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Antiplasmodial activity, in silico ADME and mammalian cell cytotoxicity of a synthetic protoberberine alkaloid, coralyne

§ [1,2]**Nekpen Erhunse, and [1]Dinkar Sahal**

[1]Malaria Drug Discovery Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, 110067, India.

[2]Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

§**Corresponding author:** Nekpen Erhunse; Email: nekpen.erhunse@uniben.edu

Abstract

Coralyne is a synthetic protoberberine alkaloid with anticancer activity and selectivity superior to that of berberine, its congener. As berberine is gifted with antiplasmodial activity, this study assessed the antiplasmodial activity of coralyne against erythrocytic stages of the malaria parasite in culture. Parasites were cultured by adopting the method described by Trager and Jensen in 1976. Following this, parasites were exposed at ring stage to increasing doses of coralyne to enable us compute the IC_{50}. Further, given that berberine is a substrate of the efflux transporter permeability glycoprotein (P-gp), in silico techniques were used to study the pharmacokinetics of oral coralyne. Coralyne showed excellent potency (IC_{50}*Pf3D7*: 0.52 µg/ml) against chloroquine sensitive strain and a little less potency (IC50*PfINDO*: 1.15 µg/ml) against the chloroquine resistant malaria parasite strain (Resistance index: 2.21). Further, with CC_{50}HEK: >100 µg/ml, it was non-toxic to mammalian cells. However, in silico absorption, distribution, metabolism and excretion (ADME) studies predicts that like berberine, coralyne may also have poor oral bioavailability thus limiting its usefulness as an orally deliverable antimalarial agent. Given the negative impact of low bioavailability in the development of protoberberine alkaloids as antimalarials, synthesizing analogues of coralyne with nanomolar potency against the malaria parasite and improved oral pharmacokinetics may be a good strategy for the future.

Keywords: Protoberberine alkaloids, Coralyne, Antiplasmodial activity, Cytotoxicity, ADME, Permeability glycoprotein

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INTRODUCTION

Malaria, a life-threatening disease is caused by *Plasmodium* parasites which are transmitted to humans via the mosquito vector. Once in the human host, it migrates to the liver to initiate the exoerythrocytic cycle. Here, it forms liver schizonts containing many merozoites which are released as merosomes that disintegrate releasing merozoites which infect red blood cells to begin the erythrocytic cycle (Pudêncio *et al*., 2006; Erhunse and Okomayin, 2022). At

this point, the infected individual shows malaria-related symptoms. Compounds which possess blood-stage antiplasmodial activity act by interfering with the parasite growth at this stage thus preventing the completion of the erythrocytic cycle.

Isoquinoline-protoberberine alkaloids are present in several medicinal plants indigenous to Africa including *Annickia affinis* (Erhunse and Sahal, 2022; Erhunse *et al*., 2023; Erhunse *et al*., 2024), *Annickia chlorantha* (Bourdat-Deschamps *et al*., 2004; Imieje *et al*., 2017), *Argemone mexicana* (Simoes-Pires *et al*., 2014), and *Berberis spps* (Belwar *et al*., 2020) amongst others. These alkaloids have impressive biological properties such as anti-inflammatory (Tillhon *et al*., 2012; Zou *et al*., 2017), anticancer (Maiti and Kumar, 2010), antiviral (Abookeleesh *et al*., 2022), antimicrobial (Tillhon *et al*., 2012), antiplasmodial (Wright *et al*., 2000) and antidiabetic (Din, 2011; Węgierek-Ciuk *et al*., 2021). As a result, semi-synthetic and synthetic derivatives have been synthesized in a bid to improve on their potency and selectivity (Pal *et al*., 1998; Węgierek-Ciuk *et al*., 2021).

While a number of naturally occurring protoberberine alkaloids are known to exhibit potent *in vitro* blood-stage antiplasmodial activity (IC_{50} < 5 µM range) (Hsieh *et al*., 2004; Imieje *et al*., 2017; Phillipson and Wright, 1991; Vennerstrom and Klayman, 1988), they seem to possess poor oral bioavailability as many of them have been proven to be substrates of the drug transporter protein P-gp (Maeng *et al*., 2002; Tarabasz and Kukula-Koch, 2020). For example, apart from having poor intestinal absorption, poor aqueous solubility, extensive metabolism and wide tissue distribution (Liu *et al.,* 2010; Tan *et al.,* 2013) are also contributing factors that result in the low plasma concentration of berberine. This poor drug-likeness may have impacted negatively on the development of protoberberine alkaloids as oral antimalarials. Interestingly, gut microbiota was seen to convert the poorly bioavailable berberine to dihydroberberine which is 5-fold more bioavailable than berberine (Wang *et al*., 2015). Thus, gut microbiota (Wang *et al*., 2015; Ai *et al*., 2021) may provide a good strategy to overcome the poor oral pharmacokinetics of this class of alkaloids for their use as blood-stage antiplasmodial agent.

