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Antiplasmodial activity of extracts and characterization of the most active extract from *Commiphora wightii*: A data deficient medicinal plant

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ABSTRACT

Commiphora wightii is a celebrated plant in ethnohistory of medicine possessing multifarious medicinal potentials, of which much remain still unexplored. This study was undertaken to evaluate the antiplasmodial potential of *C. wightii*. Extracts from *C. wightii* stem obtained by sequential fractionation using n-hexane, dichloromethane, ethyl acetate, ethanol and water (AQ) were screened for *in vitro* antiplasmodial activity against chloroquine (CQ)-sensitive *Plasmodium falciparum* (3D7) strain at 25 µg/ml final concentration. Of all the extracts tested, *C. wightii* AQ extract showed highest parasite inhibition (89.33±7.64%) and hence, was selected for further studies. The extract was classified as good to moderate antimalarial agent (IC_{50} =5.34 µg/ml). This extract did not exhibit any cytotoxicity on HEK-293 cell lines (IC_{50} >50 µg/ml) or hemolytic activity on human erythrocytes (HC_{50} >500 µg/ml). Phytochemical analysis revealed the abundance of saponins and amino acids in this extract along with alkaloids, carbohydrates and phenolics. An abundant compound similar to phenylenthanoid glycoside Scroside D, a characteristic constituent of antimalarial plant *Stachytarpheta cayennensis* was identified from LC-MS-QTOF analysis of this extract which is proposed to confer antiplasmodial activity to *C. wightii*.

KEYWORDS: Antimalarial, Antiplasmodial, *Commiphora wightii*, Cytotoxic, Malaria, *Plasmodium.*

INTRODUCTION

Commiphora wightii (Arn.) Bhandari a shrub from the *Burseraceae* family, is a highly valuable medicinal plant from Indian traditional systems of medicine and also has a nice status in the modern drug system. The earthy aromatic fragrance with mossy and skunk-like notes is distinctive of *C. wightii*. The plant runs around 4m in height and has numerous coloured, crooked and spirally ascending branches ending in sharp spines. The green barks of the plant are enclosed under shiny, ash to yellowish-white papery cover, which peels off in rolls. The leaves are deciduous, sessile, simple or trifoliate with 1-5 cm long ovate leaflets which are leathery and shiny green on top, greyish from bottom and have irregularly serrated edges. To further embellish the plant, small brownish-red sessile single or fascicles of flowers and berry-like red drupe ripe fruits of 7-8 mm diameter are present. It is disturbed in arid and semi-arid zones of northern Africa and tropical Asia.[1] In India it grows wild in Rajasthan, Gujarat (mostly Aravallis) and Karnataka.[2] It derives its generic name from Greek words 'phora' and 'kommis' which means gum bearer. Commonly known as 'Indian bdellium' in English, Mahisaksha, Guggulu, Amish, Palanksha and Pur in Sanskrit, it is popular as Guggul in most of the Indian languages.[3] The oleo gum resin, 'gum-guggul' or 'Indian myrrh' exuded by this plant is useful in the treatment of arthritis, gout, inflammation, lipid disorders, nodulocystic acne, arthritis, and obesity.[4] This myrrh when brunt has a pronounced calming effect on mind and is an important smudging incense in India and Pakistan. A commercial product 'Guglip' is marketed by CIPLA in India since 1988. Unfortunately, the plant has become endangered due to its slow-growing nature, poor seed setting, lack of cultivation, poor seed germination rate and excessive, crude and unscientific tapping of its gum resin by pharmaceutical industries and religious people.[5] It entered 1997 IUCN Red List as 'vulnerable' and 2004 IUCN Red List as 'data deficient'.[6] Being a plant from data deficient category *C. wightii* has attracted researchers in the recent past to generate information on various aspects of this plant including its medicinal properties.[7] *Commiphora* genus plants are widely used to treat malaria by the Maasai community in Kenya and Tanzania traditionally.[8, 9] But there is an absence of documents on ethnomedicinal information as well as scientific evaluation of the antiplasmodial potential of *C. wightii*. Concurrently, the emergence of resistant strains of *P. falciparum* to the frontline drugs including those used under Artemisinin-based Combination Therapy (ACT), which remains the most effective method to treat the resistant parasite[10], calls for the urgent discovery of new antimalarials. The importance of plants in antimalarial drug discovery has been evidenced since the origin

of two first-line antimalarial drugs- quinine and Artiminsisnin derivatives from medicinal plants.

