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Andrographolide effect on both *Plasmodium falciparum* infected and non infected RBCs membranes

O.I. Zaid[1*], R. Abd Majid[2], M.N. Sabariah[3], M.S. Hasidah[4], K. Al-Zihiry[2], M.F. Yam[5], R. Basir[1*]

[1]*Pharmacology Unit, Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*

[2]*Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*

[3]*Department of Hematology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*

[4]*School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, UKM-Bangi, Selangor, Malaysia*

[5]*School of Pharmaceutical Science, Universiti Sains Malaysia, 11800, Pulaou Pinang, Malaysia*

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ABSTRACT

Objective: To explore whether its antiplasmodium effect of andrographolide is attributed to its plausible effect on the plasma membrane of both *Plasmodium falciparum* infected and non-infected RBCs.

Methods: Anti-plasmodium effect of andrographolide against *Plasmodium falciparum* strains was screened using the conventional malaria drug sensitivity assay. The drug was incubated with uninfected RBCs to monitor its effect on their morphology, integrity and osmotic fragility. It was incubated with the plasmodium infected RBCs to monitor its effect on the parasite induced permeation pathways. Its effect on the potential of merozoites to invade new RBCs was tested using merozoite invasion assay.

Results: It showed that at andrographolide was innocuous to RBCs at concentrations approach its therapeutic level against plasmodia. Nevertheless, this inertness was dwindled at higher concentrations.

Conclusions: In spite of its success to inhibit plasmodium induced permeation pathway and the potential of merozoites to invade new RBCs, its anti-plasmodium effect can't be attributed to these functions as they were attained at concentrations higher than what is required to eradicate the parasite. Consequently, other mechanisms may be associated with its claimed actions.

1. Introduction

In spite of the achieved progress to eradicate the parasitic ailments, malaria is still a major therapeutic challenge and a significant economic burden in the developing countries due to plenty of factors, such as emergence of drug resistant strains of *Plasmodium falciparum* (*P. falciparum*), pesticide resistance among the mosquitoes vectors, the debilitating adverse effects of the conventional anti-malarials, the limited success in developing a potential vaccine and paucity of effective drugs alternatives for conventional anti-malarials [1,2].

Intra-erythrocytic ubiquity of plasmodium compromises the RBCs membrane (RBCM) integrity and enhances its permeability to biochemical entities. Moreover, RBCs aggregation and their susceptibility to hemolytic agents would be propagated as well. During the trophozoite stage (approximately after 12–15 h post invasion), PRBCs start expressing unique protein channels, known as new permeation pathways (NPP). NPPs mediate acquisition of nutrients and excretion of waste products as they mediate efflux and influx of various biochemical entities, *viz*, organic anions, amino acids, sugars, nucleotides and inorganic ions [3–5].

Andrographolide is a member of isoprenoid family of natural products and belongs to labdan diterpenoide lactones. It was first

*Corresponding author: O.I. Zaid, Pharmacology Unit, Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
E-mail: z_79ph@yahoo.com
R. Basir, Pharmacology Unit, Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
E-mail: rusliza1909@gmail.com
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isolated by Boorsma from different parts of *Andrographis paniculata*. The later had been used extensively in Siddhu, Ayurvedic and tribal medicine of India as well as in other countries for many clinical applications, *viz*, respiratory tract infections, ulcerative colitis, rhematoidal arthritis, cardiovascular disorders and cancer treatment. Recently, some studies have pointed out to the antiretroviral, antibacterial pro-apoptotic, anti-inflammatory and antioxidant effects of andrographolide [6–9].

Alteration of RBCs shape or membrane properties may retard the parasite growth. Previous studies revealed that different terpenoides may adversely affect biological membrane functions through changing their permeability to different entities, induction of membrane micro-domains and ATP dependent homeostatic mechanisms that maintain cell shape and volume [10].

In this study, we assessed the anti-plasmodium potential of andrographolide and its effect on the membrane of uninfected RBCs (nRBCs), solute induced lysis of PRBCs and parasite invasiveness.

