Experimental Parasitology 211 (2020) 107851

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Antimalarial alkaloid from *Hypoestes forskaolii*

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ARTICLE INFO

Keywords:
Hypoestes forskaolii (Vahl) R. Br.
Antimalarial
Phenanthro-quinolizidine alkaloids
K1
FCR3
MRC5

ABSTRACT

Following on from previous studies, we brought further our quest for anti-malarial agents isolated from plants grown in the Saudi Arabian Peninsula. Methanolic extracts were prepared from eighteen Saudi plants and then tested *in vitro* to assess their anti-malarial effects on *Plasmodium falciparum* K1, (a chloroquine-resistant strain) as well as their cytotoxicity on MRC5 (human diploid embryonic lung cell line) cells. Moderate anti-malarial activity was observed in extracts prepared from *Hypoestes forskaolii* (Vahl) R. Br. (IC_{50} value of 5.5 μg/ml) and *Rhus retinorrhaea* (IC_{50}: 7.71 μg/ml). The remaining sixteen plant extracts appeared to be inactive (IC_{50} > 12.5 μg/ml). A novel phenanthro-quinolizidine alkaloid, 15β-hydroxycryptopleurine-N-oxide, was isolated from *H. forskaolii* using bio-guided fractionation procedures. Chloroquine-resistant (K1) and chloroquine-sensitive (FCR3) strains of *P. falciparum* appeared very sensitive to the anti-malarial activity of 15β-hydroxycryptopleurine-N-oxide, giving IC_{50} of 6.11 and 5.13 nM respectively. It showed cytotoxicity against MRC5 "IC_{50} of 24.45 nM" with selectivity indices of 4.0 and 4.76 against K1 and FCR3 strains, respectively. It is our understanding that this is the first account on phenanthro-quinolizidine alkaloids anti-malarial activity on a chloroquine-resistant *P. falciparum* strain.

1. Introduction

Malaria, the most ancient disease globally, is a parasitic disease spread to healthy human individuals by female *Anopheles* mosquito that inoculates *Plasmodium* species harvested from an infected person blood. Among the five *Plasmodium* species, *P. falciparum* is the most prevalent malaria parasite in the WHO African Region. Yearly diagnosis of new malaria cases has reached the 300–500 million mark, with the occurrence of 1.5 million deaths, children being the most affected (Guinovart et al., 2006). In 2017, there were 219 million cases of malaria, most cases and deaths occur in sub-Saharan Africa (200 million or 92%) (WHO, 2018).

Evolution and emergence of multidrug-resistant strains of *P. falciparum* are responsible for the rise in malaria incidences, triggering the necessity for the development of new and effective anti-malarial agents. Historically, quinine, a quinoline-type alkaloid isolated from *Cinchona* bark, has been a powerful anti-malarial drug and it is likely that it will retain a role in disease management. However, new anti-malarial compounds are needed and natural extracts from plants or other organisms are designated as a tantalizing source (Beaufay et al., 2018). In fact, expectations for isolating new and potent anti-malarial agents were elevated after discovering sesquiterpene lactone artemisinin, and its derivatives, from *Artemisia annua* (Mishra et al., 2017).

Following from previous findings from our lab (Abdel-Sattar et al. 2008, 2009, 2010), eighteen plants were chosen from diverse locations of the Saudi Arabian peninsula, and their methanolic extracts were prepared. The extracts' anti-malarial efficacy was evaluated in chloroquine-sensitive (FCR3) and chloroquine-resistant (K1) strains of *P. falciparum*. The results revealed a promising activity for *Hypoestes forskaolii* (Vahl) R.Br. (Acanthaceae).

H. forskaolii (Vahl) R.Br. (Acanthaceae) is a herbaceous widely distributed in many African countries as well as in the southern region of Saudi Arabia. In Saudi Arabia the plant has several popular names, among them "Nadgha", "Majra", "Qumaylah" (Andriamihaja et al., 2001). It is not edible plant for animals, and has several uses such as natural insecticide, its decoction used to wash the goats infested by flea as anthelmintic (D'Ambola et al., 2018). The fresh leaves are used as wounds healing and to kill head lice and to destroy its eggs.

