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Antiplasmodial and larvicidal compounds of *Toddalia asiatica* root bark

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Abstract. From the *n*-hexane, ethyl acetate and methanol extracts of *Toddalia asiatica* root bark were isolated eight compounds (**1**–**8**) which were identified on the basis of both spectroscopic and physical data as well as comparison with already published results. The crude extracts and isolated compounds showed moderate *in vitro* antiplasmodial activity against D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) strains of *Plasmodium falciparum*. The extracts and isolates also exhibited larvicidal activities against *Aedes aegypti* and coumarins were identified as the active compounds.

Keywords. *Toddalia asiatica*; *Rutaceae*; coumarins; alkaloid; antiplasmodial; larvicidal activities.

1. Introduction

The plant *Toddalia asiatica* (L) Lam (Rutaceae) is a woody liana reaching a height of 10 m in forests as it uses other plants for support.[1] 1t is widely used by various ethnic groups in Eastern Africa for treatment of myriad ailments including cough, bronchitis, cold, fever, influenza, malaria, bacterial infections, stomachache and snake bite.[2] Recently, the plant leaves and root bark extracts were evaluated *in vivo* for antimalarial activity in mice against chloroquine-tolerant *Plasmodium berghei* NK 65 either alone or in combination with chloroquine.[3,4] Significant parasitaemia suppressions of 31.7–59.3% was observed for root bark alone while the leaves and root bark in combination with chloroquine statistically improved suppressions within the range of 45.5–85.1%. Previous phytochemical studies on the plant have led to the isolation and identification of coumarins,[5–8] alkaloids[9–12] and terpenoids.[13] In this communication, we report the isolation of eight compounds from the plant root bark, of which compounds **4–6** have their ^{13}C NMR data being documented for the first time. The antiplasmodial and larvicidal activities of these secondary metabolites are also presented here.

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2. Experimental

2.1 *General experimental procedures*

Melting points were determined using Gallenkamp melting point apparatus and the values are uncorrected. Ultraviolet-Visible spectra were recorded on a PYE UNICAM SP 8-150 UV/VIS spectrophotometer. Mass spectral data were obtained on a MAT 8200 A Varian Bremen Instrument and acquired at 70 eV ionization. Infrared data were recorded on Perkin-Elmer FTIR 600 series. NMR data were measured in $CDCl_3$ and $CDCl_3$-DMSO-d_6 (as dissolving solvents) on a Brucker NMR Ultrashield TM operating at 500 and 125 MHz, respectively.

2.2 *Plant material collection and preparation*

The roots of *T. asiatica* were collected from the wild at Kajulu location in the outskirts of Kisumu City, Kisumu County, Kenya in April 2010. Standard herbarium specimens were taken to the Department of Botany, University of Nairobi. Identification was done by Mr. Mathenge after comparison with authentic voucher specimens. The root barks removed from the roots were air dried under a shade and the dried plant materials were ground to fine powder.

2.3 *Extraction of T. asiatica root bark*

One kilogram of the ground root bark was cold extracted sequentially using *n*-hexane, ethyl acetate and methanol (three litres each) with occasional shaking using an orbital shaker set at 150 revolutions per minute. The extracts were concentrated using a rotary evaporator *in vacuo* to yield yellow, orange and brown in colour materials with the yields of 18.0 g, 39.0 g and 100.0 g, respectively.

2.4 *Isolation and characterization of compounds from n-hexane and ethyl acetate extracts*

Thin-layer chromatography (TLC) profiles of both the extracts revealed that compounds extracted by *n*-hexane were also present in the ethyl acetate. Therefore, 15 g of the former extract was combined with 30 g of the latter for column chromatography. Approximately 45 g of the combined extract was dissolved in a small amount dichloromethane (CH_2Cl_2) and adsorbed onto silica gel for column chromatography (5.0 × 80 cm, 200 g). Fractionation of the extract using gradient of *n*-hexane-ethyl acetate mixture, ethyl acetate and MeOH afforded 150 fractions (each 100 ml) whose compositions were monitored by TLC using solvent systems; *n*-hexane-ethyl acetate (9:1, 4:1, 2:1, 1:1) and CH_2Cl_2- methanol (99:1 and 95:5), respectively. Those showing similar TLC profiles were combined resulting into four pools (I-IV). Pool I (fractions 5–26, 7 g) crystallized out and was separately filtered and recrystallized (*n*-hexane-ethyl acetate, 9:1) to give dihydronitidine (**1**, 290.5 mg). Fractions 27–40 constituted pool II (20 mg) which also crystallized out and after recrystallization (*n*-hexane-EtOAc, 9:1) afforded aculeatin (**2**, 147.5 mg). The mother liquors from pools I and II upon further purification by repeated low pressure chromatography using *n*-hexane-EtOAc (9:1) followed by the same solvent system in the ratios 4:1 and 3:2, collecting 20 ml each afforded a further **1** (200.0 mg), **2** (87.5 mg), toddaculin (**3**, 194.5 mg) and suberosin (**4**, 45.0 mg), respectively.

