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Article

In vitro assessment for cytotoxicity screening of new antimalarial candidates

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In antimalarial research there are no standard procedures to determine the toxicity of a drug candidate. Among the alternatives available, *in vitro* cytotoxicity assays are the most widely used to predict toxic effects of future therapeutic products. They have the advantage over the *in vivo* assays, in that they offer the possibility to restrain the number of experimental variables. The objective of the present study was to compare *in vitro* cytotoxic methods by testing various compounds currently used to treat malaria against different cell lines. Neutral red (NR) uptake and methylthiazoletetrazolium (MTT) colorimetric *in vitro* assays were used to determine preliminary toxicity of commercially available antimalarial drugs against tumor and non-tumor cells lines. Toxicity through brine shrimp lethality bioassay and hemolytic activity were also evaluated. Significant differences were observed in the tests measured by NR uptake. The tumor cell lines TOV-21G and HepG2 and non-tumor WI-26VA4 cells showed relatively uniform toxicity results, with TOV-21G being the most sensitive cell tested, presenting the lowest concentration to cause death to 50% of viable cells (CC_{50}) values. The results of this study support the use of TOV-21G, HepG2 and WI-26VA4 cells lines as the choice for cytotoxicity tests to evaluate potential bioactive compounds.

Key words: Antimalarial. Cytotoxicity assay. MTT. Neutral red.

INTRODUCTION

In vivo and *in vitro* bioassays are carried out for the evaluation of different aspects of viral, bacterial, fungal, and parasitic pathogens. They are also key in testing drug efficacy and in the development of new drugs. One of the mandatory steps in the drug development process is the investigation of toxic effect on various biological systems (Parasuraman, 2011).

Prediction of toxicity has been performed since the 1950s, although its approach has been modified over the decades. The toxicity evaluation aims to anticipate harmful effects that an organism may suffer following exposure to a given compound (Cazarin, Corrêa, Zambrone, 2004; Bhattacharya *et al*., 2011).

Animal models continue to be used in preclinical drug development studies despite the ethical debates surrounding their use (Cazarin, Corrêa, Zambrone, 2004; Shanks, Greek, Greek, 2009; Bhattacharya *et al*., 2011; Bailey, Thew, Balls, 2014). The *in vivo* models has been re-evaluated, aiming to reduce the need to rely on animals, minimize their suffering and to ultimately replace their use altogether through alternative methods whenever possible (Alves, Colli, 2006). This reassessment was based on the principle of the 3Rs (Replacement, Reduction and Refinement) as proposed in 1959 by William M. S. Russel and Rex L. Burch (Russell, Burch, 1959).

A variety of *in vitro* assays are available to assess toxicity of a compound. These include the trypan blue

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test, the tetrazolium salt assays (MTT, MTS, XTT, or WST), neutral red (NR) and the lactate dehydrogenase (LDH) test (Putnam, Bombick, Doolittle,2002). Despite the limitations that still exist around the validations of toxicity tests, *in vitro* methods have several advantages over the *in vivo,* such as the possibility to restrain the number of experimental variables as well as the possibility to obtain meaningful data in an easier manner and in a shorter period of time (Rogero *et al*., 2003).

Malaria remains one of the major health problems in the world, mainly because of the emergence and spread of drug-resistance in the causative agents (WHO, 2018). The approaches used to discover new antimalarials include the search of compounds from natural sources, the chemical modifications of existing antimalarials, the development of hybrid compounds, the molecular modeling using virtual screening technology and docking, and even the testing of commercially available drugs prescribed for other diseases, such as cancer (Aguiar *et al*., 2012).

Although a wide number of protocols were already described for evaluating *in vitro* cytotoxicity of drug candidates, there is no gold standard model to assess toxicity in novel antimalarial research. In this regard, differences in toxicity testing can result in discrepancies between studies concerning the cytotoxicity of the new antimalarial candidates, which is one of the most important drug safety information. The aim of the present study was to compare different *in vitro* cytotoxicity assays, using distinct cell lines, in order to contribute to the development of a standardized protocol for the screening of new antimalarial compounds.