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https://doi.org/10.1016/j.exppara.2020.107851
Received 3 June 2019; Received in revised form 15 January 2020; Accepted 1 February 2020
Available online 06 February 2020
0014-4894/

(Padysakova et al., 2013). It has cytotoxic activity (Almehdar et al., 2012), and piscicidal effect and used for fishing (Ubaha et al., 2012). Previous phytochemical studies of *H. forskaolii* identified fusicoccane tricyclic diterpenes as the major class of secondary metabolites (Muhammad et al., 1998, 1997).

Hypoestes forskaolii was chosen among the tested plants for further detailed study to isolate its antimalarial bioactive. A bio-guided fractionation of the methanolic extract led to the isolation of anti-malarial compound identified as of 15β-hydroxycryptopleurine-N-oxide from the active fraction.

2. Materials and methods

2.1. General

A JASCO FT/IR-230 IR spectrometer was used to show IR spectrum. Samples were dissolved in MeOH and the measurement of mass spectra was performed on JEOL JMS-700 Mstation. The measurement of NMR spectra was achieved with a Varian Unity-400 machine using $CDCl_3$. HPLC specification (pump: Shimadzu LC-6A liquid chromatograph, UV Detector: Shimadzu SPD-6A spectrophotometric detector, column: CAPCEL PAK C18 AQ (20 × 250 mm, Shiseido Co., Ltd, Tokyo, Japan), UV detector used at 254 nm, and the flow rate was 4 mL/min. Column chromatography was possible using Silica gel 60 (230–410 mesh, Merck, Darmstadt, Germany) and NH Silica gel (Fuji. Silysia Chemical, Tokyo, Japan). Analytical TLC was executed on Silica gel 60 F254 precoated plates (Merck, Darmstadt, Germany), and chromatograms visualized were performed under UV light at 254 nm and/or sprayed with Dragendorff's reagent.

2.2. Plant materials

Between February and April 2014, eighteen plants (Table 1) were collected from the Western region of the Kingdom of Saudi Arabia and identified by our colleagues in the Department of Plant Taxonomy (King Abdulaziz University, Kingdom of Saudi Arabia). These plants sample specimens were deposited at the Herbarium of the Department of Natural Products, Faculty of Pharmacy (King Abdulaziz University, Saudi Arabia). The plants aerial portion were air dried away from direct sunlight. The plants were powdered and stored in appropriate containers and were kept till use.

2.3. Extraction

Dried powdered plant materials (aerial parts, 30 g each) were extracted with methanol (3 × 100 mL). The solvent was distilled off under reduced pressure and the methanolic extracts were kept at 4 °C for further biological *in vitro* tests.

2.4. Bio-guided fractionation of the bio-active extract prepared from H. forskaolii

Extract from 300 g *H. forskaolii* powdered aerial parts was obtained with methanol (3 × 1000 mL) and then organic solvent was evaporated under reduced pressure resulting in 50 g final dry residue. This dry extract was suspended in 300 mL water, prior to being subjected to further fractionation with petroleum ether and then with ethyl acetate. Aqueous layer pH was adjusted to 10 with NH_4OH to enable $CHCl_3$ extraction and subsequent bio-guided fractionation as reported in scheme 1 (Fig. 1). The active fraction was subjected to chromatographic purification using combined open column chromatography on normal Si gel or NH Si gel columns using $CHCl_3$/MeOH mixtures and HPLC on ODS column using MeOH/aqueous ammonia mixture (Fig. 1). *In vitro* testing of all fractions and sub fractions was carried out in K1 and FCR3 *P. falciparum* strains anti-malarial models. This approach resulted in the isolation of compound **1** (1.5 mg) with high antimalarial activity from fr. **18**. Further amount of compound **1** (2 mg) was separated from the $CHCl_3$ fraction using the same bio-guided chromatographic isolation of the EtOAc fraction (Fig. 1).