Table 1. ^{1}H NMR of compounds **1–7** from *T. asiatica* root bark.

Carbon	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	7.66 s						
2							
3		6.21 d (9.6)	6.21 d (9.6)	6.25 d (9.8)	6.26 d (9.8)	6.16 d (9.7)	6.25 d (9.7)
4	7.50 s	7.85 d (9.6)	7.81 d (9.6)	7.83 d (9.8)	8.10 d (9.8)	7.98 d (9.7)	7.98 d (9.7)
4a							
4b							
5				7.20 s			
6	4.28 s						
6a							
7	7.10 s						
8		6.62 s	6.60 s	6.80 s		6.61 s	6.83 s
8a							
9							
10	7.47 s						
10a							
11	7.69 d (8.5)						
12	6.91 d (8.5)						
12a							
1′		2.87 m	3.33 d (6.9)	3.22 d (6.7)		6.74 d (8.4)	2.78 m
2′		2.87 m	5.12 t (6.9)	5.25 t (7.1)	7.60 d (2.4)	6.54 d (8.4)	3.54 m
3′					6.98 d (2.4)		
OCH_2O	6.03 s						
Me-3′		1.39 s	1.66 s	1.65 s		1.28 s	1.13 s
Me-3′		1.27 s	1.76 s	1.76 s		1.28 s	1.13 s
OMe	3.91 s	3.84	3.81 s	3.90 s	4.14 s	3.95 s	3.85 s
OMe	3.86 s	3.89 s	3.86 s		4.15 s	3.95 s	3.86 s
NCH_3	2.58 s						
OH-3′						4.63	
OH							4.10 d (5.4)
OH							4.10 d (5.4)

"s", "d", and "m" represents singlet, doublet and multiplet

Pool III (fractions 45–75, 15 g) upon repeated fractionation using *n*-hexane-ethyl acetate (4:1, 3:2 and 3:1) over silica gel column yielded a further **4** (15.0 mg), **2** (80.0 mg) and isopimpinellin (**5**, 70.0 mg).

Pool IV (fractions 76–100, 22 g) yielded a gummy material after evaporation *in vacuo* and was further subjected to repeated low pressure chromatography using *n*-hexane-EtOAc (3:2 and 1:1) followed by CH_2Cl_2-MeOH (99:1 and 95:5) to give more of **5** (45.0 mg) and toddalenol (**6**, 43.0 mg), respectively.

2.5 *Extraction and purification of MeOH extract*

The residue from EtOAc extraction was further extracted with MeOH (3 L) in the cold for seven days, filtered and solvent removed using a rotary evaporator to give a brown material (100 g). A portion of the extract (65.0 g) pre-adsorbed on silica gel was fractionated through a silica gel column (3 × 60 cm, 250.0 g) using CH_2Cl_2-MeOH gradient to pure MeOH affording 110 fractions of 50 ml each. The composition of the fractions were monitored by TLC using solvent systems CH_2Cl_2-MeOH (4:1, 3:2 and 1:1) and those that exhibited similar TLC profiles were combined to constitute two major pools (V-VI). Fractions 14–80 (pool V, 12 g) was further purified by flash chromatography using CH_2Cl_2-MeOH (97:3) followed by the same solvent system in the ratio 19:1 to afford more of **6** (87.0 mg) and toddalolactone (**7**, 65.0 mg).

Similarly, fraction 81–110 (pool VI, 8.1 g) upon further purification using low pressure chromatography eluting with CH_2Cl_2-MeOH (17:3) followed by 4:1 gave non-UV active colourless powder which was identified spectroscopically as sucrose (**8**, 300.0 mg).