2. Materials and methods

2.1. Materials and chemicals

Human O+ erythrocytes were obtained from blood drawn from the first author. RPMI-1640 medium, albumax II, were procured from Gibco BRL (Grand Island, NY, USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), triton X-100, sorbitol, hypoxanthine, (100×) phosphate buffered saline (PBS), frusemide and chloroquine diphosphate (CQ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gentamicin was purchased from Jiangxi Dongxu Chemical Technology Co., Ltd. while andrographolide was purchased from Indofine Biochemical company Inc. (Cat No.: A-003).

2.2. Parasite culturing, maintenance and synchronization

Both CQ sensitive and resistant strains of *P. falciparum* strain 3D7 were cultured in O+ red blood cells suspended in a Complete Malaria Culture Medium (cMCM) containing RPMI-1640, 25 mM HEPES (pH 7.4), 0.75 mM hypoxanthine, 0.5% albumax, 24 mM sodium bicarbonate, 11 mM glucose and 50 μg/L gentamicin. pH was maintained at 7.4 and the hematocrite level at 2%. The culture was incubated at 37 °C in a micro-aerophilic atmosphere containing 90% N_2, 5% CO_2 and 5% O_2. Furthermore, the medium was changed every 24 h and checked using Giemsa stained thin blood smears [11,12]. Before starting drug screening, the parasites were synchronized using sorbitol synchronization technique described by Vanderberg [13].

2.3. Stock solution preparation

Stock solutions of 100 mM of each of CQ and andrographolide were prepared using PBS (pH 7.4) for the first and methanol for the second (methanol was preferred over DMSO as the later is not baneful to plasmodium).

2.4. Malaria drug sensitivity assay

Malaria drug sensitivity assay was performed according to Mathias *et al*., 2010. Drug containing flat bottomed 96 well microtiter plates, featured serial dilution of CQ (1 nm–1 μM) and andrographolide (1 nm–1 mM) were incubated for 48 h at 37 °C with PRBCs (synchronized at the ring stage with parasitemia of 1% and hematocrite 1%). Control wells containing nRBCs, PRBCs and drug solution were allocated as well. Serial drug dilution was performed using cMCM as a diluent. Three plates were prepared for each drug and each dilution was done in triplicate. At the end of the incubation period, the plates had been freeze-thawed for 1 h then the well were loaded with 100 μL of SYBR green-I lysis buffer [20 mM Tris-HCL, 5 mM EDTA, 0.008% saponin and 0.008% (v/v) triton-100]. The mixture was incubated in dark at room temperature for 1 h and finally fluorescence was measured after 15 s of plate agitation twice in Victor Plate reader (Perkin Elmer, Salem, MA) at an excitation/emission wavelength of 485/535 nm. The geometric mean of the first and second pass was used to exclude any measurement error [14]. Eventually both IC_{50} and IC_{90} for CQ against *P. falciparum* 3D7 were determined according to the recommended protocol using Microsoft excel 2007 software.

2.5. Effect of andrographolide on merozoites invasiveness

Andrographolide effect on merozoite invasion was performed as previously described [4]. Briefly, non infected RBCs were treated at 37 °C for 2 h with different concentrations of the drug (1 μM/mM) dissolved in the incomplete malaria culture medium (RPMI-1640, HEPES-tris buffer 25 mmM and gentamicin 20 μg/mL). Then the exuberant andrographolide was washed twice and to each 300 μL of the treated RBCs, 100 μL of PRBCs at parasitemia >15% and rich in schizont stage (after >35 h of parasite synchronization) was added and Hct was adjusted at 4%. The mixture was incubated at the mentioned incubation conditions for 20 h, the time point at which the microscopic determination of parasitemia was done. In this test, the amount of merozoites that could have invaded the treated RBCs was compared to the positive control that contains only non treated RBCs. The percentage of parasite duplication inhibition at each concentration was calculated using the following equation

$$\%\ of\ parasitemia\ duplication\ inhibition = \frac{Pa - Pbx}{Pa} \times 100$$

wherein *Pa* and *Pbx* represents the parasitemia of the positive control and that of the culture containing RBCs treated with x concentration of andrographolide. The percentage of each concentration was plotted versus log concentration to determine the dose response curve.