Some studies have reported synthetic derivatives of berberine with optimum therapeutic effect against different diseases (Filli *et al*., 2022). Thus, in one such study (Bahar *et al*., 2011), a semi-synthetic berberine analogue 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride has shown greatly enhanced antiplasmodial activity (IC_{50} 36 nM) in comparison to berberine (IC_{50} 0.85 µM). The dialkyl substitution on C-8 of the berberine skeleton has been suggested to cause the more than 20-fold increased antiplasmodial activity for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride.

Coralyne (13-methyl [1,3] benzodioxolo [5,6-c]-1,3-dioxolo [4,5-i]-phenanthridium) is a synthetic analogue of berberine possessing a tetracyclic structure similar to berberine while differing only in its substituents (Figure 1). The opening of the dioxole ring in the benzodioxole moiety and the presence of an extra methyl group make coralyne bind DNA better and hence makes it a more potent anticancer agent than berberine (Megyesi *et al*., 2010; Węgierek-Ciuk *et al*., 2021). Indeed, complex formation between coralyne and the nucleoside based bacterial second messenger cyclic diadenosine monophosphate has resulted in an interesting simple fluorescent turn-on assay (Zhou *et al*., 2014). In spite of a common molecular skeleton for all protoberberine alkaloids, while the *in vitro* antiplasmodial and *in vivo* antimalarial properties of berberine have been studied (Silikas *et al*., 1996; Wright *et al*., 2000; Bapna *et al*., 2015), information on the antiplasmodial activity of coralyne is lacking. This study thus set out to assess the antiplasmodial activity of coralyne against different strains of *Plasmodium falciparum*. In view of the fact that being substrates of the human Permeability glycoprotein (P-gp) a number of protoberberine alkaloids including berberine have been reported to have limited oral bioavailability, we also used *in silico* methods to predict the oral bioavailability of coralyne.

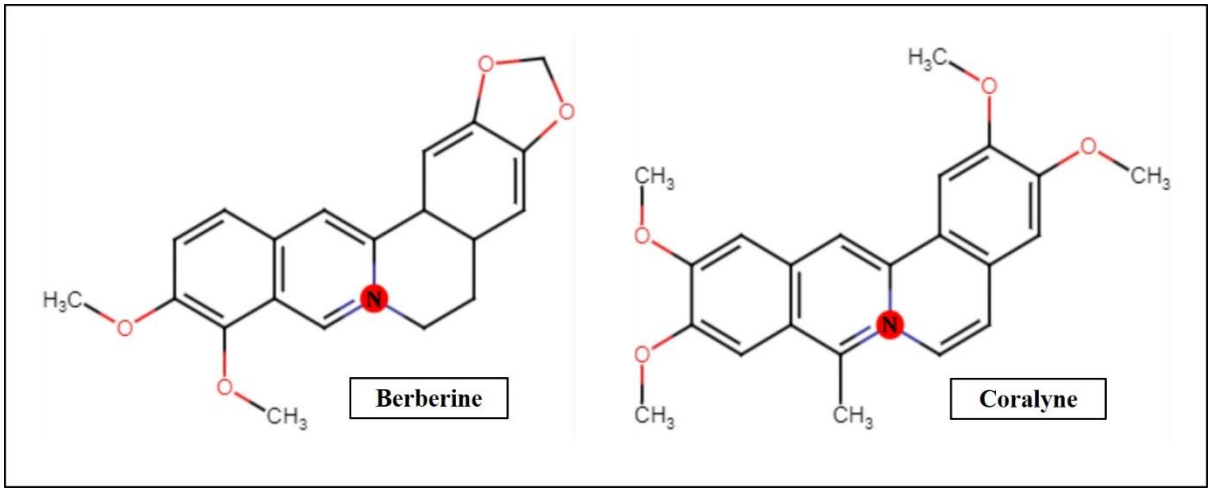


Figure 1: Berberine and its synthetic analogue, coralyne. Images were sketched using Marvin ChemAxon.

MATERIALS AND METHODS

Materials

Malaria parasite strains used for this study {*Pf*3D7 (MRA102) and *Pf*NDO (MRA819)} were gotten from Malaria research and reference reagent resource centre (MR4) Virginia, USA and maintained in our laboratory. Human endothelial kidney (HEK293T) cell line used for cytotoxicity study was obtained from American tissue type culture collection (ATCC). Coralyne chloride hydrate (R278122) was obtained from Aldrich® chemistry, Milwaukee, USA. Dimetylsulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), SYBRgreen and chloroquine diphosphate were all sourced from Sigma Aldrich, India. All other reagents used were of analytical grade.