2.6 *Physical and spectral data of the compounds isolated from T. asiatica*

2.6a *Dihydronitidine **(1)***: Colourless crystals; Mp 172–173°C (from 10% EtOAc in *n*-hexane); UV (CH_3CN) λ_{max} nm: 319.0, 284.0, 212.5; IR (KBr) ν_{max} cm^{-1}: 2950, 2888, 1598, 1494, 1463, 1415; ^1H and ^{13}C NMR: see tables 1 and 2, respectively; ESI-MS: m/z 350.3 $[M+H]^+$.

Table 2. ^{13}C NMR data of compounds **1–7** from *T. asiatica root bark*.

Carbon	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	100.70						
2	148.00	160.98	161.27	160.80	160.47	159.99	160.14
3	152.20	116.69	112.16	113.10	112.81	110.18	111.65
4	104.30	138.82	138.97	142.63	139.40	138.81	139.54
4a	130.80	107.11	107.03	109.22	107.59	102.76	106.50
4b	126.30						
5		155.95	155.11	127.60	144.27	155.22	155.89
6	48.71	112.38	120.22	120.22	114.75	105.46	119.01
6a	123.70						
7	118.60	161.68	161.62	160.38	149.99	160.93	161.83
8	146.10	95.35	95.29	97.77	128.16	91.56	95.27
8a		155.08	154.57	155.43	143.66	152.57	154.14
9	147.40						
10	110.90						
10a	126.20						
10b							
11	120.10						
12	124.20						
12a	142.70						
OCH_2O	101.00						
1′		23.45	22.63	23.50		113.06	25.76
2′		63.08	122.07	121.80	145.10	142.45	75.67
3′		59.07	132.01	133.00	105.08	69.62	71.83
Me-3′		24.69	17.79	18.00		30.23	25.39
Me-3′		18.85	25.67	25.00		30.23	25.54
OMe	55.80	63.54	56.00	56.00	60.80	56.20	62.94
OMe	61.00	56.00	63.10		61.70	56.20	56.24
N-CH_3	41.30						

2.6b *Aculeatin (2)*: Colourless crystals; Mp 115–117°C (from 5% EtOAc in *n*-hexane); $[\alpha]_D^{20}$ - 13.6° (c 0.5, MeOH); UV (CH_3CN) λ_{max} nm: 324.5, 252.5, 243.0, 223.0 and 204.5; IR (KBr) ν_{max} cm^{-1}: 3200–3000, 2984, 2954, 2925, 2851, 1728, 1640; ^1H and ^{13}C NMR: see tables 1 and 2, respectively; ESI-MS (DCI-NH_3): m/z 308.2 $[M+NH_4]^+$, 291$[M+H]^+$.

2.6c *Toddaculin (3)*: Cream crystals; Mp 94–96°C (from 40% EtOAc in *n*-hexane); UV (CH_3CN) λ_{max} nm: 326.0, 253.0, 223.0, 204.5; IR (KBr) ν_{max} cm^{-1}: 3000–3200, 2920, 2856, 1733, 1650, 1550; ^1H and ^{13}C NMR: see tables 1 and 2, respectively; ESI-MS (DCI-NH_3): m/z 566.4 $[2M+NH_4]^+$, 292.2 $[M+NH_4]^+$, 275.2 $[M+H]^+$.

2.6d *Suberosin (4)*: Light yellow crystals; Mp 183–185°C (from 10% EtOAC in *n*-hexane); UV (CH_3CN) λ_{max} nm: 328.0, 250.0, 207.5; IR (KBr) ν_{max} cm^{-1}: 2960, 2845, 1726, 1660, 1570, 1475; ^1H and ^{13} C NMR: see tables 1 and 2, respectively; ESI-MS: m/z 245.3 $[M+H]^+$.

2.6e *Isopimpinellin (5)*: Golden yellow crystals; Mp 147.5–148°C (from 30% EtOAc in *n*-hexane; UV (CH_3CN) λ_{max} nm: 309.5, 267.0, 248.0,241.0, 222.5, 195; IR (KBr) ν_{max} cm^{-1}: 3420, 3151, 3082, 3011, 2949, 2841, 1719; ^1H and ^{13}C NMR: see tables 1 and 2, respectively; ESI- MS (DCI-NH_3): m/z 510.3 $[2M+NH_4]^+$, 246.12 $[M+NH_4]^+$, 247 $[M+H]^+$.