2.6. Effect of andrographolide on sorbitol induced PRBCs hemolysis

Andrographolide effect on parasite induced permeability pathway was investigated as previously described [15]. Briefly, PRBCs predominated with trophozoites (after 30 h of synchronization) at 5% parasitemia were washed with cMCM and 100 μL of the washed cells were loaded into 24 well plates, featured two folds serial dilution of andrographolide or frusemide, a well-known inhibitor of plasmodium induced NPPs, with a concentration range of (500 nM–500 μM) for each at 1 mL/well. The dilutions were done using sorbitol buffer

solutions (300 mM sorbitol in 10 nM HEPES-Tris (pH 7.4). After 30 min incubation at 37 °C, 500 µL was aliquoted from each suspension after through mixing, centrifuged at (500 *g* for 5 min) and 200 µL of the supernatants were loaded to a flat bottomed 96 well plate. The released hemoglobin was measured at 540 nm (Spectramax plus 384) and the percentage of hemolysis in each sample was compared to positive control wells (wells contain drug free buffer solution). Finally, IC_{50} (drug concentration required to inhibit 50% of parasite induced permeability pathway) was obtained by linear regression analysis using Microsoft excel 2007 software.

Time dependent effect of sorbitol induced hemolysis was measured through withdrawing aliquots of 100 µL from each well after 5, 10, 15, 30, 40, 50 and 60 min of sorbitol buffer incubation. The time profile of the andrographolide treated cultures was compared with that of the positive control.

2.7. Effect of andrographolide on non infected RBCs membrane

Impact of andrographolide on nRBCs membrane was tested through incubating type O +ve human RBCs with Incomplete Culture Medium (RPMI-1640, 25 mM HEPES and 20 µg/mL gentamicin) containing three different concentrations of andrographolide (1 nM–1 mM) at 37 °C for 48 h using 24 well plate (1 mL/cell and kept at hematocrite 5%). Aftermath, 500 µL from each well was transferred to Eppendrof tubes after through mixing, centrifuged at 500 *g* for 5 min and two aliquots of 200 µL from of the supernatants were loaded into a flat bottomed 96 well plate and the released hemoglobin was measured at 540 nm. Results were compared with both positive and negative controls which contain RBCs suspended in 1% tween 80 and in drug free medium respectively. Tween 80 was used at 1% to induce 100% of RBCs hemolysis. Finally, the final mixture of the pelleted RBCs, that was left with 100 µL of the culture medium, was mixed thoroughly and both RBCs size and morphology were assessed using RBCs coulter heamocytometer (Sysmex KX21) and Lieshman stained smear.

2.8. Effect of andrographolide on RBCs fragility

RBC fragility test was performed as previously described [16]. Briefly, washed RBCs were suspended in series of solutions containing isotonic PBS diluted serially using D.W such that the tonicity ratio would be kept in the range of 0.0%–0.9% noticing that 0% tonicity gives 100% hemolysis. Then three sets of the mentioned solution were prepared and andrographolide was added to each one so that its final concentration would be either 1 µM or 500 µM) in each set respectively. Finally, percentage of hemolysis *vs.* tonicity curve was extrapolated for each drug concentration and was compared to that of the drug free set.

3. Results

3.1. Anti-plasmodium effect of andrographolide

Drug sensitivity assay showed that 3D7 was quite sensitive to CQ [IC_{50} = (22.3 ± 0.76) nM, IC_{90} = 7.14] and less sensitive to andrographolide [IC_{50} = (1 039.3 ± 45.76) nM, IC_{90} = (2 102.4 ± 56.2) nM].

3.2. Andrographolide induced merozoit invasion inhibition

Andrographolide had significantly ($P < 0.05$) reduced the percentage of ring infected RBCs in the merozoite invasion assay as compared to control (percentage of ring stage parasitized RBCs was increased by 11 and 7 folds in both drug free and andrographolide enriched suspensions at a concentration of >125 nM respectively (Figure 1).

3.3. Effect of andrographolide on nRBCs

Slight hemolysis was obtained when nRBCs were incubated with high concentrations of andrographolide (>250 nM) meanwhile the effect was negligible at lower concentrations. Meanwhile, it enhanced nRBCs sensitivity to the hypotonicity as it raised the tonicity threshold at which maximum nRBCs hemolysis incur (maximum hemolysis started at tonicity equivalent to 0.45% NaCl in the absence of andrographolide and 0.6% in its ubiquity (Figures 2 and 3).