This study aimed to evaluate the antiplasmodial activity of extracts from *C. wightti*. The analysis of potential antiplasmodial extract for cytotoxic activity and hemolytic activity was performed. We also identified the constituents from this extract using phytochemical analysis and LC-MS-QTOF analysis.

MATERIALS AND METHODS

Plant collection and extract preparation

Fresh samples of *C. wightii* were collected during the months of May and June from Vadodara, Gujarat. The plants were authenticated at the herbarium of The Maharaja Sayajirao University of Baroda (Biodiversity Collection Index Code: BARO), Vadodara, Gujarat where the voucher specimen of this plant namely, *C. wightii* was deposited under registration ID: JKS2. The plant stem was rinsed thoroughly to remove dirt and it was air-dried at room temperature. Extracts were prepared by subjecting 20 g of dried and ground plant material to sequential solvent extraction in the order of increasing polarity, using 200 ml each of n-hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (EtOH) and water (AQ) for 24 h at 40˚C. After filtration, the extracts were concentrated under reduced pressure at 40˚-45˚C using rotary evaporator (Buchi) and then stored at 4˚C until further used.

In vitro antiplasmodial activity

Plasmodium falciparum Chloroquine (CQ)-sensitive 3D7 strain was procured from NIMR, Delhi. The parasite culture was done using methods from Moll *et al.*[11]. Briefly, the parasite was maintained in freshly collected O^+ human RBC's at 4% hematocrit in RPMI 1640 with the addition of albumax and hypoxanthine at 37˚C in an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. The level of parasitemia was maintained at 2%. The synchronization of the parasite to the ring stage was done using 5% sorbital treatment method. Assays were performed in 96-well microtiter plates in triplicate. For initial screening, ring-stage synchronized parasite at 2% parasitemia and 4% hematocrit was treated with extracts at the final concentration of 25 µg/ml. The stocks for extracts were prepared in RPMI and the dissolution was assisted with DMSO (final concentration <1%). Culture samples with CQ diphosphate at the final concentration of 1.5 ng/ml were used as positive controls and those which did not receive any treatment served as negative controls. After 72 h of incubation, parasitemia was calculated as the average from four random sectors by microscopically observing Geimsa stained slides.

The percentage inhibition of the parasite growth was calculated according to the following equation: % Parasite inhibition = [1 - (P_S/P_{NC})] x 100, where P_S is the parasitemia of test sample or positive control, P_{NC} is the parasitemia of negative control. The extract(s) showing more than 80% inhibition of parasite growth were selected for further studies. The selected extract(s) were assessed for their IC_{50} values using dose-response analysis.

***In vitro* cytotoxic activity**

Assessment of cytotoxic activity of plant extract was done against Human embryonic kidney 293 cell lines (HEK- 293) by an assay performed in triplicate. Briefly, 200 µl (10,000 cells/well) cells were seeded in 96-well microtiter plates. After 24 h of incubation, the spent medium was aspirated. Then 200µl of various concentrations of test extract prepared in medium, ranging from 10-50 µg/ml final concentration was added to the respective wells and the plates were incubated. Cells treated with Cisplatin at the final concentration 15 µg/ml were used as standards and untreated cells were kept as negative controls. Wells with the medium without cells were kept as blanks. After 24 h, 10% MTT reagent was added to the wells to a final concentration of 5 mg/ml for 3 h, thereafter formazan crystals formed were collected and solubilized in 100 µl of DMSO. The final absorbance was determined after subtraction of background absorbance taken at 630 nm, from the MTT absorbance taken at 570 nm. After substracting the blank absorbance, the percentage cell cytotoxicity was calculated according to the following equation: % Cytotoxicity = [1 - (A_S/A_{NC})] x 100, where A_S is the absorbance of test sample or standard, A_{NC} is the absorbance of negative control. The IC_{50} values of extract on cell growth was calculated from dose-response curve. The selectivity of extract for parasite versus HEK-293 cells expressed as selectivity index (SI) was determined by the ratio of the IC_{50} value for HEK-293 cells to the IC_{50} value for *Plasmodium falciparum* 3D7.

