 7.7519$ (1) Å, $c = 18.2571$ (4) Å, $\beta = 103.7510$ (11)°, $V = 1703.18$ (6) Å³, $Z = 4$, $F(000) = 760$, $D_x = 1.417$ g cm⁻³, $\mu = 0.10$ mm⁻¹, specimen = $0.59 \times 0.22 \times 0.10$ mm (pale brown plate). 35751 reflections were measured to $2\theta_{\text{max}} = 55^\circ$ and merged to 3876 unique data. Final $R = 0.040$ [for 2968 reflections with $F^2 > 2\sigma(F^2)$], $wR = 0.103$ (all data), $S = 0.98$.

The isolated and identified compounds **1-5** were studied for their pharmacological activities.

2.3. Bioassay methodology

2.3.1. Antiplasmodial assay

Compounds **1-5** were tested *in vitro* against a multidrug resistant K1CB1 strain and a wild type chloroquine and antifolate sensitive TM4/8.2 strain of *Plasmodium falciparum*. The method described by Trager and Jensen (1976) was used for maintaining the parasites in human red blood cells in RPMI 1640 medium supplemented with 25 mM of HEPES, 0.2% of

sodium bicarbonate, and 8% human serum in a 3% carbon dioxide gas incubator maintained at 37 °C. The test samples were made up in DMSO solution and the *in vitro* antiplasmodial activity testing was carried out using the Microdilution Radioisotope Technique (as detailed in Wangchuk et al., 2011). The test sample (25 µL, in the culture medium) was placed in triplicate in a 96-well plate where parasitised erythrocytes (200 µL) with a cell suspension (1.5%) of parasitemia (0.5-1%) were then added to the wells. Generally, the ranges of the final concentrations of the samples varied from 2×10^{-5} to 1×10^{-7} M or up to 1×10^{-4} g/mL with 0.1% of the organic solvent. Due to the poor solubility of some samples, only about 10^{-5} g/mL final concentration could be tested. The plates were then cultured under standard conditions for 24 h after which ^3H -hypoxanthine (25 µL, 0.5 µCi) was added to the culture medium. The culture was incubated (18-20 h) after which the DNA from the parasite was harvested from the culture onto glass fibre filters and a liquid scintillation counter was used to determine the amount of ^3H -hypoxanthine incorporation (Desjardins et al., 1979; Kamchonwongpaisan et al., 2004). The inhibitory concentration of the sample was determined from its dose-response curves or by calculation. The assay was performed in at least three replicates. Chloroquine (Sigma company), pyrimethamine (Sigma company) and cycloguanil were used as positive controls for both plasmodial strains (Table 2). DMSO (0.1%) and distilled water were used as controls to rule out the solvent effects on the bioassay results of the test samples. All the experiments were performed three times in duplicate (3x2).

2.3.2. Cytotoxicity assay

Normal Vero cells from kidney of African green monkey, *Cercopithecus aethiops* and human oral carcinoma KB cells were maintained and cultured in MEM/EBSS supplemented with heated-inactivated fetal bovine serum (10%), NaHCO_3 (2.2 g/L) and of sodium pyruvate

(1%). KB cell lines were cultured in DMEM/low glucose supplemented with heated-inactivated fetal bovine serum (10%), NaHCO₃ (3.7 g/L) and non-essential amino acids (1%). Cytotoxicity was evaluated by the sulforhodamine B (SRB) assay (OD510 nm) as reported (Wangchuk et al., 2012a). Doxorubicin and ellipticine were used as positive control drugs for cytotoxicity activities (Table 2).

3. Results and Discussions

Since the crude extracts of a Bhutanese antimalarial medicinal plant, *M. simplicifolia* has exhibited significant antiplasmodial activity (Wangchuk et al., 2011), we examined compounds **1** and **3-5** for their antiplasmodial and cytotoxic activities (Table 2). Because of solubility problems, the IC₅₀ values could not be accurately determined for compounds **3-5** and compound **6** was not tested due to its limited quantity.

The new protoberberine alkaloid, simplicifolianine (**1**) showed the most potent antiplasmodial activity against the *P. falciparum* strains, a wild type chloroquine and antifolate sensitive strain-TM4/8.2 and a multidrug resistant strain-K1CB1 with IC₅₀ values of 0.78 µg/mL and 1.29 µg/mL, respectively. This potent antiplasmodial activity was of similar range to that of the parent crude chloroform alkaloid extract against TM4/8.2 but nearly six times more potent than the crude extracts against K1CB1. Therefore, we have identified simplicifolianine (**1**) as a potential new drug lead on which a patent can be filed.

The fact that the low cytotoxicity of compound (**1**) and the crude extract against human oral carcinoma KB cells and normal Vero epithelial cells makes the activity more interesting and demonstrated the plant's potential safety for use in BTM.

Table 2

Antiplasmodial activity (IC₅₀ in µg/mL) of the alkaloids **1** and **3-5** isolated from *M. simplicifolia*. Also reproduced in the table are the data previously reported on the crude extracts and protopine.