15β-hydroxycryptopleurine-N-oxide (1)

Faint brownish amorphous powder; IR (KBr) ν[cm^{-1}]: 3450, 1675, 1535, 1445, 1265, 1245; UV (MeOH): 263 nm; Positive FABMS *m/z* 410.1963 $[M+H]^+$ (calcd 410.196749 for $C_{24}H_{28}NO_5$), ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) are reported in Table 2.

Table 1
Antimalarial activity of the methanolic extracts of some Saudi plants against *P. falciparum* (K1) and their cytotoxicity on MRC5 cell line.

Plant Name	Family	SN[a]	IC_{50} (μg/ml)		SI[c]	Score[d]
			K1[b]	MRC-5		
1. Amaranthus viridis L.	Amaranthaceae	AV-1175	> 12.5	47.89	-	1
2. Atriplex leucoclada Boiss. var. mandavillea Al-Turki, Omer & Ghafoor	Chenopodiaceae	AL-1180	> 12.5	-	-	1
*3. Cichorium intybus*L.	Astraceae	CI-1057	> 12.5	4.03	-	1
4. Euryops arabicus Steud. ex Jaub. & Spach	Astraceae	EA-1066	> 12.5	16.19	-	1
5. Heliotropium arbainense G.Don	Boraginaceae	HA-1022	> 12.5	70.31	-	1
6. Heliotropium curassavicum L.	Boraginaceae	HC-1179	> 12.5	52.65	-	1
7. Heliotropium longiflorum Phil.	Boraginaceae	HL-1021	> 12.5	22.79	-	1
8. Heliotropium ramosissimum Sieber ex DC.	Boraginaceae	HR-1020	> 12.5	65.92	-	1
9. Hypoestes forskaolii (Vahl) R.Br.	Acanthaceae	HF-1005	5.5	0.8	0.14	2
10. Ipomoea aquatica Forssk.	Convovulaceae	IA-1073	> 12.5	-	-	1
11. Lactuca serriola L.	Astraceae	LS-1050	> 12.5	-	-	1
12. Rhus retinorrhaea	Anacardiaceae	RR-1012	7.71	52.2	6.8	2
13. Schinus mole L. fruit	Anacardiaceae	SM-1178	> 12.5	46.6	-	1
14. Schinus mole L. leaves	Anacardiaceae	SM-1178	> 12.5	23.01	-	1
15. Solanum villosum Forssk.	Solanaceae	SV-1176	> 12.5	32.58	-	1
16. Teucrium polium L.	Lamiaceae	TP-1104	> 12.5	35.57	-	1
17. Trichodesma africanum R. Br.	Boraginaceae	TA-1016	> 12.5	15.34	-	1
18. Achyranthes aspera L.	Amaranthaceae	AA-1010	> 12.5	40.8	-	1

[a] SN: Specimen number.
[b] K1: chloroquine-resistant strain.
[c] SI: selectivity index; is defined by the ratio of the IC_{50} value on MRC5 cells to that on *P. falciparum*.
[d] Score: Any extracts having score 3 (IC_{50} < 1.56 μg/ml) are considered strongly active, samples of score 2 (IC_{50} < 12.5 and > 1.56 μg/ml) are considered active, while extract showing score 1 of activity (IC_{50} > 12.5 μg/ml) is considered inactive (WHO, TDR guidelines).