RBCs exposure to a high dose of andrographolide for 48 h produced some morphological changes characterized by ubiquity of higher proportion of cells with spherocytosis and echinocytosis as compared to the untreated control (Figure 4).

The change in RBCs indices [mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH)] as detected by Coulter hemocytometer are demonstrated in Table 1.

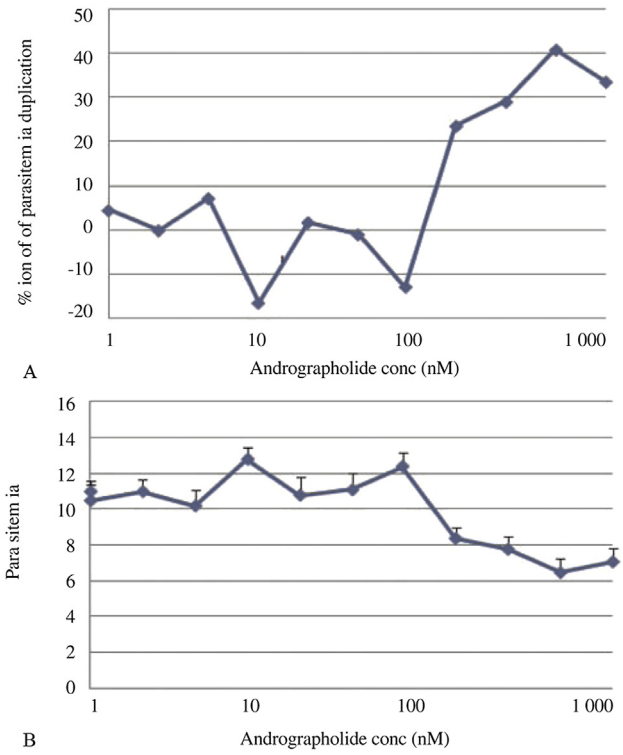

Figure 1. Effect of andrographolide on parasite invasion.
A) Represents percentage of merozoite invasion inhibition for each concentration as compared to control versus andrographolide concentration in nM. The percentage started to decrease to about 30% at a concentration of 125 nM and stayed at this level up to 1 mM. B) Represents the plot of the obtained parasitemia during the ring stage of the subsequent cycle versus andrographolide concentration in nM. * Represents statistical significance as compared to control (0 concentration of andrographolide).

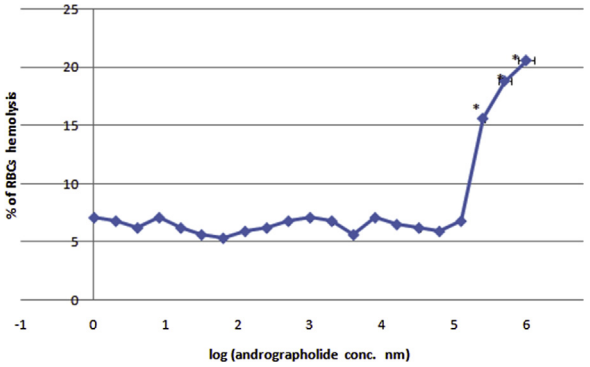

Figure 2. Effect of different concentrations of andrographolide on RBCs stability.
* represents statistically significant difference as compared to the negative control.

3.4. Effect of andrographolide on parasite induced permeation pathways

Andrographolide produced a significant effect on parasite induced new permeation pathways ($P < 0.05$) in a dose dependent manner with an IC_{50} value about >30 times more than its IC_{50} against plasmodial growth (Figure 5). Hemolysis time profile study shows that maximum sorbitol induced hemolysis was obtained after 20 min incubation with the iso-osmotic sorbitol while addition of both sorbitol and andrographolide at their IC_{50} dose had reduced hemolysis up to the end of the incubation period (Figure 5).

Table 1
RBCs indices.