MATERIAL AND METHODS

Antimalarial drugs

Cytotoxicity tests were carried out on the following antimalarial drugs: Artesunate (ART), Artemether/Lumefantrine (A/L), Chloroquine (CQ), Primaquine (PQ) and Quinine (QN). All antimalarial agents were diluted in DMSO 10% to provide a stock solution of 10,000 µg/mL. Tests solutions were prepared in RPMI-1640 culture medium, in order to obtain concentrations of the drugs ranging from 31.3 to 1,000 µg/mL. DMSO was used as positive control in serial dilutions (100% to 3.13% v/v).

Cell lines cultures

The cytotoxic effects of the antimalarial drugs were assessed against three tumor-derived cell lines: HepG2 (hepatocellular carcinoma ATCC HB-8065), HeLa S3 (cervical carcinoma ATCC CCL2) and TOV-21G (ovarian adenocarcinoma ATCC CRL-11730); and against two non-tumor immortalized cells lines: BGMK (Buffalo green monkey kidney cells) and WI-26VA4 (lung fibroblast ATCC CCL-95.1). HepG2 and BGMK were obtained from Instituto René Rachou (Fiocruz Minas, Brazil); and HeLa S3, TOV-21G and WI-26VA4 were obtained from Fundação Ezequiel Dias (Belo Horizonte, MG, Brazil).

Cells were cultured in 25 cm^2 flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 40 mg/L gentamicine and maintained at 37 ºC in a humidified incubator with 5% CO2.

Human monocytes preparation

Briefly, 25 mL of blood, kindly donated by the Hematology and Hemotherapy Center Foundation of Minas Gerais (Hemominas, Technical Cooperation 020/15), were added to 25 mL Monopaque gradient (density = 1.08) and centrifuged at 1000 xg for 40 min at room temperature. Two phases were obtained, separated by an interface ring of monocytes, which was transferred to another tube and washed twice with PBS (pH = 7.3) at 800 xg for 15 minutes. After this experimental procedure a final concentration of 1 $x10^6$ cells in 180 µL was obtained by resuspending the pellet with PBS.

In vitro cytotoxicity assay

For the *in vitro* tests, the confluent cell lines monolayer was detached with trypsin 0.25% (Sigma®), washed with culture medium, distributed in a flat-bottomed 96-well culture plates ($1×10^6$cells/well), in which they were homogenized in RPMI 1640 medium

supplemented with FBS and antibiotics. The cells were then incubated for 18 h at 37°C to ensure cell adherence.

Cytotoxicity assays were performed incubating the cell lines and the monocytes, with 20 µL of the different antimalarial drugs concentrations (31.3-1,000 µg/mL) for 24 h at 37°C, 5% CO_2 and 95% humidity. The tested antimalarials were prepared as a stock solution using DMS0 10% v/v. The final concentration of DMSO in the plated cells did not exceed 1%. All experiments were run in triplicate. Negative controls were not treated with antimalarial drugs.

Cell viability after drug exposure was measured by tetrazolium MTT test and neutral red uptake assay (NR). Monocyte viability was evaluated by the MTT assay.

Cytotoxicity of the tested antimalarial drugs was expressed as the cytotoxic concentration of the extracts to cause death to 50% of viable cells (CC_{50}). The calculation of CC_{50} was performed by a nonlinear regression dose-response curve to the drugs using the Origin software GrapPad8.0.

The MTT assay

The cytotoxic potential of the antimalarial drugs was determined by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay, as previously described (Mosmann, 1983; Carmichael *et al*., 1987). After antimalarial drug exposure, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 3 h. The supernatant was then carefully removed, followed by the addition of 100 µL/well of DMSO to dissolve the formazan crystals. Finally, the optical density at 570 nm was measured on an ELISA reader (SpectraMax340PC^{384}, Molecular Devices).

The Neutral Red assay

The neutral red assay (NR) was performed as previously described, with some modifications (Borenfreund, Buerner, 1984). After *incubation* of cell cultures with the antimalarial drugs, the supernatants from each well were aspirated. Immediately, 100 µL of the neutral red solution (40 µg/mL) was added to each well and incubated for 3 h. The neutral red solution was then removed and 200 µL of a solution containing formaldehyde (0.5% v/v) and $CaCl_2$ (1%) were added. Following 5 min incubation, the supernatant was removed, and cells were subject to 100 µL of a mixture of acetic acid (1%) and ethanol (50%). The absorbance was read at 540 nm using an ELISA reader (SpectraMax 340PC^{384}, Molecular Devices).