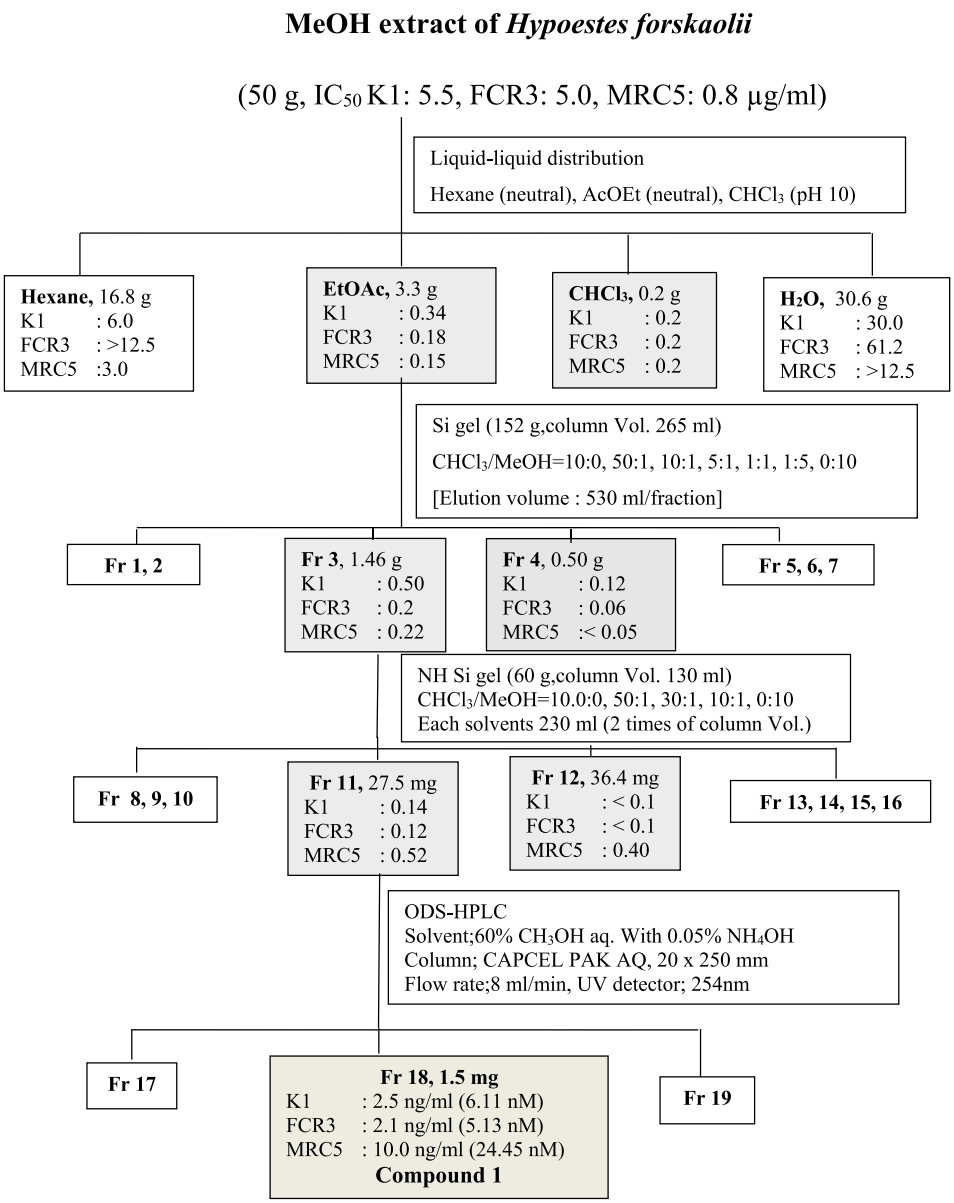


Fig. 1. Chromatographic separation of compound **1** and the antimalarial activity and cytotoxicity of the different chromatographic fractions from the methanolic extract of *H. forskaolii*.

2.5. Biological assays

The antimalarial activity of the prepared extracts, and isolated compound as well as standard antimalarial drugs was performed on chloroquine-resistant K1 strain and chloroquine-sensitive FCR3 strain of *P. falciparum* as reported previously (Abdel-Sattar et al., 2009). Detailed procedures is available in the supplementary file.

3. Results and discussion

3.1. Antimalarial activity and cytotoxicity of plant extracts

Eighteen plants belonging to nine families and thirteen genera were evaluated for their antimalarial activity according to the screening protocol of the Research Centre for Tropical Diseases, Kitasato Institute, Tokyo, Japan, and the protocol regulated and proposed by the TDR program (WHO) (Abdel-Sattar et al., 2009).

