Evaluating the Time Dependent Anti-plasmodium Activity of Andrographolide and Chloroquine on Different Stages of the Intraerythrocytic Cycle of Plasmodium Falciparum 3D7 in Vitro

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Research

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Abstract

Background: The increasing incidence of drug resistance among various strains of Plasmodium falciparum has compelled researchers to search for new improved therapeutic alternatives to current antimalarials. Consequently, the study aimed to investigate the effect of varying the duration of andrographolide exposure on its anti-plasmodial effect against intra erythrocytic stages of the P. falciparum 3D7 parasite. Although andrographolide has demonstrated prior anti-plasmodial effect against P. falciparum 3D7, its time-dependent effect subsequent to different durations of drug exposure in addition to the impact of relevant pharmacologically active concentrations on the cellular morphology of various intraerythrocytic stages of the P. falciparum 3D7 parasite cycle are limited.

Methods: P. falciparum 3D7 parasites cultivated in vitro in blood cultures were individually incubated with different concentrations of andrographolide, chloroquine and drug-free parasite culture which served as the representative control. Suppression of parasite growth was determined by parasite lactate dehydrogenase (pLDH) based drug sensitivity assay. The inhibition of parasite growth and changes in morphology of intraerythrocytic parasites subsequent to treatment initiation with andrographolide or chloroquine were assessed upon commencement of a synchronized cycle at 12, 24 and 48 h respectively.

Results: Andrographolide showed satisfactory growth inhibitory effect however its inhibitory activity was substantially lower when compared to that of chloroquine. Unlike chloroquine which showed maximal inhibitory activity within the first 12 h of the cycle, suppression of parasite growth by andrographolide was most prominent during the development of early trophozoites (viz the second 12 hours). Andrographolide failed to produce any effect on the morphology of ring stage parasites, it however produced a noticeable change in the morphological appearance and sizes of mature trophozoites. Whereas, with chloroquine notable changes to ring and trophozoite stages of the parasites were evident.

Conclusion: The data obtained indicates the potential role of andrographolide as an adjunctive treatment in malaria subject to further clinical evaluations.

Background

Malaria infection is a leading cause of morbidity and mortality in tropical and subtropical regions of the world including Africa, Southeast Asia, and Eastern Mediterranean regions [1]. Infection is caused by any of the five known plasmodia that infect humans namely Plasmodium vivax, P. falciparum, P. malariae, P. ovale or the more recently characterized simian malaria parasite Pknowlesci [2]. Over the past few decades, significant resurgence of malaria cases and associated mortality have been reported despite judicious implementation of rational eradication programmes [1]. The malaria situation is hypothesized to have deteriorated commensurately with the emergence of multi drug resistant strains of the plasmodium parasite.

Hitherto, there are a few classes of drugs used to treat malaria infection. The various classes of anti-malarial drugs act against different stages of the Plasmodium parasite's life cycle [3] viz the sporozoites, hepatic schizonts or stages of the intraerythrocytic cycle. Generally, sporocytocidal drugs are given as prophylactic agents against malaria infection while blood schizontocidal drugs, including, chloroquine (CQ), quinine, mefloquine, primaquine, proguanil (chloroguanide) and pyrimethamine are given during the intraerythrocytic cycle which is a crucial stage targeted for parasite eradication and for arresting the progression of disease [4]. Aside from their schizonticidal properties chloroquine (CQ), 8-aminoquinolines are also utilized to reduced or prevent reoccurrence of relapsing
malaria caused by *P. vivax* or *P. ovale*. Both of the aforementioned species have hepatic schizonts that may persist for long intervals of time intra-hepatically.

The discovery of novel antimalarial principles from plant sources in the past coupled with the emergence of drug-resistance among species of *P. falciparum* to current antimalarials has prompted researchers to extensively explore phytochemicals as potential sources of anti-malarial pharmacophore candidates against antigenically variant malaria parasites as potential substitutes for the conventional anti-malarial drugs. Ethnomedicinal plants offer potential as a source of viable cost-effective anti-malarial principles in the realm of malaria chemotherapy particularly in underprivileged populations where access to healthcare facilities is limited.