In vitro hemolysis assay

Hemolysis assay was performed as described by Wolfgang, Pfannenbecker and Hoppe (1987). Each test drug (20 µL) in concentrations from 15.63 to 500 µg/mL was incubated with 180 µL of a human erythrocytes suspension (2% hematocrit) at 37 °C for 30 min in a shaking water bath. Red blood cells incubated with PBS and 0.05% saponin was used as negative and positive control, respectively. The mixtures were centrifuged at 1,000 xg for 10 min and the absorbance of the supernatants was measured at 540 nm in an ELISA reader (SpectraMax 340PC384, Molecular Devices). The percent hemolysis was calculated using the following formula:

$$\% \text{ hemolysis} = 100 \times \frac{\text{Absorbance of test drug - Absorbance of negative control}}{\text{Absorbance positive control - Absorbance of negative control}}$$

Artemia salina lethality bioassay

The brine shrimp bioassay was performed according to the method described by Meyer *et al.* (1982), with slight modifications. *A. salina* (brine shrimp) nauplii were obtained from eggs incubated in artificial seawater (3.8%, w/v AquaSalt-Aqua One) under light at room temperature. After hatching, the nauplii were collected and used in bioassays conducted in 96-well microplates. In each experiment, 15 nauplii were exposed to the antimalarials in different concentrations (1,000, 500, 250, 125, 62.5 and 31.3 µg/mL). After 24 h, the number of deaths was counted under stereoscope microscope in order to determine the survival rates (%).(%). Each drug concentration, including Thymol 10% (Sigma-Aldrich) as a positive control and artificial seawater as negative control, had three replicates. Lethal concentration for 50% of *A. salina* nauplii (LC_{50}) was calculated using Origin® 8.0 software.

Statistical analysis

Data were analyzed using the nonparametric Mann-Whitney, Kruskal-Wallis tests and Dunns methods. The values of $p<0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Malaria is a serious disease caused by *Plasmodium* spp. parasites and can be fatal if left untreated. There is an arsenal of antimalarial compounds available; however, drug-resistant parasites have emerged worldwide, including those resistant to Artemisinin derivatives (WHO, 2018). Therefore, the continued search for new antimalarial agents and products of any origin (natural or synthetic) remains a priority for different research groups.

The discovery of a new drug depends on the availability of screening assays capable of identifying drug candidates before these can move on to clinical trials (Hughes *et al*., 2011). In addition to tests to assess the effectiveness, dose and solubility of a compound, safety must also be determined to obtain preliminary information about its toxic potential. Criteria were established to calculate a mathematical relationship between activity and toxicity for hits and leads. It's accepted that for a hit the effector concentration for half-maximum response (EC_{50}) should be <1 μM for sensitive and multiple resistant strains of *Plasmodium* spp. CC_{50} for the mammalian cell line must be greater than 10 fold the EC_{50} (effector concentration for half-maximum response). However, in the case of a lead, an EC_{50}<100 nM and a value greater than 100 fold between CC_{50} and EC_{50} is required (Katsuno *et al*., 2015). Assays to evaluate antiplasmodial activity are well established, but there are no standard protocols to determine cytotoxicity. The results reported in the literature use a wide variety of cells lines and there is no consensus on which one would be most suitable for this type of assay (Ashok, Ganguly, Murugesan, 2014; Cargnin *et al*., 2018; Jonet *et al*., 2018). Thus, further screening methods to investigate the toxic effects of drug on cells should be tested to establish the best possible procedure to test cytotoxicity under standardized conditions.

The cytotoxic activity of antimalarials was evaluated by MTT and Neutral Red (NR) assays. The MTT assay is based on reduction of the tetrazolium dye by NAD(P)H-dependent cellular oxidoreductase enzymes, determined through the number of viable cells present in each assay condition. The NR is based on the ability of viable cells to incorporate and bind the supra vital dye neutral red in the lysosomes. These two methods were employed as a tool to assess the toxicity of different antimalarial compounds over a panel of cell cultures. The toxicity profile of the antimalarials was evaluated by comparing the CC_{50} values obtained using MTT and NR.