Out of the eighteen plant extracts tested on chloroquine-resistant strain K1 (Table 1), sixteen were inactive (IC_{50} > 12.5 µg/ml, score 1) and only the methanolic extracts of *Rhus retinorrhaea* and *H. forskaolii* were active with score 2 (IC_{50} of 7.71 and 5.5 µg/ml, respectively). The methanolic extract of *H. forskaolii* (50 g) was subjected to a bio-guided fractionation (Fig. 1) against the K1 and FCR3 strains. Fractions or subfractions with the least cytotoxicity against MRC5 were chosen for further evaluation. The highest activities were demonstrated by $CHCl_3$ and EtOAc fractions (Fig. 1). *In vitro* antimalarial test of chromatographic subfractions of EtOAc extract (Fig. 1) revealed two active subfractions; fr. **3** and fr. **4.** Fraction **3** (IC_{50} K1: 0.5 µg/ml, FCR3: 0.2; MRC5: IC_{50} 0.22 µg/ml) was selected for further chromatography due to its less cytotoxicity than fr. **4** (MRC5: IC_{50} < 0.05 µg/ml). This resulted in the isolation of an active antimalarial compound (**1**) from subfraction **18**, as shown in Fig. 1 (K1: IC_{50} 2.5 ng/ml (6.11 nM), FCR3: IC_{50} 2.1 ng/ml (5.13 nM), MRC5: IC_{50}10 ng/ml (24.45 nM).

3.2. Structure elucidation of new compound

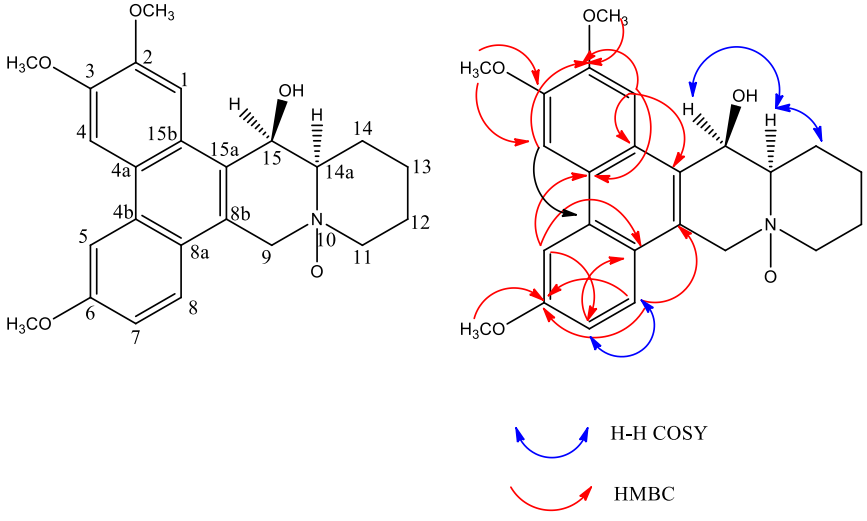
Compound **1** (Fig. 2) was isolated as a faint brownish amorphous powder, positively responded to test for alkaloid on TLC using

Table 2
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for Compound (d, ppm $CDCl_3$).

position	δ_H	δ_C
1	7.59 (1H, s)	104.9
2		149.1
3		150.0
4	7.86 (1H, s)	103.6
4a		125.6
4b		131.5
5	7.86 (1H, d, 2.5)	105.1
6		158.3
7	7.16 (1H, dd, 9.5, 2.5)	115.3
8	7.61 (1H, d, 9.5)	124.2
8a		122.2
8b		118.6
9	4.75 (1H, d, 15.5) 5.26 (1H, d, 15.5)	70.2
11	3.35 (1H, br. d, 12.0) 3.83 (1H, br. d, 12.0)	69.8
12	1.79 (1H, br. d 14.0) 2.57 (1H, qt, 13.5, 3.0)	20.6
13	1.62 (1H, qt, 13.0, 3.0) 2.09 (1H, br. d, 13.0)	22.9
14	1.94 (1H, br. d, 13.0) 2.95 (1H, qd, 13.0, 4.0)	23.4
14a	3.41 (1H, dt, 12.5, 2.5)	67.5
15	4.98 (1H, d, 2.5)	67.1
15a		127.3
15b		124.4
OMe-2	4.08 (3H, s)	56.1
OMe-3	4.12 (3H, s)	56.2
OMe-6	4.02 (3H, s)	55.5