***In vitro* hemolytic activity**

The hemolytic effect of plant extract was evaluated using human erythrocytes in a triplicate assay. In brief, erythrocytes collected from Suraktam Blood Bank (Vadodara, Gujarat) were suspended in Phosphate Buffer Saline (PBS). The erythrocytes suspension (2% hematocrit) was then treated with plant extract at different concentrations (50-500 µg/ml) at 37˚C for 1 h. After incubation, erythrocytes were pelleted at 3000 g for 10 min at 4˚C. To quantify the amount of hemoglobin released due to erythrocyte lysis if any, the supernatant was collected and absorbance at 450 nm was read spectrophotometrically. Erythrocytes suspended in PBS

were considered as negative controls, having 0% hemolysis and erythrocytes treated with Triton X-100 (0.1%) were kept as positive controls having 100% hemolysis of the cells. The percentage hemolysis was determined as the percentage relative to the hemolysis caused by Triton X-100 using the following equation: % Hemolysis = $[(A_S-A_{NC})/(A_{PC}-A_{NC})] \times 100$, where A_S is the absorbance of test sample, A_{NC} is the absorbance of the negative control and A_{PC} is the absorbance of the positive control. The concentration of extract which exhibited 50% hemolysis, (HC_{50}) was calculated from dose-response curve. Extract with HC_{50} value lower than 200µg/ml was considered as hemolytic.

Qualitative and Quantitative analysis of phytochemical constituents

Screening and identification of major phytochemical constituents of potential antiplasmodial plant extract(s) were carried out using standard procedures as described by Harborne[12], Sofowora[13] and Trease & Evans.[14]

LC-MS-QTOF analysis

The separation and mass analysis of the components of *C. wightii* AQ extract which exhibited promising antiplasmodial activity was performed with LC system (Agilent Technologies) using a C-18 column equipped with a photodiode array detector, coupled with a Q-TOF mass spectrometer and 6200 series TOF/6500 series Q-TOF B.08.00 (B8058.0) data processing software. A gradient of water: methanol was used as the mobile phase, at a temperature of 25˚C at a flow rate of 0.4 ml/min, where 90% water and 10% methanol started the gradient. The detection wavelength was kept from 190 to 400 nm. The mass spectrometry analysis was performed in positive mode with the mass scan over the range of 100-1500 m/z. The fragmentation pattern and the base peaks in the mass spectra generated were matched against the public databases such as NIST and Pub chem as well as literature available was referred for putative identification of compounds.

Statistical analysis

Data was expressed in terms of mean±standard deviation. For comparison of data and determination of statistical significance, one-way ANOVA and two-tailed t-test (GraphPad Prism 6.0, GraphPad Software Inc., U.S.A.) were used. IC_{50} and HC_{50} values were calculated by using dose-response analysis (GraphPad Prism 6.0, GraphPad Software Inc., U.S.A.).

RESULTS AND DISCUSSION

In vitro antiplasmodial activity

The antiplasmodial activity of *C. wightii* extracts was evaluated against CQ-sensitive *P. falciparum* 3D7. For initial screening, 25 µg/ml concentration of the plant extracts was considered (Fig. 1). Overall, the antiplasmodial activity was found to increase gradually along with the increasing polarity of the extract fractions. Among all the extracts tested, *C. wightii* AQ extract showed the highest parasite inhibition with the extent of 89.33±7.64% with no significant difference from CQ diphosphate treated control ($P \leq 0.05$), suggesting that the antiplasmodial principles were most concentrated in the AQ extract. The study ahead was focused on this extract only. The IC_{50} value for this extract was calculated as 5.34 µg/ml and the IC_{50} value of the pure compound control, CQ diphosphate was found to be 0.27 ng/ml (Fig. 2). Based on the classification given by Rasoanaivo *et al.*[15] for antimalarial activity, the extract was classified as class III antimalarial, with good to moderate antimalarial activity.