The results for ART are shown on Table I. The CC_{50} ranged from 174.03 ± 37.55 μM (TOV-21G) to 399.87 ± 115.99 μM (WI-26VA4). The ART was significantly more toxic to TOV-21G cells, followed by the HepG2, than the other tested cell lines in both NR and MTT methods. When the two methods were compared, significant differences for the cells WI-26VA4 and BGMK were observed.

The results found to CQ (Table II) were similar among the different tested cell lines, except for HeLa S3, in which the CC_{50} value was about three times higher than the others. The MTT and NR methods presented similar results for all evaluated cell lines.

The CC_{50} values of A/L (Table III) ranged from 386.07 ± 155.6 μM to 100.51 ± 42.37 μM, with no significant differences among the different cell lines and methods (MTT and NR).

The CC_{50} values of PQ were significantly higher in WI-26VA4 cells in both methods evaluated and for HeLa S3 cells in the NR assays, resulting in reduced cytotoxic effect. The other values were in agreement among the different cell lines tested and the evaluated methods (Table IV).

The QN CC_{50} values obtained from MTT were similar in most evaluated cell lines (Table V). There was a greater variation in CC_{50} values with NR assay. HeLa S3 and WI-26VA4 were shown to be the cell lines with higher values.

The DMSO was used as positive control, concentrations below 25% were not toxic (Table VI). The results CC_{50} were similar in most cell lines evaluated in the *MTT* or NR assays.

Results of the NR and MTT assays were compiled as shown in figure 1. Despite the differences among the compounds, the presence of DMSO did not inhibited cell growth. These results corroborate the hypothesis which states that the differences in the results between those assays may be related to the mechanism of action of the compounds.

TABLE I - Cytotoxicity of Artesunate (ART) against different cell lines determined by MTT and NR assays and expressed as the cytotoxic concentration for 50% of cells (CC_{50}) in µM.

Cell line	CC_{50} ART µM - mean ± SD	
	MTT	NR
WI-26VA4	399.87 ± 115.99	707.65 ± 67.69#
BGMK	490.18 ± 302.35	208.81 ± 13.16*#
HepG2	205.10 ± 33.97*	160.38 ± 94.70*
TOV-21G	174.03 ± 37.55*	192.99 ± 41.16*
HeLa S3	302.54 ± 180.70	598.46 ± 30.31#

*Significantly different among the evaluated cell lines; #Significantly different values between tests; *# $p<0.05$; MTT - 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide; NR - Neutral Red. SD- standard deviation.

TABLE II - Cytotoxicity of Chloroquine (CQ) against different cell lines determined by MTT and NR assays and expressed as the cytotoxic concentration for 50% of cells (CC_{50}) in µM.

Cell line	CC_{50} CQ µM - mean ± SD	
	MTT	NR
WI-26VA4	259.19 ± 51.75	279.25 ± 7.14
BGMK	162.65 ± 46.4	240.71 ± 27.32
HepG2	176.63 ± 33.34	337.99 ± 48.30
TOV-21G	143.88 ± 22.95	250.64 ± 79.29
HeLa S3	1,022.4 ± 131.31*	754.84 ± 57.10*

*Significantly different among the evaluated cell lines; * $p<0.05$ MTT - 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide. NR - Neutral Red. SD- standard deviation.

TABLE III - Cytotoxicity of Artemether/Lumefantrine (A/L) against different cell lines determined by MTT and NR assays and expressed as the cytotoxic concentration for 50% of cells (CC_{50}) in µM.

Cell line	CC_{50} A/L µM - mean ± SD	
	MTT	NR
WI-26VA4	386.07 ± 155.6	199.48 ± 2.96
BGMK	205.85 ± 53.08	152,41 ± 20.95
HepG2	132.09 ± 22.98	277.28 ± 26.08
TOV-21G	218.18 ± 63.57	100.51 ± 42.37
HeLa S3	171.93 ± 27.03	310.84 ± 59.72

MTT - 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide. NR - Neutral Red. SD- standard deviation.