In vitro antiplasmodial activity testing against *P. falciparum* parasites

P. falciparum strains were cultured by the prescribed method (Trager and Jensen, 1976). The parasite culture was synchronized using 5% sorbitol (Lambros and Vanderberg, 1979). SYBR green dye method (Smilkstein *et al*., 2004) was used to monitor the growth of parasite in the enucleated human red blood cells. Briefly, 96 µl of ring stage parasites at 1 Percentage parasitemia (% p), 2% hematocrit were exposed to 4 µl of varying concentrations of coralyne (0, 0.20, 0.39, 0.78, 1.5, 3.13 and 6.25 µg/ml) in triplicate wells. Chloroquine (40 µM) was used as zero growth control whereas, parasites exposed to 0.4% DMSO represented 100% growth. The 96-well plate was incubated for 48 hr at 37 ^{o}C under reduced O_2 (5% O_2, 5% CO_2, and 90% N_2). At the end of the 48-hr incubation, cells were treated with 100 µl of SYBR Green I solution in lysis buffer {2 µl of 10,000 X SYBR Green I per 10 ml of lysis buffer (20 mM Tris buffer pH 7.5, 5 mM Ethylenediamine tetraacetic acid (EDTA), 0.008% saponin and 0.08% v/v Triton X-100)} so as to lyse the cells and stain the parasite's DNA. This was thereafter thoroughly mixed after which the 96-well plate was incubated in the dark at normal culture conditions for 1 hr. Following this, fluorescence was estimated on a 96-well fluorescence plate reader (Victor, Perkin-Elmer), with excitation and emission wavelengths of 497 and 520 nm, respectively. Concentration of coralyne inhibiting parasite growth by 50% (i.e. IC_{50}) was computed using the IC Estimator-version 1.2 software (http://www.antimalarial-icestimator.net/MethodIntro.htm) (Free Software Foundation, Boston, MA, USA). Results were validated using microscopy of Giemsa-stained blood smears.

In vitro cytotoxicity testing against mammalian cell line

Cytotoxicity was monitored using the method described by Mosmann (1983). Human endothelial kidney (HEK) cells were cultured using complete DMEM (cDMEM) i.e. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS). When full confluence was achieved, the cells were detached by trypsin treatment, seeded (10^4 cells / 100 µl) in a 96-well plate and allowed a 12-hr incubation. Following this, spent media 90 µl was replaced with 86 µl of fresh cDMEM. To these wells, 4 µl of coralyne at varying concentrations (0 - 100 µg/ml) were

added in triplicate whereas for control wells, 4 µl of 0.4% or 10% DMSO were added to 2 sets of triplicate wells representing 100% growth and 0% growth respectively. The plate was placed in a CO_2 incubator at 37°C for 24 hr. Thereafter, MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (5 mg/ml) was prepared in 1X Phosphate buffered saline (PBS) from which 20 µl (100 µg) was added to each well. The plate was then wrapped in foil and incubated for 3 hr. Then, 120 µl corresponding to cDMEM, drug or DMSO and MTT was aspirated following which 200 µl DMSO was added to each well to dissolve the formazan crystals formed. Formation of formazan which indicates cell growth was measured on a microplate reader (Versa Max) at 570 nm. Concentration of coralyne inhibiting HEK growth by 50% (i.e. CC_{50}) was computed using the IC Estimator-version 1.2 software (http://www.antimalarial-icestimator.net/MethodIntro.htm) (Free Software Foundation, Boston, MA, USA).

In silico Prediction of ADME

Prediction of ADME (absorption, distribution, metabolism and excretion) was done using SwissADME web tool which is freely available at http://www.swissadme.ch (Tripathi *et al*., 2019; Al Azzam *et al*., 2022). Briefly, compounds were sketched using Marvin chemaxon which afforded the generation of their simplified molecular-input line-entry system (SMILES) algorithm that was thereafter used to evaluate their pharmacokinetics.

RESULTS

Antiplasmodial activity and cytotoxicity of Coralyne

Coralyne showed excellent activity (IC_{50} 0.52 µg/ml) against the chloroquine sensitive strain of the malaria parasite. Its IC_{50} at 1.15 µg/ml against the chloroquine resistant strain, although ~ two-fold higher was nevertheless quite promising giving a resistance index of two (Figures 2 & 3). Further, its CC_{50} against the human epithelial kidney cell line was >100 µg/ml (Figure 4) implying a Selectivity index of >85.