2.6f *Toddalenol (6)*: Greenish-yellow crystals; Mp 224–225°C (from 20% EtOAc in *n*-Hexane); UV(CH_3CN) λ_{max} nm: 317.0, 288.5, 259.0, 216.5; IR (KBr) ν_{max} cm^{-1}: 3450, 3000–3200, 2950, 2840, 1723, 1645, 1445; ^1H and ^{13}C NMR: see tables 1 and 2, respectively; ESI- MS (DCI-NH_3) m/z: 308.2 $[M+NH_4]^+$, 273.1$[M\text{-}OH]^+$; EI-Ms (70 eV) m/z: 290 (3), 272 (21).

2.6g *Toddalolactone (7)*: Colourless Crystals; Mp 141–141.5°C (from 1% methanol in DCM); UV (CH_3CN) λ_{max} nm: 326.0, 253.0, 242.5, 224.0, 205.0; IR (KBr) ν_{max} cm^{-1}: 3300, 2930, 2860, 1732, 1635; ^1H and ^{13}C NMR: see tables 1 and 2, respectively; ESI- MS (DCI+NH_3) m/z: 634.5 $[2M+NH_4]^+$, 617.4 $[2M+H]^+$, 326.3 $[M+NH_4]^+$, 309.2 $[M+H]^+$; EIMS (70 eV) m/z: 308 $[M]^+$ 914, 290 $[M\text{-}H_2O]^+$ (23), 272 $[M\text{-}2H_2O]^+$ (100).

3. Bioassay

3.1 *In vitro mosquito larvicidal assay*

The larvicidal activities of crude extracts and pure isolates of *T. asiatica* were evaluated against 2nd instar larvae of *Aedes aegypti* according to WHO larval susceptibility test method.[14] The larval bio-efficacy of test samples was determined by making serial dilutions of 100, 50, 25, 12.5, 6.25 3.125 µg/ml and the bioassays were performed within a temperature range of 27°–30°C and relative humidity of 75.5 ± 5% under light and dark cycles of 12 h each.

For control, 1 ml of ethanol was added to 19 mL of water and for each concentration, the experiment was carried out in triplicates. A group of 20 larvae were counted and placed in each of the transparent plastic cups (300 ml) that were used in carrying out the tests.

The number of the dead and moribund larvae were counted and recorded after 24 h and 48 h. The larvae were considered dead or moribund if they were unresponsive to light within 3 min even after prodding with a needle.[15] Mortality was corrected for the natural mortality observed in the negative controls using the formula $P = (P1 - C)/1 - C$,[15] where P1 denotes observed mortality and C represents the natural mortality observed from the negative control. The data obtained was subjected to log-probit regression analysis to calculate the median lethal concentrations (LC_{50}) values.

3.2 *In vitro antiplasmodial assay*

The samples (crude extracts and pure compounds) were assayed using an automated micro-dilution technique to determine the IC_{50} (50% growth inhibition of cultured parasites).[16]

The two strains of *P. falciparum* used were chloroquine resistant *Indochina* (W2) and chloroquine sensitive *Sierra Leone* D(6). Quinine, chloroquine and mefloquine were used as the reference drugs.

Test compounds (5 mg) were dissolved in 5 ml dimethyl sulphoxide (DMSO) to give stock solutions of 1 mg/ml. To reach the desired starting concentration, the stock solutions were diluted in the media RPMI 1640 and 350 µL of the test compounds or drug solutions loaded on to the wells of the first column of a sterilized 96 well flat bottom culture plates. This was followed by two-fold serial dilutions using a Biomek Automated Laboratory work station.

The last column of the plate was left for negative and positive control. The positive control consisted

of three complete medium with serum (CMS) containing parasites whereas the negative control consisted of CMS and erythrocytes with no parasites. A 200 μL of the parasitized blood (1% parasitemia) and 6% haemacrit were added to each and incubated for 24 h at 37°C in 6% CO_2. These resulted in the first wells of each compound to be tested at a concentration of 50 μg/ml. A 25 μL of [3H] hypoxanthine was added per well and incubated for a further 18 h at 37°C, then frozen at −20°C for maximum lyses of erythrocytes. The content of the plates were harvested using an automated cell harvester from Puckered Bioscience and then left to dry. Scintillation fluid was added to the plates, sealed and then counted on a liquid scintillation counter and this gave raw data representing the parasites counts.

Computation of the concentration of drugs causing 50% inhibition of (G-3H) hypoxanthine uptake (IC_{50}) was carried out by interpolation after logarithmic transformation uptake of both concentration and radioactivity in counts per minute (CPM) values using the formula indicated.