TABLE IV - Cytotoxicity of Primaquine (PQ) against different cell lines determined by MTT and NR assays and expressed as the cytotoxic concentration for 50% of cells (CC_{50}) in µM.

Cell line	CC_{50} PQ µM - mean ± SD	
	MTT	NR
WI-26VA4	920.8 ± 17.4*	972.29 ± 11.65
BGMK	237.90 ± 84.64	219.36 ± 18.78*
HepG2	196.71 ± 51.33	283.22 ± 84.81*
TOV-21G	191.93 ± 50.16	235.33 ± 30.08*
HeLa S3	277.57 ± 63.85	1,934.6 ± 195.19#

*Significantly different among the evaluated cell lines; # Significantly different values between tests; * # P <0.05 MTT - 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide. NR - Neutral Red. SD- standard deviation.

TABLE V - Cytotoxicity of Quinine (QN) against different cell lines determined by MTT and NR assays and expressed as the cytotoxic concentration for 50% of cells (CC_{50}) in µM.

Cell line	CC_{50}QN µM - mean ± SD	
	MTT	NR
WI-26VA4	334.12 ± 136.7	1,254.1 ± 236.45*#
BGMK	513.80 ± 130.35	319.94 ± 66.44
HepG2	387.23 ± 142.93	441.33 ± 55.24
TOV-21G	232.17 ± 116.17	159.44 ± 38.12
HeLa S3	315.95 ± 139.91	929.09 ± 40.94*#

*Significantly different among the evaluated cell lines; # Significantly different values between tests; * # P <0.05 MTT - 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide. NR - Neutral Red. SD- standard deviation.

TABLE VI - Cytotoxicity of Dimethylsulfoxide (DMSO) against different cell lines determined by MTT and NR assays and expressed as the cytotoxic concentration for 50% of cells (CC_{50}) in %.

Cell line	CC_{50} DMSO - mean ± SD (%)	
	MTT	NR
WI-26VA4	21.47 ± 8.64	22.97 ± 10.46
BGMK	29.15 ± 3.34	55.17 ± 7.89
HepG2	27.01 ± 1.65	22.55 ± 5.31
TOV-21G	20.07 ± 8.29	45.02 ± 16.22
HeLa S3	43.07 ± 30.70	56.95 ± 5.88

MTT - 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide. NR - Neutral Red; SD- standard deviation.

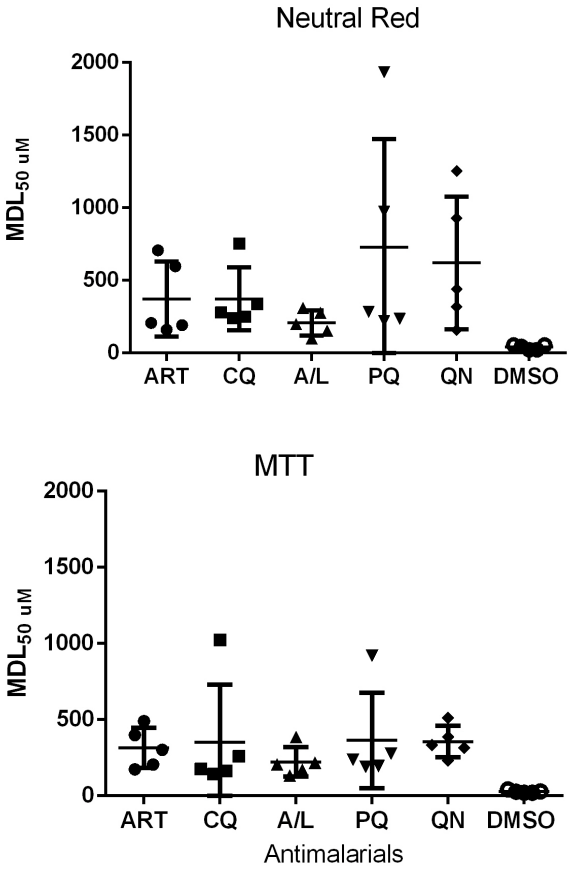

FIGURE 1 - CC_{50} results obtained with antimalarials tested against different cell lines and measured by Neutral Red and MTT assays.

