PHARMA SCIENCE MONITOR
AN INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES

ANALYSIS OF *IN-VITRO* ANTIMALARIAL ACTIVITY OF ANDROGRAPHOLIDE AND 5-HYDROXY-7,8-DIMETHOXYFLAVONE ISOLATED FROM *ANDROGRAPHIS PANICULATA* AGAINST *PLASMODIUM BERGHEI* PARASITE

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ABSTRACT

In the present investigation, *in-vitro* antimalarial activity of two active constituents of *Andrographis paniculata* was analyzed against *Plasmodium berghei* infection, propagated in Balb/c mice by injecting $1x10^5$–$1x10^7$ parasitised red blood cells in citrate saline. Results indicated that chloroform soluble fraction of methanolic extract significantly ($P < 0.05$) inhibits parasitaemia (74 ± 3.8)% at 1 mg. mL^{-1} in comparison to methanolic extract (54.9 ± 4.8)%, n-butanol (56.5 ± 2.8)% and ethyl acetate (47.8 ± 3.6)% soluble fractions of methanolic extract, respectively. However, it was also significantly ($P < 0.05$) different from chloroquine phosphate (82.6 ± 3.8)% inhibition at 0.39 mM. Moreover, andrographolide showed significantly ($P < 0.05$) better inhibition (53.9 ± 3.1)% against parasitaemia than 5-hydroxy-7, 8-dimethoxyflavone (15.4 ± 2.9)%, but it was lower ($P < 0.05$) than that of chloroquine phosphate (61.5 ± 3.1 at 0.039 mM)%. Therefore, andrographolide, an active constituent of *A. paniculata* warrants thorough *in-vitro* and *in-vivo* study for scale up the technology.

Keywords: Andrographolide, 5-hydroxy-7, 8-dimethoxyflavone, Antimalarial.

INTRODUCTION

Malaria is one of the most widespread and vicious diseases affecting the mankind. The disease is caused by *Plasmodium* (Protozoa) of which four species *P. falciparam, P. vivax, P. ovale* and *P. malariae* are infectious to humans [1]. The disease is recognized as a wide spread epidemic in tropical and sub-tropical areas of the world, especially because of the remorseless rise in the resistance of *P. falciparum* to commonly used antimalarial drugs like chloroquine, mefloquine, mepacrine, pyrimethamine, primaquine and sulphadoxin [2,3]. Moreover, *P. falciparum* has acquired significant resistance and become prevalent in different parts of the world, which is a matter of serious concern [4,5]. Further, mefloquine resistance among *P. falciparum* has become more common in some of the

eastern countries [6]. Even resistance against the novel drug - quinine has been recently reported from certain parts of South East Asia and Brazil [7]. The developing resistance towards conventional antimalarial agents has created serious threat for the treatment of malaria. Consequently, the attention has been focused on natural products and the search of an effective antimalarial agent. Chinese herbal drug artemether from *Artemisia annua*, is the out come of such efforts [8]. *Andrographis paniculata*, belonging to family *Acanthaceae* found mostly in tropical and subtropical regions of the world was used as bitter and remedy for malaria [9-11]. The active constituents of *Andrographis paniculata* are andrographolides and xanthones, which are isolated, characterized [12-15]. Andrographolide and 1,2-dihydroxy-6,8-dimethoxy-xanthone possess significantly higher activity in comparison of other isolated xanthones [9]. However, limited information is available for antimalarial activity of isolated xanthones. Hence, the objectives of the present investigation were the phytochemical screening of *A. paniculata* and evaluation of antimalarial activity of andrographolide and isolated xanthone, 5-hydroxy-7,8-dimethoxy flavone against *P. berghei in vitro*.