Andrographolide (AG) is a labdane diterpenoid derivative present abundantly in the herbaceous plant *Andrographis paniculata* which is extensively cultivated in Southern Asia, China and some parts of Europe. AG is an active principle of *A. paniculata*, and several publications have appeared in recent years highlighting the biological characteristics of AG including its antimicrobial, anti-inflammatory and antioxidant properties.

The *in vitro* and *in vivo* anti-plasmodial activity of andrographolide was screened previously by Mishra et al., Additionally, Zaid et al., (2015) reported the probable mechanism of action of AG as via permeation pathways channels visible on the membrane of infected RBCs as well as its impact on merozoite invasion. However, the impact of AG against different stages of the plasmodium parasite has not been sufficiently explored.

This study aimed to determine the time-dependent effect(s) of AG and CQ on the various stages of the *P. falciparum* 3D7 parasite’s intraerythrocytic cycle (as a means to assess the stage of the parasite’s reproductive cycle wherein maximum antiparasitic effect can be achieved) in addition to its impact on size and morphology of parasite forms (variants) constituting the intraerythrocytic cycle.

**Methods**

**Red blood cells**

Uninfected RBCs (type O- negative) was donated by the first author under the supervision of a haematologist. The blood was mixed with citrate phosphate buffer as anticoagulant (in the ratio of 1:9 anticoagulant/ blood); the blood was subsequently washed thrice using washing medium to remove plasma and white blood cells and resuspended to obtain a suspension of RBCs. The washing medium contained RPMI-1640, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethan-sulphonic acid) buffer (pH 7.4), 24 mM sodium bicarbonate, 11 mM glucose and 50 µg/L gentamicin. The standard protocol for blood washing was adopted as previously described.

**Chemicals and consumables**

Human O- erythrocytes were pelleted from blood suspended in RPMI-1640 medium. Albumax II was procured from Gibco BRL (Grand Island, NY, USA). Whereas, HEPES, triton X-100, EDTA, saponin, sorbitol, hypoxanthine, (100X) phosphate buffered saline (PBS), Giemsa stain and chloroquine diphosphate (CQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gentamicin was purchased from (Jiangxi Dongxu Chemical Technology Co., Ltd) while AG was purchased from Indofine Biochemical company Inc. (Cat No.: A-003).

**Parasite**
*P. falciparum* 3D7 (a CQ sensitive strain) was procured as cryopreserved specimens (at -160 °C) under liquid nitrogen from the Department of Medical Microbiology and Parasitology, Faculty of Medicine, Universiti Malaya, Kuala Lumpur, Malaysia. The cryovials were thawed at 37 °C using the standard thawing technique for malaria cryopreserved specimens. The parasites were subsequently mixed with the RBCs suspension at a haematocrit of 2%-5% and cultured per standard cultivation conditions viz micro-aerophilic environment (5% CO₂, 37 °C). Recovery of the cryopreserved parasites was carried out, using the modified protocol described by Trager and Jensen [13].

**Drug sensitivity assay and time-dependent effect**

Malaria drug sensitivity assay was carried out using Plasmodium Lactate DeHydrogenase-Technique (pLDH) as described by Makler et al., [14]. Stock solutions of 10 mM AG and CQ were prepared in DMSO and PBS (pH = 7.4) respectively. Fifty microliters (50 µL) of the synchronized culture was loaded into flat-bottomed 96-well microtiter plates featuring different concentrations of each drug (1 nM to 1 mM). The mixtures were prepared in triplicate and incubated at standard conditions for parasite cultivation over different time intervals; 12 h, 24 h, and 48 hours post-cultivation. The final parasitemia and hematocrit were maintained at 1% with parasitized RBCs (PRBCs) synchronized at the ring stage. The plates were deep frozen at − 80 °C for 1 hour after incubation and parasite growth was screened using Malstat reagent mixed with NBT/PES reagent (4:1 Malstat, NBT/PES) [14]. The sensitivity of the parasite to either AG or CQ was estimated through extrapolation of the log (dose) response curve. The concentrations required to inhibit the parasite growth at 20%, 50%, and 90% (IC₂₀, IC₅₀, and IC₉₀) respectively were identified.