Dragendorff's spray reagent. It has a molecular formula of $C_{24}H_{27}NO_5$, as deduced from the quasi-molecular ion peaks at *m/z* 410.1963 $[M+H]^+$ (calcd 410.196749 for $C_{24}H_{28}NO_5$) in positive FAB-MS, and as supported by the ^{13}C-NMR data (Table 2). Examination of the ^{1}H and ^{13}C-NMR spectra of **1** revealed a great similarity to those of phenanthro [9,10-*b*] quinolizidine-type alkaloids (PQA) such as cryptopleurine and (−)-(15*R*)-hydroxycryptopleurine (Cai et al., 2006; Huang et al., 2002; Ju et al., 2004; Suzuki et al., 1995; Wang and Wang, 2010). These findings suggested that **1** is a PQA derivative having 2, 3, 6-trisubstituted system with three aromatic methoxy groups [δ_H: 4.02 (3H, s, -OCH_3-6), 4.08 (3H, s, -OCH_3-2), and 4.12 (3H, s, -OCH_3-3)]. This observation was further confirmed by the presence of two singlets at δ_H 7.59 (1H, s) and 7.86 (1H, s) for the two aromatic protons H-1 and H-4 respectively, an AMX proton spin system at δ_H 7.86 (1H, d, J = 2.5 Hz, H-5), 7.16 (1H, dd, J = 2.5 and 9.5 Hz, H-7), and 7.61 (1H, d, J = 9.5 Hz, H-8). ^{1}H-^{1}H COSY showed correlation between H-7/H-8; H-15/H-14a and H-14a/H-14. The presence of two meta-coupled protons in the tetra-substituted aromatic of ring A was confirmed through HMBC correlations between H-1 and C-2, C-3, C-4a and C15b, in addition to the correlations between H-4 and C-2, C-15b. Moreover, the structure of compound **1** as shown in Fig. 2. In addition, the ^{13}C-NMR spectrum of **1** revealed the presence of nine quaternary carbons, seven methines, five methylenes, and three methoxy groups. Among them, two methylenes (δ_C 70.2 and 69.8) and one methine (δ_C 67.5) were ascribed to those carbons bearing a nitrogen atom; C-9, C-11 and C-14a (Morita et al., 2004). The signals at δ_H 4.75 (1H, d, J = 15.5 Hz) and 5.26 (1H, d, J = 15.5 Hz) were assigned to H-9α and H-9β protons, which showed direct correlation in ^{1}H-^{1}H COSY spectrum. The signals at δ_H 4.98 (1H, d, J = 2.5 Hz, H-15) and δ_C 67.1 (C-15) in the ^{1}H and ^{13}C-NMR spectra, respectively, confirmed the presence of a hydroxyl group at C-15, which further confirmed from the long correlation between H-9a, 9b and C-11, C8a, C-8b and C-15a. The oxygenation pattern of the molecular formula $C_{24}H_{27}NO_5$ and the downfield shifts of C-9, C-11 and 14a (δ_C 70.2, 69.8 & 67.5) from the respective carbons in hydroxycryptopleurine (δ_C 55.0, 57.0 & 64.0) (Cai et al., 2006), confirmed the presence of *N*-oxide at *N*-10 (Ohyama et al., 2000). This was further confirmed by observing absorption peak of an $N^{\oplus}$ group at 1265 cm^{-1} in the IR spectrum of **1** (Wiley and Wakefield, 1960).