Andrographolide concentration	MCV in fl	MCH in pg
1 mM	69.3 ± 3.5	29.6 ± 1.6
500 μM	74.0 ± 2.6	29.2 ± 2.0
250 μM	75.3 ± 3.4	30.3 ± 2.1
125 μM	82.0 ± 2.1	33.8 ± 1.0
0	81.3 ± 2.0	33.7 ± 0.5

4. Discussion

Andrographolide is a diterpenoid lactone that belongs to isoprenoid family of natural products. It is the main bioactive constituent of stem and leaves of *Andrographis paniculata*. It is an interesting pharmacophore with an antioxidant, anticancer, anti-inflammatory [8], anti-rheumatoidal, anti malaria anti-HIV [6], antibacterial and immune-modulatory effects were studied extensively. The antitumor and anti-inflammatory effects are attributed to its pro-apototic and cell cycle arrest actions [9]. It

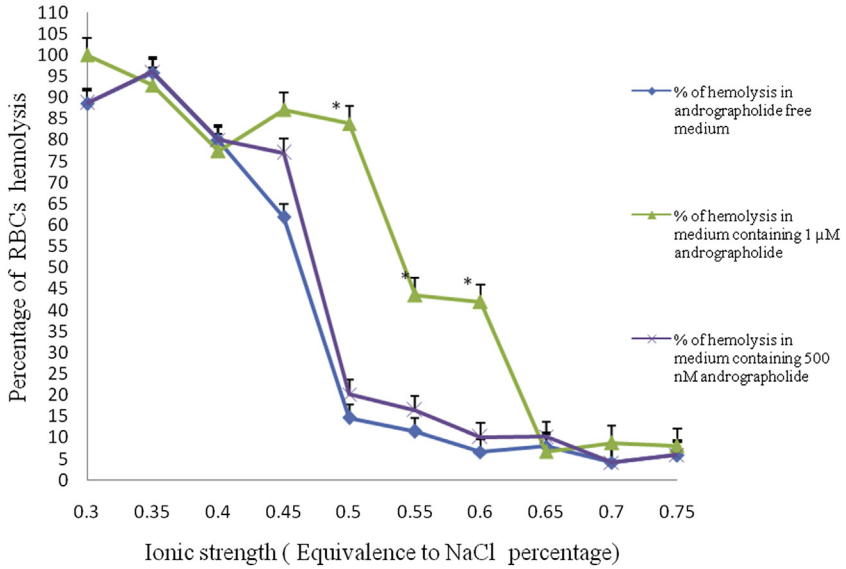

Figure 3. Osmotic fragility profile of RBCs incubated in drug free medium and media containing three different concentrations of andrographolide; 500 nM, 1 μM and 500 μM respectively.
The test was performed in triplicate and * indicates statistical significance ($P < 0.05$) as compared to the degree of hemolysis in the drug free medium.

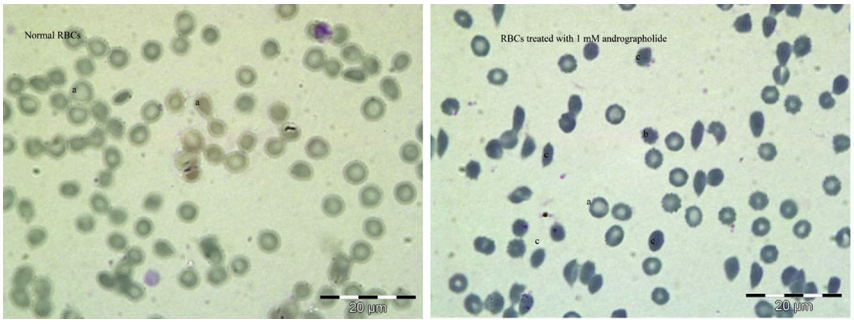

Figure 4. RBCs morphology of normal untreated (right hand side) andrographolide treated (left hand side).
a, b & c represent normal discoid shaped, spherocytic and and echinocytic RBCs respectively.