To calculate IC_{50}, the mid point (Y_{50}) was calculated by the formula below.

$$Y_{50} = [(\text{PRBC} - \text{CPM value}) - (\text{UNPRBC} - \text{CPM value})]/2$$

$$IC_{50} = \text{antilog}\left[\log X_1 + (\log Y_{50} - \log X_1) \times (\log X_2 - \log X_1)\right]/\log Y_2 - \log Y_1,$$

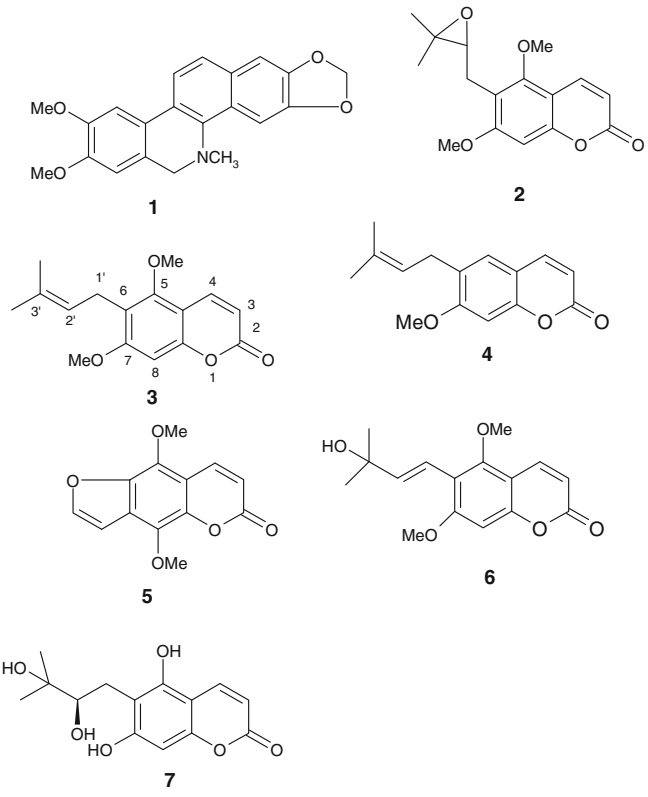
where IC_{50} = 50% growth inhibition of cultured parasites.

X_1 and X_2 = lower and higher concentration of the samples, respectively. Y_1 = CPM value which corresponds with X_1. Y_2 = CPM value which correspond with X_2.

4. Results and discussion

4.1 *Phytochemical studies*

Chromatographic fractionation of *n*-hexane, ethyl acetate and MeOH extracts of *T. asiatica* root bark afforded eight compounds characterized as dihydronitidine (**1**), aculeatin (**2**), toddaculin (**3**), isopimpinellin (**5**), suberosin (**4**), toddalenol (**6**), toddalolactone (**7**) and α-D-sucrose (**8**). The isolates were identified using spectroscopic methods (UV, IR, ^{1}H and ^{13}C NMR, and MS) as well as comparison with already documented literature.[6,12,17–21]



4.2 *Larvicidal activity of crude extracts and pure isolates from T. asiatica against Aedes egypti*

n-Hexane, ethyl acetate and methanol extracts and isolated compounds from the root bark were tested for their potency against mosquito larvae and the result are as shown in table 3. The *n*-hexane extract showed the best activity with LC_{50} value of 9.4 ± 1.0 μg/ml after 48 h exposure as compared to ethyl acetate 13.8 ± 3.5 μg/ml and methanol 50.1 ± 3.0 μg/ml after the same exposure duration.

The isolate toddalenol (**6**) was the most toxic with LC_{50} value of 6.4 ± 1.0 μg/ml after 48 h exposure to the test organisms. The LC_{50} values for others were as follows: isopimpinelin (**5**) 7.6 ± 1.0 μg/ml, toddaculin (**3**) 10.2 ± 2.0 μg/ml, dihydronitidine (**1**) 11.8 ± 2.5 μg/ml, toddalolactone (**7**) 497 ± 4.5 μg/ml after same exposure duration. Aculeatin (**2**) did not show any activity even at a high concentration of 100 μg/ml. The larvicidal potency of *T. asiatica* and its constituents against *Aedes aegypti* is reported for the first time. All the coumarins isolated had same skeleton but with varied biopotency. The differences in toxicity could be due to varied substituents on the parent molecules. From the test results, it can be noted that at 48 h test

Table 3. LC_{50} of *T. asiatica* extracts and constituents against 2nd instar larvae of *Aedes Aegypti* after 24 h and 48 h exposure, respectively.