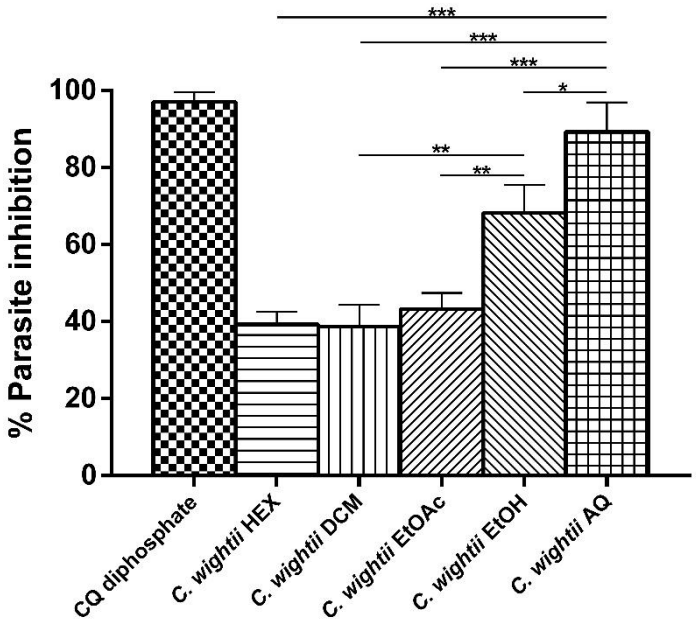

Fig. 1: Percentage inhibition of *P. falciparum* 3D7 following a 72 h exposure to *C. wightii* extracts *in vitro* at the final concentration of 25 µg/ml.

Data represents Mean±SD (n=3); *, ** and * indicate statistically significant difference at P≤0.05, 0.01 and 0.001 respectively.**

Water boiled extracts from *C. schimperi* (O. Bergman) Engl. have been used by the Maasai community of Kenya to treat malaria and the methanol derived chloroform extract from this plant was reported to have antiplasmodial activity[8]. In addition to the current study where

antiplasmodial activity was found in *C. wightii* AQ extract, these reports suggest the presence of antiplasmodial agents in the *Commiphora* genus which are polar in nature.

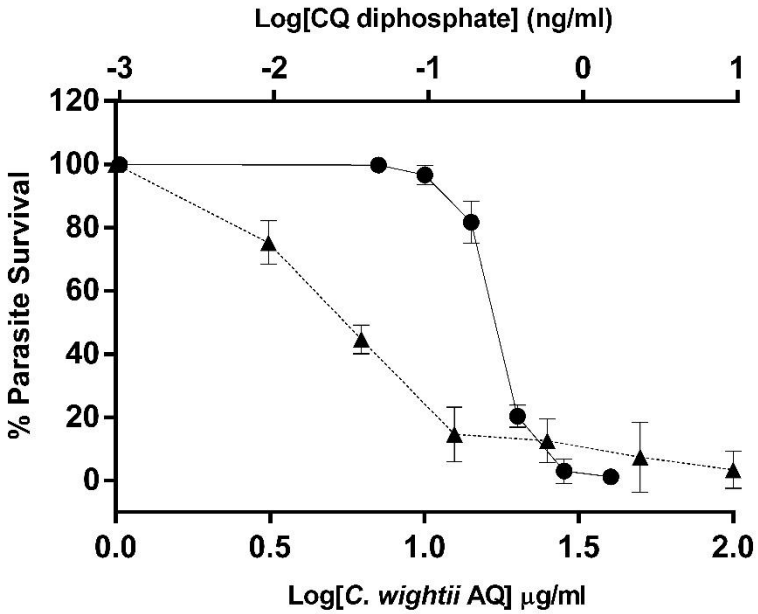

Fig. 2: IC_{50} determination of Control Chloroquine diphosphate and *C. wightii* aqueous extract against *P. falciparum* 3D7 *in vitro.*

Data represents Mean±SD (n=3); IC_{50} for Chloroquine diphosphate (-●-) was calculated as 0.27 ng/ml, IC_{50} for *C. wightii* aqueous (-▲-) extract was calculated as 5.34 µg/ml.

***In vitro* cytotoxic activity**

C. wightii AQ extract did not exhibit any cytotoxic activity against HEK-293 cells and the IC_{50} value of the extract was found to be > 50 µg/ml (Fig. 3). Absence of significant cytotoxicity even at the highest test concentration 50 µg/ml, indicated a SI > 10. Toxicities from *C. wightii* have been reported only from resin derived extracts which include, mild gastrointestinal discomfort, thyroid problems, generalized skin rash.[16] Although around 53% of the resin components are water soluble[4], no cytotoxicity from AQ extract derived from *C. wightii* was observed in our study. A SI value > 10 suggests that the antiprotozoal activity of the plant extract is not due to general cytotoxicity and the extract is promising for further phytochemical analysis.[17] The absence of cytotoxicity suggested *C. wightii* AQ extract to be highly selective for the parasite and recommended its further analysis.