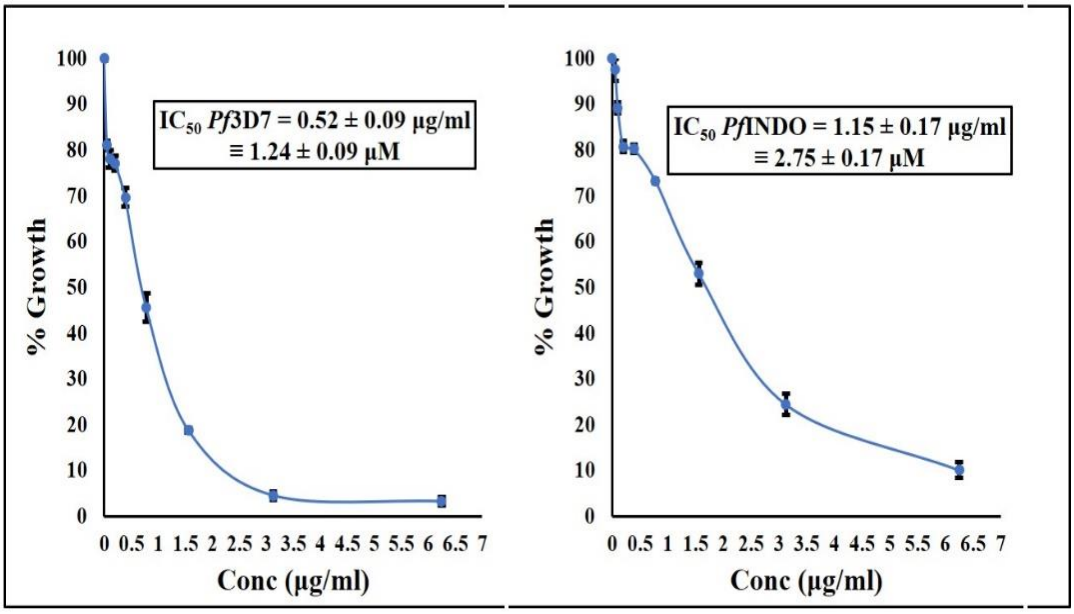


Figure 2: Concentration-response curve of coralyne against chloroquine sensitive *Pf*3D7 (left panel) and chloroquine resistant *Pf*INDO (right panel). With a resistance index (RI) (IC_{50}*Pf*3D7/IC_{50}*Pf*INDO) of 2.21, coralyne is a little more potent against the chloroquine sensitive strain (*Pf*3D7) than chloroquine resistant strain (*Pf*INDO) of the parasite used in this study. Data points shown represent mean with standard deviations of three replicates.