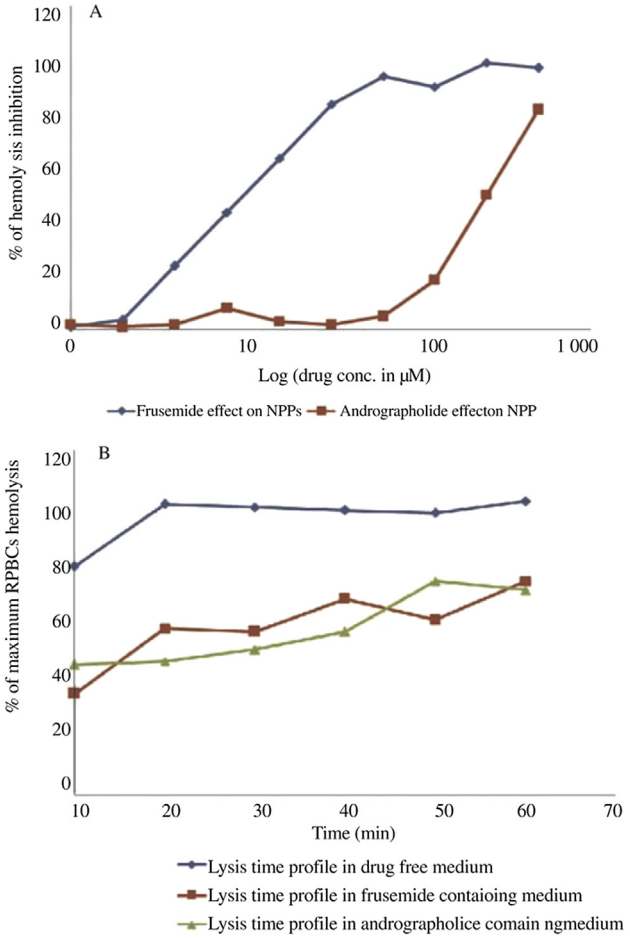

Figure 5. A) Dose response curve of andrographolide against *Plasmaodium falciparum* induced new permeation pathway. Maximum hemolysis was obtained in drug free medium and the percentage of hemolysis inhibition was plotted on Y-axis versus drug concentration on X-axis. Results of andrographolide induced inhibition was compared with that of frusemide; a well known standard inhibitor of *Plasmodium falciparum* NPPs. IC_{50} of the inhibitory effect of andrographolide was about 25 times that of frusemide. B) PRBCs hydrolysis time profile in media containing the IC_{50} dose of each of frusemide and andrographolide for NPPs inhibition and in drug free medium as well. Maximum sorbitol induced hemolysis was obtained after 20 min in the drug free medium.

was found that andrographolide inhibits P27 and cyclin dependent kinase enzymes in tumor cells [7]. Furthermore, it inhibits IL2, TNF-α production in immune cells [8].

Due to their amphipathic nature, terpenoides are expected to accumulate in membranous structures resulting in a loss of their integrity and ease their disruption during stressful situations [17,18]. Although moderate concentrations of andrographolide failed to induce RBCs hemolysis in the isotonic environment, its adverse impact on RBCs membrane was more obvious when the latter was incubated in a hypotonic environment. Andrographolide could have increased the tonicity threshold at which hemolysis commences indicating that slighter drop in isotonic strength is enough to produce complete RBCs hemolysis in the presence of andrographolide. This indicates that andrographolide compromises RBCs membrane integrity, reduces their life span and inhibits their capability to resist the hypotonicity induced spherocytosis.

Blood smears showed that during incubation some RBCs have undergone morphological changes characterized by spherocytosis and echinocytosis. The former is characterized by changing the morphology of RBCs from the normal discoid biconcave shape with a central palor in the middle area into darker smaller spherocytes. Meanwhile the latter is associated with appearance of series of round protrusions, speckles or crenations on RBCs surface. These crenations evolve from the imbalance in the RBCs membrane which results in changing the area between the two layers and creation of these crenations. Echinocytosis was attributed also to change in the conformational stability of the RBCs membrane structural proteins which are responsible for maintenance of cells morphology. In contrast to echinocytosis, stomatocytosis may occur which occurs when the inner leaflet of the RBCs membrane is affected resulting in creation of curvatures toward the intracellular compartments and creation of stomata like structures [19]. At higher concentrations, andrographolide has increased the incidence of both the echinocytosis and spherocytosis due to disturbing hinocytogenic factor that acted upon the outer leaflet of the RBCs membrane. This action may be attributed to its tendency as a lipophilic amphiphile to accumulate in the double layered plasma membrane and disturb its stability or due to its action on the structural proteins that constitute the RBCs cytoskeleton.