Samples	Antiplasmodial		Cytotoxicity	
	TM4/8.2	K1CB1	Vero cells	KB cells
MeOH extract	<12.5 ^a	12.5 ^a	> 10 ^a	> 10 ^a
CH ₂ Cl ₂ extract	15.50 ± 1.95 ^a	12.80 ± 2.63 ^a	> 25 ^a	> 25 ^a
CHCl ₃ alkaloid extract	0.40 ± 0.00 ^a	6.39 ± 2.73 ^a	> 25 ^a	> 25 ^a
Simplicifolianine (1)	0.78 ± 0.14	1.29 ± 0.54	> 3.79	> 3.79
Protopine (2)	1.45 ± 0.53 ^b	1.38 ± 0.31 ^b	> 3.50 ^b	> 3.50 ^b
Norsanguinarine (3)	> 0.32	> 0.32	> 0.32	> 0.32
Dihydrosanguinarine (4)	> 3.33	> 3.33	> 3.33	> 3.33
6-Methoxydihydrosanguinarine (5)	> 3.63	> 3.63	> 3.63	> 3.63
Chloroquine ^c	0.010	0.089		
Cycloguanil ^c	0.009	0.810		
Pyrimethamine ^c	0.020	7.700		
Ellipticine ^d			0.093	
Doxorubicin ^d				0.56

^a original activity taken from Wangchuk et al. (2011).

^b original activity taken from Wangchuk et al. (2012).

^c reference drugs for antiplasmodial activity.

^d reference drugs for cytotoxicity activity.

Protopine (**2**) has been isolated from different plant species and in our earlier studies, we established its antiplasmodial activity against the same strains as highly significant (Wangchuk et al., 2010; 2012b) (Table 2) and has been also reported to have broad range of

biological activities (Vacek et al., 2010). Due to limitation in solubility of compounds **3-5**, the highest concentration tested were 0.32, 3.33 and 3.63 $\mu\text{g/mL}$, respectively. At such concentrations, compound **3** and **4** did not show any significant antiplasmodial activities nor the cytotoxicities. However, compound **5** exhibited about 10-20% inhibitory effect against both parasite strains with no cytotoxicity against the mammalian cells.

Since compounds **1** and **2** exhibited highly significant antimalarial activities and that protopine (**2**) was the major alkaloid present, it can be deduced that these alkaloids, either alone or in combination, may be responsible for the major antiplasmodial activities of the extract of this plant. Considering the increased resistance of the parasites to the conventional antimalarial drugs (Dondorp et al., 2009; Marti et al., 2010), these findings are timely as they provide a new potential drug lead targeting malarial infections. The Bhutanese traditional formulae involving this plant have potential to become an alternative treatment regimen for malaria and could potentially lead to the development of new hybrid of antimalarial drugs based on the lead compound identified here. Reports on compound **5** indicated antiproliferative effects on K549 human lung cancer cells, PC3 human prostate cancer cells, MCF-7 human breast cancer cells and A562 human leukemia cells (Cho, 2001) and showed an inhibitory effect on the growth of human colon carcinoma cells and induced apoptosis (Lee et al., 2004). However, this compound exhibited no cytotoxicity against KB and Vero cells in our study at the highest concentration tested. This compound was also reported to display anti-platelet aggregation activity (Chen et al., 2001).

From our earlier studies of the crude extracts of this plant, we concluded that this plant had only weak antibacterial properties against *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRSA), *Bacillus subtilis* and *Helicobacter pylori* (Wangchuk et al., 2011). Based on these results, the pure isolates from this plant were not tested for their antimicrobial activities.

However, Navaroo and Delgado (1999) and Feng et al. (2011), reported that dihydrosanguinarine (4) displayed varying antimicrobial activities against *S. aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Candida albicans*, *Botrytis cinerea*, *Phytophthora capsici* and *Alternaria solani*. Compound 5 (6-methoxydihydrosanguinarine or 6-methoxysanguinarine or often reported as 8-methoxydihydrosanguinarine) was also reported to have moderate to weak antimicrobial activity against MRSA (Choi et al., 2010), *S. aureus*, *E. coli* and *A. hydrophila* with MIC values ranging from 12.5-50 µg/mL (Miao et al., 2011). Compound 3 exhibited antifungal activity against phytopathogenic fungi *A. brassiciola* and *C. maculans* with 75-80% inhibition at 200 µg/mL (Singh et al., 2009) and compound 4 showed significant antiparasitic effect against *Ichthyophthirius multifiliis* in richadsin with an IC₅₀ value of 5.2 µg/mL (Yao et al., 2011).

4. Conclusion and future directions

In summary, this phytochemical study of *M. simplicifolia* found the following: 1) a new protoberberine type alkaloid which we named as simplicifolianine (1); 2) protopine (2) was identified as the major alkaloid constituent; 3) compound 1 showed significant *in vitro* antiplasmodial activity with low cytotoxicity and therefore, we have identified it as a potential antimalarial drug lead; and 4) the *in vitro* bioassay results of the crude extracts and the pure compounds 1 and 2 were proportionate with the ethnopharmacological uses of this plant and thus substantiated its usage in a crude drug form in BTM, individually or in combination with other medicinal ingredients, to treat malaria.

In future, scale-up isolation of the minor alkaloids, non-alkaloids and the essential oil components and the evaluation of their antiplasmodial and anticancer activities will be

undertaken. As resistance to the front line antimalarial drugs appears to be increasing, further work including the *in vivo* studies on compounds **1** and **2** which showed strong antiplasmodial activities is essential. Assessing the antimalarial and anticancer activities of formulations comprising mixtures of various ratios of the two most active compounds could also potentially give interesting results and could shed light on their synergism.

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