**Parasite cultivation and maintenance**

The procured parasites were cultivated in a culture suspension containing O- negative RBCs and suspended in a complete RPMI-1640 malaria culture medium (CMCM) containing 25 mM HEPES, 0.75 mM hypoxanthine, Albumax 5%, 24 mM sodium bicarbonate, 11 mM glucose, and 20 µg/ml gentamicin at pH and hematocrit levels of 7.4 and 2%, respectively. Subsequently, the culture was incubated at 37 °C in a micro-aerophilic environment containing (5% CO₂). The medium was changed daily, and parasite growth was monitored using Giemsa stained thin blood smears. The parasites were passed to other culture media upon attaining a threshold parasitaemia of 2%.

**Parasite synchronization**

The process of culture (parasite) synchronization at the ring stage of the intraerythrocytic cycle was carried out prior to the drug sensitivity assay. The process of synchronization was performed using the sorbitol synchronization technique as described by Lambros and Vanderberg [15]. Briefly, the pelleted PRBCs were incubated with sorbitol for 5 min and then cultivated as described above (section Parasite cultivation and maintenance) after washing out the sorbitol solution [15].

**Effects of andrographolide and chloroquine on cell morphology and intraerythrocytic cycle progression of *P. falciparum* 3D7**

The impact of AG and CQ on the development of various stages of the *P. falciparum* 3D7 parasite’s intraerythrocytic cycle was performed by exposing the synchronized PRBCs to each drug at its IC₂₀, IC₅₀ and IC₉₀ concentrations for a period of 48 hours in 75 cm² cell culture flasks at the standard cultivation conditions as described above (section parasite cultivation and maintenance). The flasks were prepared in triplicate, and the haematocrit (Hct) was maintained at 2%. During the incubation, inoculums of the cultures were taken after 12, 24
and 48 hours, respectively. The parasite growth for each of the AG and CQ exposed flasks was compared with that of the non-exposed flasks representing the untreated control. Giemsa-stained thin blood smears were used to monitor the impact of each concentration on the cell count of each stage of the intraerythrocytic cycle as well as the impact against cell size and cell morphology [16]. The percentage of parasitized cells was determined within 2000 PRBCs with the aide of an optical microscope. The percentage of each stage of the life cycle was determined amongst infected cells while the impact of CQ or AG against cell morphology and size measurements were determined by image analysis (Olympus BX51, Olympus Corporation, Tokyo, Japan).

**Statistical analysis**

The time-dependent effect of each drug through the span of one complete intraerythrocytic cycle was calculated using GraphPad Prism 6 Software version 6.01 (Inc., La Jolla, CA, USA). The results obtained were expressed as mean ± S.E.M. Significant differences between treatment groups was determined using One-Way ANOVA followed by a Bonferroni post-hoc analysis for inter groups’ comparison using SPSS software version 23. A value of P < 0.05 was considered significant.

**Results**

Drug sensitivity assay and time-dependent effect

As shown in Table 1, AG produced moderate growth inhibitory activity at IC<sub>20</sub>, IC<sub>50</sub> and IC<sub>90</sub> which was significantly less than that of CQ (P < 0.05) at similar concentrations. Regardless of the slight growth inhibitory effect observed for AG during the initial 12 h of the cycle (evidenced by significantly higher values of growth inhibitory parameters at IC<sub>20</sub>, IC<sub>50</sub> and IC<sub>90</sub> respectively), inhibition of parasite growth was significantly diminished 24 hours after commencement of the synchronized cycle as shown in Table 1. There was a further albeit marginal decrease in growth inhibitory parameters (IC<sub>20</sub>, IC<sub>50</sub> and IC<sub>90</sub>) after 48 hours. Although this decline was insignificant as compared to that after 24 hours (P < 0.05), it remained significant compared to AG-mediated growth inhibition after 12 hours (P < 0.001).