In the present work, tumor cell lines were used under the rational that some antimalarials or their derivatives have shown anti-neoplastic activity (Ghantous *et al.*, 2010; Das, 2015).

Chloroquine was administered as an adjuvant for treatment of patients with glioblastoma as it blocks the formation of autolysosomes during autophagy inducing cell death (Geng *et al.,* 2010; Das *et al.*, 2018). Artemisinin, considered a broad-spectrum antitumor drug, exhibits antineoplastic effects on human cancer cell lines, showing a synergic effect with other antimalignant drugs with no increased toxicity toward normal cells (Das, 2015; Yu *et al.*, 2018). Cardoso *et al.* (2018) proved that Artemisinin and its derivative Artemether, produce DNA damage and induce dose dependent increase in micronuclei formation in human lymphocytes. Das *et al.* (2018) described the effects of the antimalarial drugs

Tafenoquine and Mefloquine against chronic lymphocytic leukemia (CLL) cells. Their results showed selectivity of this drug at doses slightly toxic to normal B cells and toxic to CLL cells through lysosome disruption.

Of the five cell lines used in the experiments, three are cancer derived cell lines, HeLa S3, HepG2 and TOV-21G expressing the p53 wild type gene (WT) (http://p53.free.fr), and BGMK and WI-26VA4 which do not express p53 (WT). Briefly, p53 is a gene that regulates the cell cycle and its mutation or inactivation implies the onset of cancer. A cell undergoing DNA damage activates the p53 protein, which encodes a nuclear phosphoprotein, which blocks the cell cycle by promoting DNA repair. If it cannot be recovered, the p53 protein triggers the programmed cell death mechanism, called apoptosis.

Mutations in this gene are associated with a variety of human cancers and can also result in drug resistance. In addition, the inactivation of p53 regulators, such as caspase-9, can also lead to drug resistance (Goldstein *et al*., 2011; Housman *et al*., 2014). The mechanism of apoptosis activated by DNA damage occurs in normal and cancerous cells. However, the activation of apoptosis by p53 WT in tumor cells may result in ambiguous responses inducing cell death rather than their proliferation (Kim, Giese, Deppert, 2009). Therefore, the expression of p53 in cell lines should be taken into account prior to its choice for cytotoxicity assays, a practice that is not currently seen in research.

Quinoline-based antimalarial drugs were used in the present study, such as CQ and QN and PQ. These agents act by interfering with the hemoglobin digestion in the erythrocyte stages of the parasite. *Plasmodium* spp. degrades hemoglobin into heme and polymerize into hemozoin to prevent toxicity. Quinolines bind to heme avoiding detoxification process and induce the production of free radical, capable of killing the parasite (Percário *et al*., 2012). Similar levels of cytotoxicity were expected in the current experiments, even against different cell lines. However, significant differences were observed in the tests measured by NR. CC_{50} values are greater in almost all cell lines, tumor and non-tumor, when compared with the CC_{50} values quantified by MTT.

The NR colorimetric assay is based on the incorporation of this supravital dye into the lysosomes of viable cells (Borenfreund, Buerner, 1984). Quinolines are weak bases, which enter the lysosome resulting in progressive swelling of this organelle disrupting lysosomal function. Changes in the lysosome membrane result in structural changes of the organelle, leading to fusion of small lysosomes to form larger vacuoles with greatly reduced surface-to-volume (Poole, Ohkuma, 1981). These changes in the organelle can influence the results of NR tests, overestimating CC_{50} values, compared to MTT method. Tests discrepancies in the results of NR and MTT assays can interfere in the interpretation of the cytotoxicity of the studied antimalarials agents and the results need a careful analysis.

Despite the fact that the MTT assay is widely used, it is known whether it has any limitations related to cellular metabolic and energy perturbations. It is known that glucose concentration, its uptake rate, the rate of glycolysis, the level of lactate, pyruvate, and NADH/NADPH can influence MTT reduction and consequently impact the assay readout. The Neutral Red assay is cheaper in comparison and more sensitive than MTT and other cytotoxicity tests, such as enzyme leakage and protein content (Repetto, del Peso, Zurita, 2008; Stepanenkov, Dmitrenko, 2015).