Inhibition of ring infected RBCs percentage after mixing the schizonts of the previous life cycle with andrographolide-treated-RBCs indicates that the latter had compromised the potential of merozoites to invade new RBCs. Merozoites invasion is a multi-steps process starts with RBCs recognition by the parasite, re-orientation of the merozoites on RBCs surface and junction formation, inward motion driven by the merozoites apical complex actinomyosine then finally pinching of the junction and entry of the parasite into RBCs where the parasitphorous vacuolar membrane evolution starts. Merozoites carry some specific surface antigens that bind to RBCs in sialic acid dependent manner, *viz*, merozoite surface protein −1, glycosyl phosphotidyl inositol and erythrocyte binding antigen-175. Sailic acid is a part of glycophorine molecules whose ubiquity in RBCs membrane relies on its integrity. Our results confirms the capacity of andrographolide to compromise the functional characteristics of RBCs membrane constituents as andrographolide induced inhibition of merozoite invasion indicates loss of the integrity or functional characteristics of the sialic acid content of the PRBCs membrane which is crucial for the invasion process. Meanwhile, during the invasion process, an invagination is formed on the surface of the RBCs to allow for parasite re-orientation and thence the intra-erythrocytic parasite entry. So inhibition of merozoite invasion may be attributed to the potential of andrographolide to disturb the proper flow of these steps. Such action has come in coincidence with results of the osmotic fragility test that showed a prominent loss of aptitude of the andrographolide treated RBCs to resist the hypotonicity induced spherocytosis [17,18,20].

On the other hand, the study showed that andrographolide had reduced the activity of parasite induced new permeation pathway. During the intra-erythrocytic stage, *P. falciparum*, triggers alterations in RBCs membrane resulting in changing its function for the favor of the parasite. Infected RBCs express specific protein channels called (NPP) which regulate entry of the nutrients and exodus of the waste products. PRBCs start expressing NNPs after 10–15 h post invasion and reach the plateau after 30 h. Consequently, we opted to start our experiment with trophozoites grown after 30 h of synchronization [5]. NPPs have broad specificity to various agents so they allow

passage of different types of cations, anions, Zwetter ions and non electrolytes, such as monosaccharides, small polyols, amino acids, nucleotides, mono carboxylates, quaternary ammonium compounds, inorganic cations, *viz*, calcium ion as well as large molecules, such as glutathione. PRBCs hemolyze when they are incubated with an isotonic solution of any of these species due to solute influx along with water into the intracellular compartment. Sorbitol was chosen in our assay as it is the most famous solutes that have ever been used in malaria research to synchronize the plasmodium culture and to study effects of drugs on NPPs [5,21].

In spite of its obvious inhibitory effect on NPPs function, Andrographolide was required in an amount higher than what was required to inhibit the parasite growth indicating that NPPs inhibition is not a major mechanism of andrographolide induced plasmodium growth inhibition and further investigation is recommended to explore this phenomenon. Previous studies attributed the channel blocking effect of frusemide to presence of carboxyl heads along with a sizeable hydrophobic tail [22]. Andrographolide effect on NPPs may be attributed to the direct blocking effect as it possesses a hydroxyl group head, which is also hydrophilic like the frusemide's carboxyl and a prominent hydrophobic tail or due to its potential to alter cell membrane fluidity and functions after being accumulated there. Direct inhibitory effect of drugs with amphiphile characters was proposed previously as NPPs are made up of two chains, non ionic hydrophobic and cationic chain. The former mediates ingress of non ions while the latter facilitates passage of the anionic substrates which are driven by the electrochemical gradient while the cationic ones are repulsed by the cationic chain. Previously, it was found that NPPs play a role in cellular development and growth. As an inwardly rectified channel, NPPs can regulate the membrane potential through increasing the ion conductance which in turn raises the of cell membrane linked adenyl cyclase enzyme that activates protein kinase A and trigger cellular growth [23].

Conflict of interest statement

We declare that we have no conflict of interest.

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