The discrepancy in growth inhibitory activity of CQ along the different time points assessed was less when compared to that of AG where its maximal parasite growth inhibitory activity was observed during the first 12 hours of the cycle and gradually decreased thereafter. Inhibition of parasite growth by CQ after 24 and 48 hours was slightly greater than that after 12 hours as indicated by the decrease in values of the growth inhibitory parameters at 24 and 48 hours of the cycle (Table 1). For CQ, the values at 24 and 48 hours were significantly different (P < 0.05) as compared to that after 12 hours (Table 1).

**Effects on the intraerythrocytic cycle progression of P. falciparum 3D7 following exposure to andrographolide and chloroquine**

The findings showed that CQ at IC<sub>50</sub> concentration prompted a parasiticidal effect wherein approximately 50% of the parasites were killed within the first 12 h of the cycle (Fig. 1B). Moreover, a high frequency of pyknotic cells (dead parasites) and inviable cells were observed after CQ exposure. However, AG failed to produce noticeable parasiticidal effect during this interval and the parasite development sustained a normal course which was comparable to that of the control (Fig. 1A). After 24 h of the cycle, AG showed prominent parasiticidal effect (Fig. 1C). This finding was in accordance with results of the drug sensitivity and time-dependent effect, wherein the inhibitory growth parameters of AG were prominently decreased after 24 h of the cycle as compared with those
after 12 h (Fig. 1C). Nevertheless, along with CQ exposure, there were incidences of new cell death were absent and the total amount of viable cells remained the same as that after 12 h. In addition, the rings that had been retrieved after 12 h had transformed into new trophozoites (Fig. 1B). At the end of the cycle (after 48 h), there were no dead parasites identified. The overall parasitemia remained the same as compared to that after 12 h and 24 h following commencement of the cycle for both CQ and AG, respectively (Fig. 1B and 1C).

However, a dominance of schizonts was observed in all the flasks with a total percentage less than that of the control. Furthermore, the number of trophozoites retrieved in the treated flasks was significantly decreased as compared to the control (Fig. 1A). At this stage, new rings appeared to initiate a new cycle. Their number was comparatively less in the chloroquine-treated flasks, indicating a possible role for chloroquine against merozoite rupture or ring invasion. This effect was absent when the parasite was exposed to AG (Fig. 1C).

### Table 1

| Growth inhibitory parameters (IC<sub>20</sub>, IC<sub>50</sub> and IC<sub>90</sub>) of andrographolide and chloroquine on <em>P. falciparum</em> 3D7 |
|-----------------|------------------|-----------------|-----------------|
| IC<sub>20</sub> nM | IC<sub>50</sub> nM | IC<sub>90</sub> nM |
|-----------------|------------------|-----------------|
| andrographolide | chloroquine      | andrographolide | chloroquine      | andrographolide | chloroquine      |
| 12 hours        | 13200.88 ± 1174.08***aaa | 9.43 ± 1.6 | 38691.75 ± 359.52***$$aaa | 54.44 ± 2.41* | 15747.66 ± 165.94***$$aaa | 227.38 ± 13.66** |
| 24 hours        | 2367.39 ± 76.30aaa | 6.89 ± 0.79 | 7983.78 ± 267.82aaa | 33.94 ± 1.30 | 57223.87 ± 1494.33aaa | 102.90 ± 2.09 |
| 48 hours        | 587.80 ± 52.01aaa | 7.43 ± 1.22 | 4148.61 ± 412.62aaa | 20.19 ± 1.79 | 63839.38 ± 6595.98aaa | 49.64 ± 1.42 |

(IC<sub>20</sub>, IC<sub>50</sub> and IC<sub>90</sub>) (the concentrations required to inhibit the growth of <em>P. falciparum</em> 3D7 cultivated in blood culture at the standard conditions by 20, 50 and 90% respectively). Results were expressed as (mean in nM ± SEM). ***,**, and $$$ represent a statistically significant difference in the parameter as compared to time 48 and 24, respectively for each drug. Meanwhile, a, aa and aaa represents a statistically significant difference as compared with a cohort parameter of chloroquine with (P < 0.05), (P < 0.01) and (P < 0.001) respectively.