From the foregoing discussion and the 2D-spectral data (^{1}H-^{1}H COSY, HMQC, and HMBC), and by comparison of spectral data with those of the closely related compounds (Chen et al., 2016; Cai et al., 2006; Ju et al., 2004; Stærk et al., 2002; Suzuki et al., 1995), the structure of **1** was concluded to be the *N*-oxide form of 15β-hydroxycryptopleurine The relative configuration of the hydroxy group at C-15 was determined to be β and opposite to that of H-14a on the basis of the small coupling constant (2.5 Hz) (Chen et al., 2016; Cai et al., 2006; Stærk et al., 2002). Therefore, the structure of the new compound **(1)** was elucidated as 15β-hydroxycryptopleurine-N-oxide.



Compound 1 **Compound 1 with significant H-H COSY and HMBC correlations**

Fig. 2. Chemical structure of compound 1.

3.3. Antimalarial activity and cytotoxicity of compound **1**

Recently, 11(12)-epoxyhypoestenone diterpene and 2, 6-dimethoxysavinin lignan, isolated from the whole plant of *Hypoestes verticillaris* were found to demonstrate mild anti-malarial activity against both CQ susceptible (D6) and resistant (W2) strains of *P. falciparum* (IC_{50} values of 328 μM–93 μM (Omole et al., 2019). Also, moderately active antimalarial compounds were previously isolated from the methanolic extract of the aerial parts of *Hypoestes forskaolii* (Al Musayeib et al., 2014). It is worth to mention that extract of *H. forskaolii* was found to demonstrate *in vitro* ovicidal activity against gastrointestinal nematodes of sheep (D'Ambola et al., 2018). Also, local shepherds in Saudi Arabia use decoction of its leaves to externally wash and kill insects and parasites of the sheep (Balkwill and Norris, 1985; Gushash, 2006). The methanolic crude extract of *H. forskaolii* fractions thereof and 15β-hydroxycryptopleurine-N-oxide showed promising antimalarial activity. Compound **1** represents the first phenanthro[9,10-*b*]quinolizidine-type alkaloid with potential antimalarial activity. It showed an IC_{50} values of 2.5 ng/ml (6.11 nM) and 2.1 ng/ml (5.13 nM) against K1 and FCR3, respectively. In addition, it showed cytotoxicity against MRC5 IC_{50} = 10 ng/ml (24.45 nM) with selectivity indices of 4.0 and 4.76 against K1 and FCR3 strains, respectively. To the best of our knowledge, this is the first report about antimalarial activity of phenanthro-quinolizidine alkaloids. On the other hand, number of PQA-type alkaloids were synthesized and investigated for their antitumor activities and toxicities (Ikeda et al., 2011), and their anti-inflammatory activity (Yang et al., 2006) and structure-activity studies (Gao et al., 2007) were previously reported. Accordingly, it is interesting to further study the synthesis and structure-activity relationship (SAR) of 15β-hydroxycryptopleurine-N-oxide and its analogues for developing more potent antimalarial agent(s) to selectively inhibit chloroquine-resistant *Plasmodium falciparum* strain.

4. Conclusion

The methanolic extract of *H. forskaolii* showed the most active extract among eighteen tested plant extracts for their *in vitro* antimalarial activity. Bio-guided fractionation of *H. forskaolii* extract led to isolation of a new antimalarial alkaloid identified as 15β-hydroxycryptopleurine-N-oxide with a promising activity for further detailed study, which open the hope for the synthesis of new phenanthro-quinolizidine alkaloids with therapeutic potential as antimalarial agents.

Author's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Essam Abdel-Sattar: Writing - original draft, Writing - review & editing. **Hossam Mohamed Abdallah:** Writing - original draft, Writing - review & editing. **Sahar El-Mekkawy:** Writing - original draft, Writing - review & editing. **Chikara Ichino:** Writing - original draft, Writing - review & editing. **Hiroaki Kiyohara:** Writing - original draft, Writing - review & editing. **Haruki Yamada:** Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2020.107851.

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