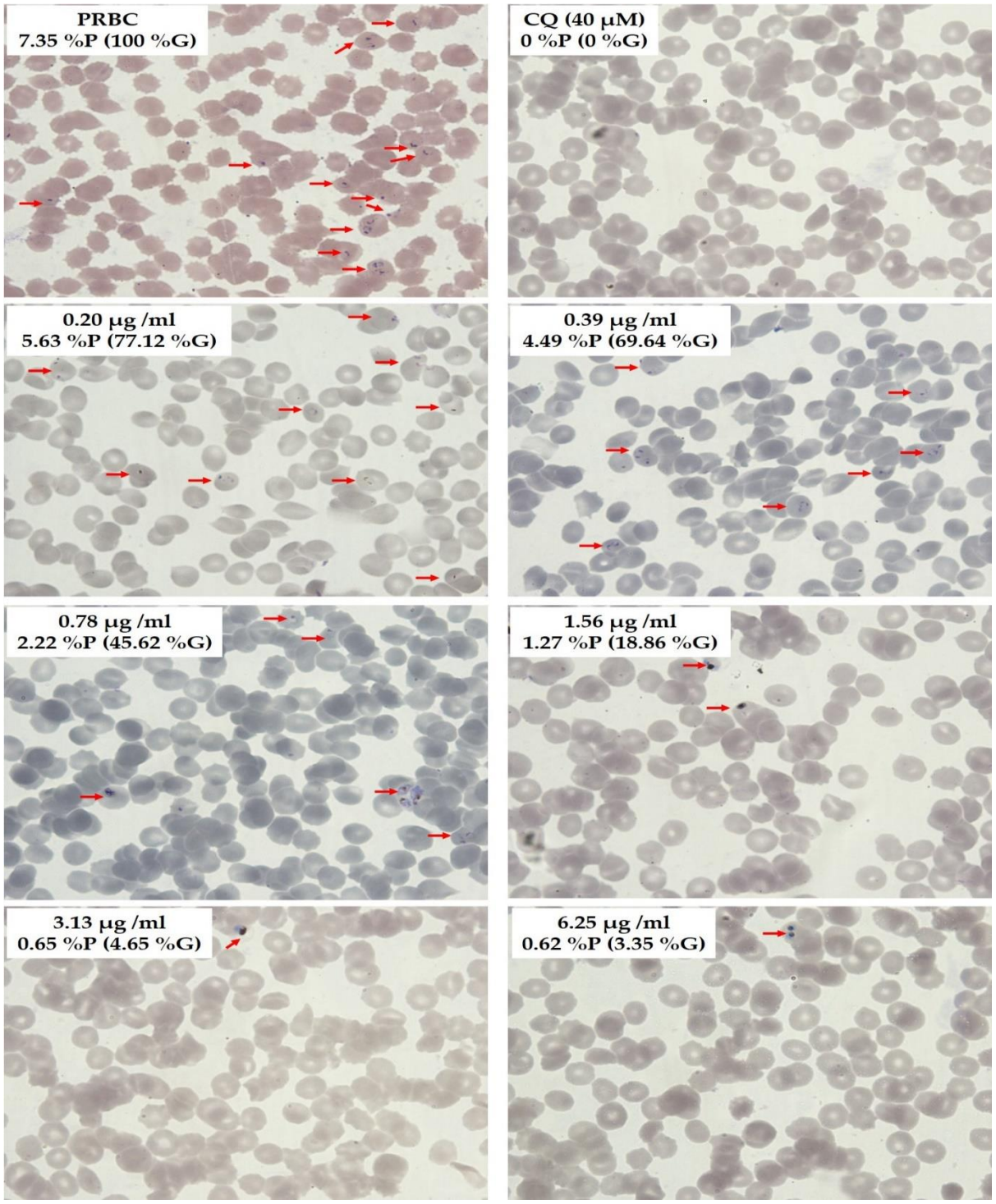


Figure 3: Representative microscopic images of 3D7 parasites exposed to various doses (0-6.25 µg/ml) of coralyne. Parasites are trapped in the trophozoite stage upon exposure to 1.56 µg/ml of coralyne. Red arrows indicate parasitized red blood cells. Key: PRBC = Parasitized red blood cell; CQ = Chloroquine; % P = % parasitemia; %G = % growth obtained after counting 3000 cells.

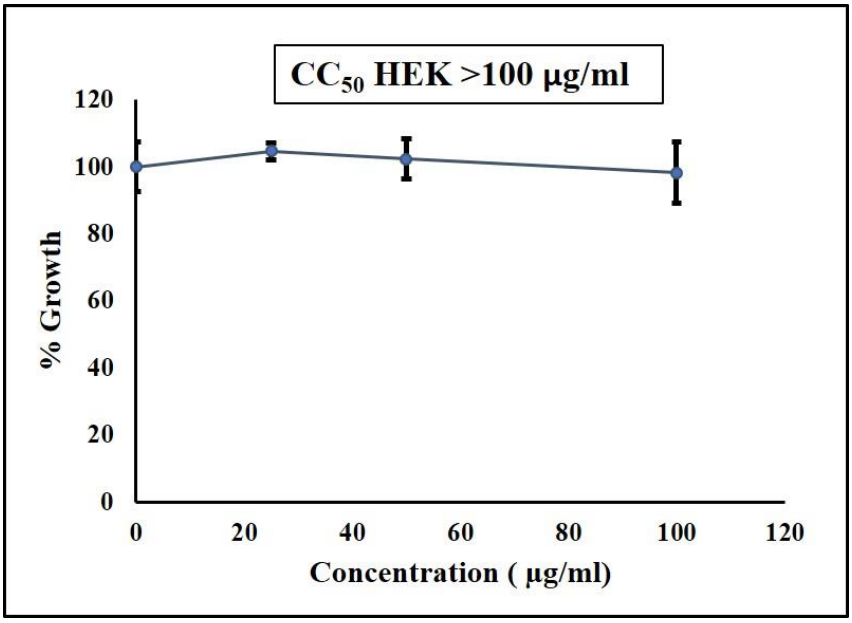


Figure 4: Concentration response curve of coralyne against human endothelial kidney cell line. Coralyne combined activity against the malarial parasite with selectivity as it was not toxic to the mammalian cell line (CC_{50}HEK >100 µg/ml) with selectivity index greater than eighty five.