**Effects of andrographolide and chloroquine on the cell morphology of <em>P. falciparum</em> 3D7**

**Ring stage morphology after 12 hours**

Unlike CQ, AG failed to produce observable effect(s) on the morphology of ring stage parasites. All ring stage parasites in andrographolide-treated flasks were morphologically comparable to those of the control group (Fig. 2). Furthermore, the sizes of ring stage parasites did not differ significantly as compared to those of the control group (Fig. 3A). On the other hand, a large proportion of ring stage parasites in the chloroquine-treated flasks appeared to have altered morphology observable within the first 12 h of the cycle. Morphological changes observed included prominent shrinkage, fragmented structure, and loss of cellular integrity. These observations are concordant with the effects of chloroquine against the ring count. Ring count was affected by CQ but not by AG (Figs. 2 and 3A).

**Trophozoite morphology after 24 hours**

Both CQ and AG produced prominent changes in the morphological appearance and sizes of mature trophozoites. Morphological changes were apparent during the 12 h interval between the 12th and 24th hour of the parasite’s (synchronized) reproductive cycle. The effect on trophozoite morphology due to CQ was more prominent
compared to AG as evidenced by the higher numbers of disfigured and shrunken cells observed in chloroquine-treated flasks (Figs. 2 and 3B).

Schizont morphology after 48 hours

Changes in the sizes and morphological appearance of schizonts were minimal to absent when exposed to either CQ or AG in the various samples assessed (Figs. 2 and 3C).

Discussion

Recent progress in antimalarial drug research has prompted the search for potent chemotherapeutic agents within the phytochemical sanctuary, particularly following the discovery of artemisinins; a potent antimalarial phytochemical discovered in *Artemisia annua* [17]. Artemisinin is now recognized as one of the conventional antimalarials, moreover Artemisinin-based combination therapy (ACTs) is now the front-line treatment for *P. falciparum* instead of mono-therapies, especially in the regions wherein multidrug resistant strains of *P. falciparum* persist [18].

It is worthy of mention that several phytochemicals have been investigated for their anti-plasmodial activity in the past, however, the majority of these phytochemicals failed to produce considerable antimalarial effects comparable to that of existing antimalarial drugs. Nevertheless, the possibility exists of developing therapeutically active pharmacophores from phytochemical precursors. In addition to a compound's antimalarial activity its selectivity toward the parasite and safety to individuals infected with malaria are also of major importance [19]. Several studies fail to assess the time-dependent effects of pharmacologically active antimalarial principles derived from phytochemicals on the progression of various stages of the plasmodium parasite's reproductive cycle within erythrocytes or incidence of morphologic and dimensional changes on different stages of the parasite [20].

This study screened the anti-plasmodial activity of AG at different time points during the intraerythrocytic cycle of *P. falciparum* 3D7 while simultaneously determining its impact on the development of different stages of the parasite, its morphology, and related dimensional characteristics. AG has previously been reported to exert anti-plasmodial activity against *P. falciparum* 3D7 [20, 21]. Furthermore, Megantara et al., (2015) reported the promising potential of AG as an effective pharmacophore for developing new antimalarial drugs [21].

In this experiment, the pure compound of AG showed a good anti-plasmodial activity ($IC_{50} = 4148.61$ nM). However, the anti-plasmodial activity of AG was about 1000-fold less than that of chloroquine. This suggests that further structural modifications could possibly be beneficial towards improving its anti-plasmodial activity. Furthermore, the results obtained corroborate previous observations reported by Zaid et al., [11]. The anti-plasmodial activity of AG was attributed to its inhibitory effect against plasmepsins I, II, and IV by Megantara et al., (2015) [21]. Plasmepsin is an aspartate protease enzyme present abundantly in the acidic digestive vacuoles of *Plasmodium* [22]. It takes part in the degradative pathway that breaks down globin into essential amino acids. It is well known that *Plasmodium* relies on haemoglobin as its sole source of protein [23]. Haemoglobin is broken down by hemoglobinase enzymes into globin and heme, where the former is subjected to a series of degradative aspartate proteases to be broken down into its essential amino acids [24]. This pathway was also suggested as a candidate target for drugs used in malaria chemotherapy [24].