In vitro hemolytic activity

No hemolytic activity was observed by *C. wightii* AQ extract till the concentration of 250 µg/ml but a slight hemolytic activity of 3.37±1.82% was found at 500 µg/ml extract concentration (Fig. 3). The HC_{50} value of the extract was found to be > 500 µg/ml. Although, hemolysis might have been observed in the case of *C. wightii* AQ extract due to the significant abundance of saponins[18], but interestingly the extract was found to be harmless to erythrocytes with HC_{50} >500 µg/ml, a concentration well above than that required for this extract to exhibit antiplasmodial effect or other therapeutic effects in general. Along similar lines, lack of hemolytic potency was observed in the case of boswellic acids, which are the main sapogenins of the gum resin of a plant from the *Burseraceae* family, *Boswellia* serrate[19] also known as Salai guggul in Sanskrit. This suggests the presence of boswellic acids or similar sapogenins as the main constituent aglycones of *C. wightii* saponins. Furthermore, our observation corroborates the statement that various structural aspects, such as the nature of the aglycone backbone, the complexity of sugar moieties, and the number, length, and position of sugar side chains, may affect hemolytic activity.[19]

Qualitative and quantitative analysis of phytochemical constituents

Phytochemical analysis of *C. wightii* AQ extract revealed free amino acids and saponins as abundant components of the extract along with the presence of alkaloids, carbohydrates and phenolics as other major components (Table 1).

LC-MS-QTOF analysis

Compounds from abundant classes of *C. wightii* AQ extract exposed after phytochemical analysis were identified by LC-MS-QTOF analysis. The results obtained from the LC-MS-QTOF analysis showed 14 components, out of which 8 could be identified (Table 2). Among free amino acids detected valine could be identified. A phenylenthanoid glycoside Scroside D was identified and it is worth mentioning that this compound produced the most abundant ions. Other components identified included 2 alkaloids, 2 carbohydrates, 1 phenolic compound, and 1 phenylpropanoid. Additionally, 6 saponin derivatives were also detected from the extract.

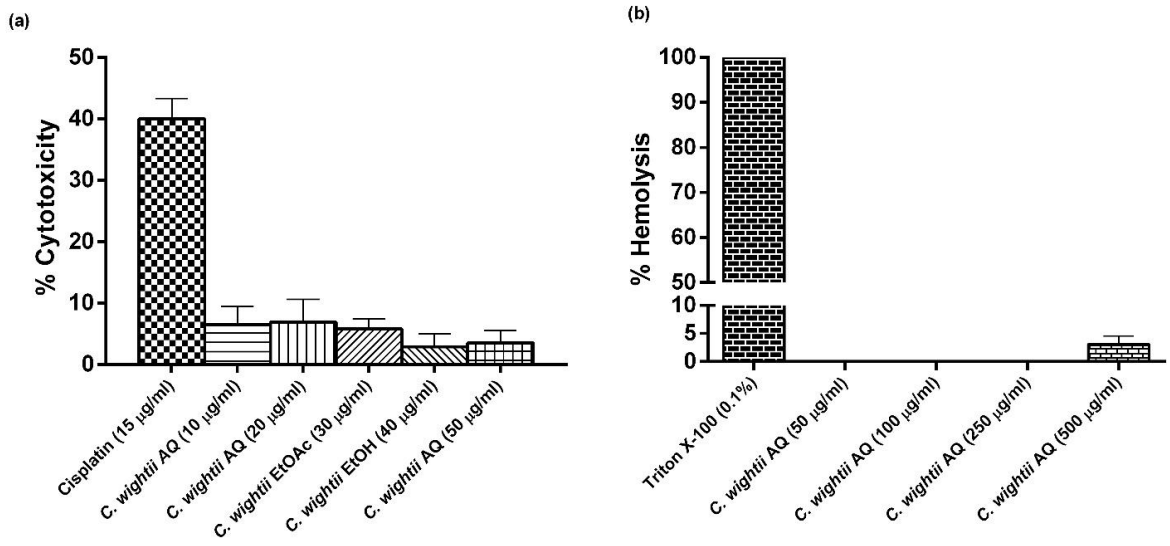

Fig. 3: Cytotoxicity assay and Hemolysis assay results of *C. wightii* aqueous extract performed *in vitro.*

(a) The percentage cytotoxicity induced upon incubation of HEK-293 Cells with *C. wightii* aqueous extract at various concentrations. Cisplatin was used as a positive control. Data are presented as mean± standard deviation from an experiment done in triplicate.

(b) The percentage hemolysis of human erythrocytes upon treatment with *C. wightii* aqueous extract at various concentrations. Triton X-100 (0.1%) was used as a positive control. Data are presented as mean± standard deviation from an experiment done in triplicate.