Sample	LC_{50}(μg/ml) in 24 h	LC_{50}(μg/ml) in 48 h
Crude extracts/isolated compounds		
n-Hexane	10.4 ± 2.0	9.4 ± 1.0
Ethyl acetate	13.8 ± 3.5	13.8 ± 3.5
Methanol	57.2 ± 5.0	50.1 ± 3.0
Dihydronitidine (**1**)	12.2 ± 3.5	11.8 ± 2.5
Aculeatin (**2**)	NA	NA
Toddaculin (**3**)	509 ± 2.5	10.2 ± 2.0
Isopimpinelin (**5**)	8.2 ± 0.5	7.6 ± 1.0
Todalenol (**6**)	6.8 ± 0.5	6.4 ± 1.0
Toddalolactone (**7**)	509 ± 5.5	497 ± 4.5

Key: NA = not active

duration, extracts and isolates showed better LC_{50} values than at 24 h exposure. This suggested that at longer test duration the samples could be more toxic to the larvae.

4.3 *Antiplasmodial activity of T. asiatica crude extracts and isolated compounds*

Chloroquine-resistant *Indochina* (W2) and chloroquine-sensitive *Sierra-Leone* (D6) strains of *Plasmodium falciparum* were used in this test. Crude extracts from the root bark were potent against the resistant strain of the parasite with the highest activity recorded with ethyl acetate extract; IC_{50} 9.4 ± 1.1 μg/ml. *n*-Hexane and methanol extracts had values of IC_{50} 26.4 ± 0.1 and 15.6 ± 2.5 μg/ml, respectively.

Among the isolates, the alkaloid dihydronitidine (**1**) was fairly active against both strains of parasite with IC_{50} values of 6.5 ± 1 and 8.7 ± 1.1 μg/ml against W2 and D6 strains, respectively. This activity is quite low compared to nitidine[8] which had IC_{50} values of 9 ng/ml and 108 ng/ml against W2 and D6, respectively. Thus, reduction of nitidine to dihydronitidine reduces its ability to bind to the DNA protein of the parasite.[22] Coumarins showed comparable activity to the alkaloid with isopimpinellin (**5**) being most active against W2 with IC_{50} value of 6.0 ± 2.0 μg/ml while toddaculin (**3**) was the most active against D6 strain with IC_{50} value of 4.5 ± 0.1 μg/ml (table 4). The biopotency of toddaculin (**3**), isopimpinellin (**5**) and aculeatin (**2**), were much better than coumarins previously reported.[7] The antiplasmodial activities of dihydronitidine (**1**), aculeatin (**2**),

Table 4. *In vitro* IC_{50} values of crude extracts and constituents of *T. asiatica* against W2 and D6 strains of *Plasmodium falciparum*.

Sample/drug	W2 (μg/ml)	D6 (μg/ml)
Extracts		
n-Hexane	26.4 ± 0.1	26.1 ± 0.3
Ethyl acetate	9.4 ± 1.1	12.4 ± 0.9
Methanol	15.6 ± 2.5	17.1 ± 1.6
Isolated compounds		
Dihydronitidine (**1**)	6.5 ± 1.6	8.7 ± 1.1
Aculeatin (**2**)	8.3 ± 1.1	7.6 ± 1.7
Toddaculin (**3**)	6.1 ± 1.3	4.5 ± 0.1
Isopimpinelin (**5**)	6.0 ± 2.0	7.0 ± 1.6
Todalenol (**6**)	NA	NA
Toddalolactone (**7**)	14.1 ± 0.1	15.6 ± 0.6
Standard drugs		
Chloroquine	0.576 ± 0.005	0.0032 ± 0.045
Mefloquine	0.009 ± 0.001	0.023 ± 0.001
Quinine	0.098 ± 0.020	0.0208 ± 0.015

Key: NA = not active

toddalolactone (**7**), isopimpinellin (**5**) and toddaculin (**3**) are being reported for the first time. The activity of the coumarins could be due to α, β-unsaturated keto group.[23] Activity observed could be attributed to the reason why the plant is used to treat malaria and its associated symptoms by traditional health practitioners.

5. Conclusion

The present study describes the compounds isolated from *T. asiatica* and their biological activities. The findings validate the use of the plant in herbal medicine against malaria. The compound structures were confirmed by IR, NMR and mass spectral data.

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