In silico ADME

The chemical structures of the naturally occurring protoberberine alkaloids (berberine, palmatine, jatrorrhizine and columbamine), the synthetic protoberberine alkaloid (coralyne), the semi-synthetic berberine derivative (5,6-didehydro-8,8-dietyl-1,3-oxodihydroberberine chloride) and artemisinin and its semi-synthetic water-soluble form artesunate (which served as standard) were drawn using Marvin chemaxon so as to retrieve their respective SMILES. These were then used to predict their oral pharmacokinetics. Unlike the artemisinin and artesunate standards, all the alkaloids were returned as substrates of the Permeability glycoprotein (P-gp) (Table 1) suggesting that they are likely to be easily extruded by the transporter when ingested via the oral route. However, a representation of passive gastrointestinal absorption and brain penetration of protoberberine alkaloids using the Brain or IntestinaL EstimateD permeation predictive model (BOILED-Egg model) shown in Figure 5 suggests that the intestinal absorption and brain penetration of the protoberberine alkaloids is not impacted by virtue of their being substrates of P-gp. Further, their bioavailability radars (Figure 6) suggest that they are orally bioavailable.

DISCUSSION

Coralyne is a synthetic protoberberine alkaloid which differs from berberine only in the substituents on its tetracyclic structure (Megyesi *et al*., 2010; Węgierek-Ciuk *et al*., 2021). One of these differing substituents is the presence of a methyl group at the C-8 position of the berberine skeleton. Although coralyne is a potent anticancer agent, its activity against the malaria parasite has not been studied. It was therefore interesting for us to find that coralyne possesses excellent blood stage antiplasmodial activity against drug sensitive *Pf*3D7 (IC_{50} 0.5 µg/ml) and drug resistant strain *Pf*INDO (IC_{50} 1.15 µg/ml) of the malaria parasite implying a resistance index of two. While an RI of 100 suggests high level resistance, a resistance index <10 suggests an intermediate resistance level (Nzila and Mwai, 2010). Hence the RI of two for coralyne observed by us is miniscule.

Table 1: *In silico* ADME of some protoberberine alkaloids

Molecule	Molecular Formula	MW	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Bioavailability Score
1. Palmatine	$C_{21}H_{22}NO_4$	352.40	H	Y	Y	Y	N	N	Y	Y	0.55
2. Columbamine	$C_{20}H_{20}NO_4$	338.38	H	Y	Y	Y	N	N	Y	Y	0.55
3. Jatrorrhizine	$C_{20}H_{20}NO_4$	338.38	H	Y	Y	Y	N	N	Y	Y	0.55
3. Berberine	$C_{20}H_{18}NO_4$	336.36	H	Y	Y	Y	N	N	Y	Y	0.55
4. Coralyne	$C_{22}H_{22}NO_4$	364.41	H	Y	Y	N	N	N	Y	N	0.55
5. 5,6-didehydro-8,8-dietyl-1,3-oxodihydroberberine chloride	$C_{24}H_{24}ClNO_5^-$	441.90	H	Y	Y	N	Y	Y	Y	N	0.85
6. Artemisinin*	$C_{15}H_{22}O_5$	282.33	H	Y	N	Y	N	N	N	N	0.55
7. Artesunate*	$C_{19}H_{28}O_8$	384.42	H	Y	N	N	N	N	N	N	0.56

*Artemisinin and artesunate served as standards. MW = molecular weight, H = high, Y = yes, N = no