Both ART and Artemether present in the compound A/L are semi synthetic metabolites of Dihydroartemisinin. Nanomolar concentrations of these compounds are toxic to plasmodial parasites, whereas in the case of mammalian cells, micromolar concentrations are required to cause toxicity (Genovese, Newman, Brewer, 2000). These compounds can cause DNA damage and cell death by activation of apoptosis by the intrinsic pathway (Lu *et al*., 2014). Guragain *et al.* (2018) studied ART and CQ as therapeutic agents against cholangiocarcinoma (CCA). The results shown that ART induced necrotic cell death and CQ induced apoptotic cell death in CCA cells. In this study, the A/L showed no difference between the CC_{50} values in MTT and NR.

For the antimalarial drugs ART, A/L, PQ and QN we found no significant difference in CC_{50} values comparing immortalized cells and primary culture (Table VII). The QN was significantly less toxic to monocytes than to tumor cells WI-26VA4, BGMK, HepG2, TOV-21G. However, the antimalarials A/L and ART showed a higher

cytotoxicity to monocytes as measured by the CC_{50} in comparison with the other antiplasmodial agents. As discussed above, the Artemisinin derivatives may be more toxic to monocytes than Quinoline derivatives because of its different mechanisms on cell death (Golenser et al., 2006; Simpson et al., 2006).

TABLE VII - CC_{50} values (μM) for all compounds in primary human monocyte lineage. Cell viability assessed by MTT

	A/L	ART	CQ	QN	PQ
CC_{50}	84.6μM	249.7μM	531.47μM	542.5μM	645μM

QN- Quinine; PQ- Primaquine; A/L- Arthemeter/Lumefantrine; ART- Artesunate; CQ- Chloroquine.MTT- 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide.

The hemolytic effect of the tested antimalarials was evaluated against human erythrocytes. The drugs were not toxic to the erythrocytes after 30 minutes of incubation, inducing a low rate of hemolysis, except for the ART, which showed values above 50% hemolysis in the higher tested concentration (1,300 μM) (Table VIII).

TABLE VIII - Hemolytic activity of antimalarials drugs

Drug concentration μg/mL	Mean rate of hemolysis (%)					
	Drugs					
	QN	PQ	A/L	ART	CQ	DMSO
15	6.95	6.91	6.95	7.72	9.98	10.04
31	6.78	6.83	7.13	6.90	10.09	10.08
62	7.04	6.92	7.23	7.05	10.14	10.14
125	6.89	7.09	8.21	7.07	10.36	10.13
250	7.18	7.52	9.33	13.97	10.33	10.54
500	7.06	8.91	11.98	85.59	10.29	16.94

QN-Quinine; PQ- Primaquine; A/L- Arthemeter/Lumefantrine; ART-Artesunate; CQ-Chloroquine. DMSO- Dimethylsulfoxide. Negative control (PBS) absorbance- 0.059 ± 0.002. Positive control (0.05% Saponin) Absorbance- 1.106±0.031

In hemolysis assays, ART was the only compound that showed toxicity above 50% at the highest tested dose. This drug selectively accumulates inside Plasmodium infected erythrocytes as compared to non-parasitized red blood cells, and its concentration it's up to 300-fold higher than those in plasma (Gu, Warhurst, Peters, 1984). Even though the other tested compounds didn't show toxicity towards the erythrocytes it is known that some antimalarials have a significant hemolytic effect (Mohammad *et al*., 2018; Recht, Ashley, White, 2018). PQ is important in the treatment for *P. falciparum* because it's a potent gamecytocide, and can also prevent relapses in *vivax* and *ovale* infections. Therefore, PQ has different approaches in the treatment of malaria; the disadvantage of this compound is related to the hemolytic toxicity in glucose-6-phosphate dehydrogenase (G6PD) deficient subjects. Even though this kind of toxicity couldn't be assessed in the *in vitro* hemolysis assay, it remains a useful screening method (Ashley, Recht, White, 2014)