It is well known that CQ produces its effect by targeting the haemoglobin degradation pathway. Its mechanism of action is slightly different from that proposed for AG. It interferes with pathways that detoxify heme, an obnoxious waste product that is released after the breakdown of haemoglobin. It is detoxified inside the digestive vacuoles of
plasmodia into hemozoin; an innocuous inert waste product of heme. Consequently, inhibition of heme
detoxification results in its accumulation and induction of a cascade of heme-induced oxidative pathways [24].

In *Plasmodium*, haemoglobin degradation pathway starts during the ring stage and continues throughout early
and mature trophozoite development. Its activity reaches its utmost level during the early trophozoite stage within
the period of 6 to 12 h after cultivation of the synchronized ring stage [25].

The results showed that most of the parasiticidal effect due to chloroquine was observed within the first 12 h of
the cycle wherein the ring stage predominated (Figs. 1 and 2). This finding is in good agreement with a report by
Zhang (1986) [26] describing the time-dependent effect of CQ during the course of the intraerythrocytic cycle.
Furthermore, CQ produced noticeable morphological changes during this period on both rings and trophozoites.
These changes were characterized by cellular shrinkage, cellular disfiguration, and loss of chromatin materials
(Fig. 3). In addition, morphological changes were absent at the schizont stage in agreement with reports by Zhang
et al., (1986). Overall, morphological changes were observable and persisted up to the end of the cycle indicating
that CQ compromised the cellular activity of the mature trophozoites without inducing prominent lethality.

The stage wherein breakdown of haemoglobin and release of heme commences is still being debated. Some
reports have pointed out to the possible incidence of this pathway during the ring stage [26], while its maximum
incidence occurs during early trophozoite development [25]. This suggests that the maximum effect of CQ should
occur during the trophozoite, not the ring stage. The study contradicts this notion as it emphasizes that a
prominent anti-plasmodium effect for CQ can be observed before the trophozoite stage and during the ring stage.
Nevertheless, there are studies reporting that inhibition of heme detoxification and release of heme is not the only
mechanism of anti-plasmodial activity of CQ. Some studies declared that CQ might interfere with the integrity of
lysosomal membranes resulting in the increased release of degrading enzymes like caspase [27]. This, in turn, may
affect the mitochondrial membrane and increase the release of cyt C, which in turns triggers cellular apoptosis
[28].

Andrographolide failed to produce similar changes as compared to CQ during the first 12 h. Its impact was
however notable after 12 h of the cycle, wherein dead parasites and morphologic changes in intact cells were
observed (Figs. 3 and 4). However, these changes were less prominent as compared to those induced by CQ. This
is because AG failed to produce noticeable morphological changes during the early stages of the parasite
inoculation and did not exhibit any effect on cellular viability during the late stages of inoculation. Also, the
delayed lethality can be attributed to its impact on targets that showed their utmost expression during this stage.
For instance, AG was suggested as a potent inhibitor of the plasmepsin enzyme [21], which is involved in
haemoglobin breakdown [29].

Andrographolide is derived from a labdane diterpenoid (a terpene derivative), Goulart *et al.*, (2004) elucidated that
some terpene compounds (nerolidol, farnesol, and linalool) show inhibitory effect(s) on the isoprenoid
biosynthesis of trophozoite and schizont of *P. falciparum* [30]. Furthermore, the mono-terpenes have been reported
to inhibit the growth of *P. falciparum* by inhibition of protein prenylation [31]. From these, it was surmised that AG
might have exerted a negative impact on the functional characters of isoprenoid synthesis pathway, which begins
in apicoplasts. Apicoplasts are relict organelles that occur as parts of the cellular structure of apicomplexan
protozoa. They (apicoplasts) possess several functions ranging from pathways inavolved in fatty acid, isoprenoid,
iron-sulphur, and heam biosynthesis [32]. The isoprenoid synthesis pathway is considered the most important
pathway within mature trophozoites and can be set as an essential drug target for malaria chemotherapy [30]. This pathway is essential, as it fuels the cell with the required energy [33].