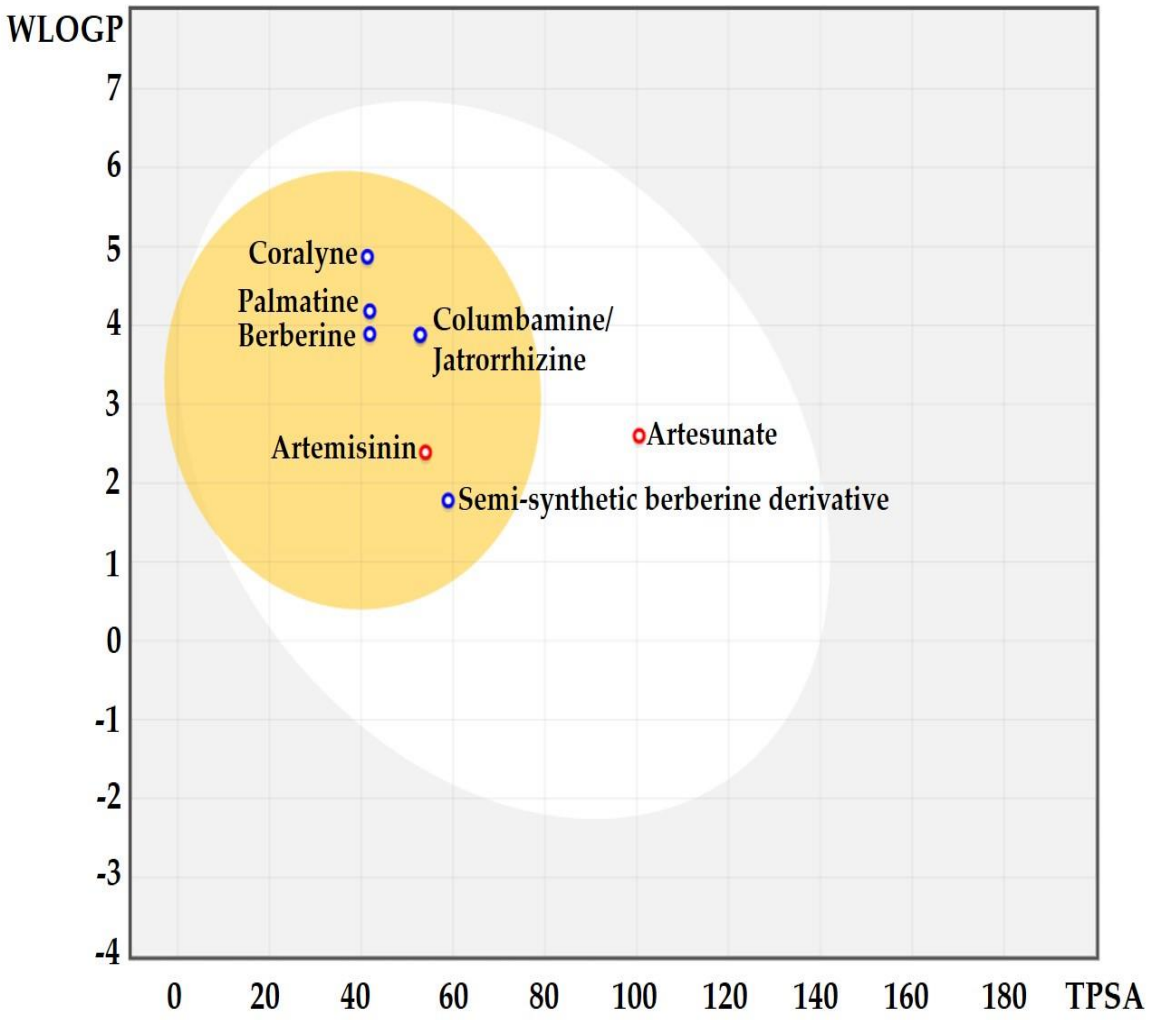


Figure 5: Schematic representation of passive gastrointestinal absorption and brain penetration of protoberberine alkaloids using the Brain or IntestinaL EstimateD permeation predictive model (BOILED-Egg model). The Swiss ADME web tool uses the inbuilt BOILED-Egg model to evaluate passive gastrointestinal absorption (HIA) (white part) and brain penetration (BBB) (yellow part). Blue and red circles denote P-gp substrates and non-substrates respectively. Protoberberine alkaloids showed good brain penetration. Artemisinin and artesunate which served as standards aren't substrates of P-gp. Further while artemisinin can cross the BBB, artesunate has higher intestinal absorption. Semi-synthetic berberine derivative = 5,6-didehydro-8,8-dietyl-1,3-oxodihydroberberine chloride.

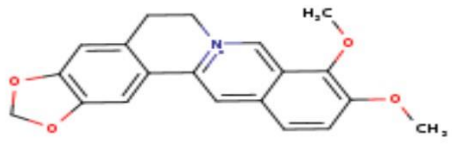
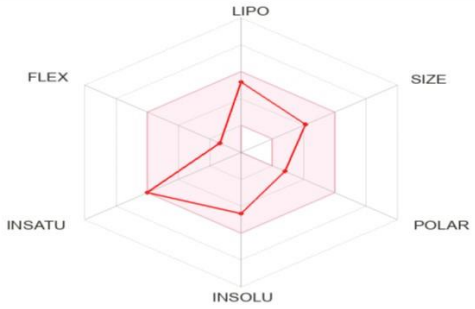
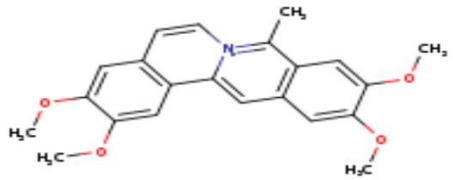
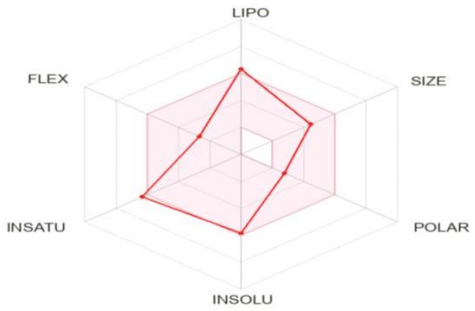
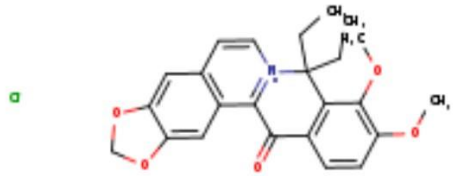
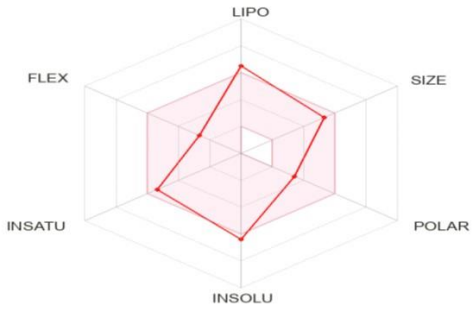
Protoberberine alkaloid	Bioavailability Radar
 Berberine	
 Coralyne	
 5,6-didehydro-8,8-dietyl-1,3-oxodihydroberberine chloride	