Scroside D, which is a phenylenthanoid glycoside (a type of phenolic compound) with powerful antioxidant activity[20] was found as one of the main components in *C. wightii* AQ extract. Phenylenthanoid glycosides are characteristic constituents of the plant *Stachytarpheta cayennensis*, which is used ethnomedically to treat symptoms of malaria.[21] This raises the possibility of Scroside D as an antiplasmodial principle from *C. wightii* which exhibits its antiplasmodial effect by virtue of its strong antioxidant potential. Besides, Scroside D presence may also contribute to the antioxidant activity of the *C. wightii*, in addition to the established antioxidants from this plant, Z-guggulsterones and E-guggulsterones.[22]

Table 1: Qualitative and quantitative phytochemical screening of *C. wightii* aqueous extract.

Phytochemical constituents	Qualitative analysis	Quantitative analysis (µg/mg)
Alkaloids	±	127.15±31.53
Carbohydrates	±	23.70±3.34
Flavanoids	-	0.00±0.00
Phenolics	±±	12.08±1.76
Proteins	-	0.00±0.00
Saponins	±±	434.42±49.22
Steroids	-	0.00±0.00
Terpenoids	-	0.00±0.00
Amino acids	±±±	318.71± 25.44
Anthraquinone	-	0.00±0.00
Phlobatannins	-	0.00±0.00

Values expressed as Mean±SD (n=3); - = absent; ± = present; ±± = moderately present; ±±± = abundantly present

Table 2: Compounds detected from *C. wightii* aqueous extract by LC-MS-QTOF analysis.

RT (Start-End in mins)	m/z	Ion type	Compound Monoisotropic mass	Formula	Compound Class	Putative identified compound
1.274-7.444	140.0684	[M+Na]+	117.079	$C_5H_{11}NO_2$	Amino acids	Valine
	203.0531	[M+Na]+	180.0634	$C_6H_{12}O_6$	Carbohydrates	Glucose, Myo-inositol
	215.0531	[M+Na]+	192.0634	$C_7H_{12}O_6$	Phenolics	Quinic acid
	224.1281	[M+H]+	223.1208	$C_{12}H_{17}NO_3$	Alkaloids	Anhalidine
	224.1281	[M+H]+	223.1208	$C_{12}H_{17}NO_3$	Alkaloids	Anhalonidine
10.662-15.588	457.1319	[M+Na]+	434.1424	$C_{18}H_{26}O_{12}$	Carbohydrates	Sorbitol hexaacetate
	501.1584	[M+Na]+	478.1686	$C_{20}H_{30}O_{13}$	Phenylenthanoid glycosides	Scroside D
	501.1585	[M+Na]+	478.1686	$C_{20}H_{30}O_{13}$		
	502.1615	[M+Na]+	478.1686	$C_{20}H_{30}O_{13}$		
	517.132	[M+K]+	478.1686	$C_{20}H_{30}O_{13}$		
25.456-27.463	163.0762	[M+H]+	162.0681	$C_{10}H_{10}O_2$	Phenylpropanoids	Methyl cinnamate
27.612-29.884	782.571	M+Na]+	759.5815	$C_{35}H_{77}N_{13}OS_2$	Saponin glycosides	Saponin derivatives
	786.6029	[M+H]+	785.5963	$C_{35}H_{69}N_{20}O$		
	810.6032	[M+H]+	809.595	$C_{35}H_{67}N_{23}$		
	811.6064	[M+Na]+	788.6198	$C_{35}H_{72}N_{20}O$		
	812.6146	[M+Na]+	788.6198	$C_{35}H_{72}N_{20}O$		
	834.6015	[M+K]+	795.6409	$C_{39}H_{85}N_7O_9$		

RT: Retention time

CONCLUSIONS

The present study explored the antiplasmodial potential of *C. wightii and* demonstrated the antiplasmodial activity of its AQ extract. The lack of toxicity observed from *C. wightii* AQ extract is another valuable information as aqueous decoctions from the *Commiphora* genus are widely used traditionally. Phytochemical analysis revealed the major components of this extract and the results along with literature propose Scroside D as the antiplasmodial principle from *C. wightii*. Studies with putative antiplasmodial principle Scroside D *in vivo* can lead us to the drugs urgently needed to restore the antiplasmodial drug

reserve. The work is an addition to the recent efforts to generate information on the medicinal properties of *C. wightii*.

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