After commencement of the synchronized cycle, the results showed that the number of viable parasites subsequent to CQ exposure was decreased compared to the control, moreover, this number remained the same after 24 h as compared to that after 12 h. In the drug sensitivity assay, only a small decline in the inhibitory growth parameters was observed. This was attributed to the pLDH assay being performed at the same time as the drug sensitivity assay, which overestimates the parasitemia when measuring the enzyme activity of non-viable parasites. The number of viable cells decreased prominently as per the results of the Giemsa-stained thin blood film, but the changes in the growth inhibitory parameters as per the results of the sensitivity assay were less. The pLDH enzyme is involved in the conversion of lactate to pyruvate, and helps in the release of energy in the form of Adenosine Triphosphate ATP. Its activity may persist even after parasite death, and is responsible for the overestimation of parasite growth while using the well-known pLDH technique [34, 35], unlike SYBR Green I Malaria Drug Sensitivity Assay: which detect the existence of malaria DNA of the parasite inside infected erythrocytes [36].

A similar situation was observed subsequent to AG exposure. However, in this case, dead parasites were absent during the first 12 h. whereas, the dead parasites started to appear after 24 h of drug exposure. This implies that the anti-plasmodial activity of AG began 12 h after commencement of the cycle. Similarly, inhibitory growth parameters due to AG were prominently lesser after 48 h as compared to that at 24 h. However, results of Giemsa-stained thin blood smears for AG treated flasks showed a comparable number of the parasites after 24 and 48 h. The morphological features of the cells exposed to CQ suggested the incidence of necrosis and apoptosis within the parasites. Meanwhile, the death crisis features of the AG-treated flasks were suggestive of a mild form of apoptosis. The incidence of apoptosis is expected when the mitochondrial membrane starts losing its integrity, resulting in the release of cytochrome C and other apoptosis initiators into the cytosol. This, in turn, induces a cascade of the apoptotic pathway that is characterized by the activation of caspase enzymes and loss of cellular content [37]. Induction of the apoptosis pathway was detected for AG in most cancer mammalian cells models [38]. In addition to that, Luo et al., (2013) mentioned that during treatment of lung cancer cells, the nuclear transcription factor-kappa B (NF-κB) targets inhibition using AG [39]. However, further studies are warranted to elucidate the role of AG in apoptotic pathways in the context of malaria.

**Conclusions**

In conclusion, this study suggests that the initial effect of AG is exerted against mature trophozoites during the 12–24 h period after commencement of the intraerythrocytic cycle. However, the effect of CQ was more prominent as compared to AG, as observed from the rings and early trophozoites within the first 12 h of the cycle. Further investigations on the molecular mechanisms underlying the effect of AG on the *Plasmodium* parasite are warranted to better understand the time course of its anti-plasmodial activity on blood stage parasites and explore its potential application as an adjuvant to existing antimalarial therapy.

**Abbreviations**

AG
Andrographolide; ANOVA: Analysis Of Variance; CQ: chloroquine; DMSO: Di-methylsulphoxide; IC<sub>20</sub>: inhibitory concentration at 20% of maximal growth; IC<sub>50</sub>: inhibitory concentration at 50% of maximal growth; IC<sub>90</sub>: inhibitory concentration at 90% of maximal growth; NBT: Nitroblue tetrazolium chloride; *P. falciparum*: *Plasmodium*
**Declarations**

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**Author Contributions:**

Conceptualization of ideas, A.A.I.A., R.A.M., and Z.O.I; experimental design, A.A.I.A., Z.O.I; article preparation, A.A.I.A., and Z.O.I; technical assistance and reviewed the findings, S.A.A., and M.N.W; revision of the manuscript content and editing, R.B., I.S.I., R.O.B., N.Z.U; final approval, all authors have read and agree to the published version of the manuscript.

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**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

approval was waived

**Consent for publication**

Not applicable.

**Disclosure**

Some aspects of this work were presented as abstract at INFECTIONS 2017 conference at Universiti Putra Malaysia, as well as the Annual Scientific Conference of Malaysian Society of Parasitology and Tropical Medicine, March 2018.

**Competing interests**

The authors declare that they have no competing interests.

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