In the *Artemia* toxicity assay, the cut-off point of LC_{50} value < 80 μg/mL is interpreted as a highly toxic compound or extract; between 80 μg/mL and 250 μg/mL, moderately toxic; and LC_{50}> 250 μg/mL, mildly toxic or non-toxic (Dolabela, 1997). Thus, antimalarial drugs exhibited no toxic effects on *Artemia* nauplii, except A/L, which demonstrated to be moderately toxic (Table IX). The brine shrimp *Artemia salina* is used to determine cytotoxicity, mainly because is simple, rapid and inexpensive, allowing a larger number of samples to be processed and tested (Parra *et al*., 2001). This assay has shown a good correlation with *in vivo* tests (Logarto *et al*., 2001). Thus, brine shrimp lethality bioassay may be useful as a pre-screening tool to assess toxicity. There is a concern that the solvent used in this assay may give false positive results. However, Wu (2014) demonstrated that Dimethylsulfoxide (DMSO) is a safe solvent to be used in this bioassay.

TABLE IX - Toxicity evaluation of antimalarial drugs using *Artemia salina* lethality assay

Drugs	Mean lethality (%) of *A. salina*						LC_{50}
	Drugs concentration (µg/mL)						
	1,000	500	250	125	62.5	31.25	
PQ	100	100	100	90	57*	12*	221.36**µM**
CQ	100	100	100	82	10*	3*	300.65**µM**
QN	69*	56*	21*	16*	15*	5*	1,369.92**µM**
A/L	100	100	100	93	41*	22*	86.06**µM**
ART	55*	23*	7*	6*	6*	5*	1,081.4**µM**
Thymol[a]	80	70	100	100	80	100	1,15%
Artificial seawater[b]	7*	7*	0*	0*	0*	3*	-
DMSO[c]	100	100	96	83	25*	7*	8.64%

QN- Quinine; PQ- Primaquine; A/L- Arthemeter/Lumefantrine; ART- Artesunate; CQ- Chloroquine.*p<0,05 compared with the positive control - [a]Thymol concentration- 10, 5, 2.5, 1.25, 0.63%. [b]3.8%, w/v AquaSalt-Aqua One; [c] DMSO concentration- 100, 50, 25, 12.5, 6.25, 3,13%.

In addition, in order to measure the toxicity of the compounds in relation to *A. salina*, several researchers use the classification in which highly toxic compounds present the inhibitory concentration to 50% of crustaceans below 100 µg/ml, those with toxicity values between 100-500 µg/mL are considered compounds with moderate toxicity, those with values between 500-1000 µg/mL are considered of low toxicity and those with values above 1,000 µg/mL are considered non-toxic (Rajabi *et al*., 2015; Karchesy *et al*., 2016). This classification usually used by researchers corroborates the claim that the majority of antimalarial drugs tested in this study had moderate or low toxicity. Lastly, the increase in mortality was proportional to the increase in concentration, which provides linearity in the dose-effect relationship of each compound.

CONCLUSIONS

The present work indicates that *A. salina* bioassay and the evaluation of hemolytic activity may be used as pre-*screening* cytotoxicity tests, due to their low costs and simplicity to perform.

The tumor cell lines TOV-21G and HepG2 and non-tumor WI-26VA4 cells showed relatively consistent toxicity results, presenting similar results between the MTT and NR assays, with TOV-21G being the most sensitive cell tested presenting the lowest CC_{50} values in both tests. The culture of primary cells is more laborious and requires human blood, nonetheless the results were comparable to those of immortalized cells.

The present study focuses in compounds used in malaria treatment and regardless of the method used for evaluation of toxicity, similar results were obtained with TOV-21G, HepG2 and WI-26VA4 cells lines. Therefore, it is reasonable to propose these cells as the choice for cytotoxicity tests, in comparison with BGMK and HeLa S3 for evaluation of potential bioactive compounds in malaria.

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AUTHOR'S CONTRIBUTION

MRE, FPV, ACCA and EMMR contributed to the research project conception and design, to the supervision of the experiments, and to the interpretation of results and writing the manuscript. MRE, SNA and ACCA maintained the cell lines, performed toxicity techniques and data gathering. All authors contributed to critically revising the content of the final manuscript. The authors declare that they have no conflict of interest.

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