Figure 6: Bioavailability radar of berberine, its synthetic analogue coralyne and semi-synthetic analogue (5,6-didehydro-8,8-dietyl-1,3-oxodihydroberberine chloride) predicts oral bioavailability since they all lie just within the pink region which represents the optimal values for each of the six properties listed: lipophilicity- X-lOGP3 -0.7 to +5.0, Molecular weight/Size- 150-500 g/mol, Polarity (TPSA)-20 to 130 $Å^2$, log S (lNSOLU) not higher than 6, Saturation fraction (lNSATU) of carbons in the sp3 hybridization not <0.25, and flexibility (FLEX)- not more than 9 rotatable bonds.

Our *in-silico* data suggests that like the naturally-occurring protoberberine alkaloids, coralyne is also a substrate of P-gp. However, in spite of it being a P-gp substrate, it also reported a high intestinal absorption for coralyne and the other alkaloids (Table 1). The resolution to this apparent contradiction is to be seen in the findings by Ogihara *et al*., (2006) who have demonstrated that P-gp substrates can be categorized into two classes based on their intestinal absorption characteristics. Thus, while on the one hand, the intestinal absorption of a class dubbed verapamil-type substrates is unimpacted by virtue of their being substrates of P-gp, intestinal absorption of vinblastine-type substrates on the other hand is compromised (Ogihara *et al*., 2006). The result of our *in-silico* ADME study predicts that the intestinal absorption of several protoberberine alkaloids may not be impacted by P-gp which is surprising in view of the fact that berberine co-administered with P-gp inhibitors reported increased absorption of the alkaloid (Pan *et al*., 2012; Tsai and Tsai, 2004). A similar observation was reported for the antioxidant anthocyanin by Tripathi *et al*., (2019). Although the web tool returned anthocyanin as a P-gp substrate, intestinal absorption was also high (Tripathi *et al*., 2019). From the BOILED-Egg result, the alkaloids investigated have a higher likelihood of entry into brain (Figure 5, yellow region). Localization in the white area of the model depicts whether the compound has higher likelihood of passive gastrointestinal absorption. Hence, localization in either compartment isn't mutually exclusive (Al Azzam *et al*., 2022).

Compared to berberine as also other protoberberine alkaloids studied, coralyne interacts with fewer of the major CYP450 enzymes (Table 1). This suggests decreased elimination of coralyne by these enzymes. The results of our *in-silico* ADME study for berberine is similar to what has been previously reported (Imenshahidi and Hosseinzadeh, 2016). Further, a bioavailability score of 0.55 suggests coralyne may have inherited the poor oral pharmacokinetics of its congener berberine (Table 1). As such, studies directed at designing analogues of these alkaloids with not only an improved potency against the malaria parasite but also, robust oral pharmacokinetics are advocated. Intriguingly, our *in-silico* study predicts an increase in the bioavailability of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (the semi-synthetic analogue of berberine) (bioavailability score 0.85) as compared to the other protoberberine alkaloids with this score at 0.55 (Table 1). However, there is need to validate the *in-silico* bioavailability data for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride and coralyne using *in vitro* and *in vivo* bioavailability models given the discrepancy in intestinal absorption result between *in-silico* and wet lab studies of berberine.

CONCLUSION

Our study reports the antiplasmodial activity of coralyne for the first time. Although it displayed good activity against the malaria parasite in culture, our *in-silico* ADME model predicts that like berberine, coralyne may also have poor oral bioavailability. However, wet lab ADME studies weren't conducted. Thus, *in vitro* and *in vivo* intestinal absorption studies are required to confidently determine its oral bioavailability.

Conflict of Interest

Authors have no conflict of interest to declare

Author contribution statement

NE: Conceived, designed and performed the experiments; Analysed and interpreted the data; Wrote the paper. DS: Conceived, designed and supervised the study; Revised the paper.

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