EXPERIMENTAL

Reagents and chemicals

The air dried whole herb of *Andrographis paniculata* was obtained from Indian Herbs and Research Supply Company, Saharanpur (UP) India, authenticated on the basis of taxonomic characters and compared with herbarium specimens available at the Museum-cum-Herbarium of the University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India. The voucher specimen (No. 971) of the plant was deposited at the Herbarium of the Institute. Chloroquine (Resochin injection, 40 g. L^{-1}, 30 mL vial) was purchased from Bayer Pharmaceuticals, India. All other chemicals used were of analytical grade.

Extraction and fractionation

The dried powdered plant material (2.15 kg) of *A. paniculata* was extracted twice with methanol (500 mL) by maceration for 96 h at room temperature. After maceration, the extract was filtered and solvent was evaporated using rotary flash evaporator (Buchi Laboratory Equipments, Switzerland) to obtain the drug extract. The extracts from two macerations were combined to obtain 73 g of methanolic syrupy residue. A portion of

this extract (5 g) was used for evaluating antimalarial activity and also for isolating pure compound, andrographolide for similar studies and remaining 68 g of methanolic extract was suspended in 1 L of distilled water and partitioned sequentially with chloroform, ethyl acetate and n-butanol (3x500 mL each). After each partition, the solvent from organic phase was recovered under reduced pressure yielding 18, 12 and 10 g of chloroform, ethyl acetate and n-butanol soluble portions, respectively. The chloroform soluble portion of methanolic extract (12 g) was column chromatographed over silica gel (300 g, 60-120 mesh) column packed in chloroform. The column was eluted using chloroform and chloroform - methanol mixture (1% *v/v*, 2% *v/v*, 4% *v/v*) in the increasing order of polarity. A total of 100 fractions, of 250 mL each were collected and those showing similar TLC pattern were combined to get a total of 30 fractions.

Extraction and isolation

The residue of the fractions was dissolved in hot hexane. The hexane soluble portion was separated and the insoluble residue was kept in a mixture of chloroform and methanol for crystallization and re-crystallization. On the other hand, the methanolic extract was refluxed with activated charcoal (150 g) for 1 h and the solution was filtered. The filtrate was concentrated under reduced pressure, crystallized and re-crystallized with methanol. The resulting crystals were collected by filtration.

Characterization of the active constituent

The scanning of the fractions was performed in UV-Visible spectrophotometer (Shimadzu, Japan) to determine the λ_{max} of active constituents in methanol, used as solvent. Fourier transform infrared spectrum (FTIR) was recorded for fractions using Spectrum BX (Perkin Elmer, USA) infrared spectrophotometer. Samples were prepared in KBr disk (2 mg sample / 200 mg KBr) with a hydrostatic press at a force of 275790.292 Pascal's for 4 min. All the ^{1}H NMR spectra were recorded with BRUKER AVANCE (Bruker Avance, USA) II 400 MHz spectrometer at 294.3 K, and the data processing was performed with TOP SPIN 1.3 standard software. All one-dimensional spectra obtained were deconvoluted with Nuts NMR data processing software (Acorn NMR Inc., USA). The proton chemical shifts of fractions were determined in $CDCl_3$ solution respectively. The chemical shift (δ) was referenced to the internal signal of

tetramethylsilane (TMS, 0.01%). The mass spectrum was obtained on VG-70S 11-250J 9CMS-DS instrument (VJ Analytical Limited, Manchester, UK).

Analysis of antimalarial activity

Experiments on animals were carried out as per the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Culture, Government of India. The Institutional Animal Ethics Committee approved the study. Adult BALB/c mice (25 ± 1 g) of either sex, bred in the Central Animal House of Punjab University, Chandigarh, fed on the standard diet and water *ad* libitum were used.

Evaluation of parasitaemia

P. berghei (NK-65 strain) was maintained by propagating infection in mice by injecting 1 x 10^5 – 1 x 10^7 parasitised red blood cells in citrate saline (a mixture of 0.85% *m/v* sodium chloride and 3.8% *m/v* sodium citrate) i.p. from infected mice to naives. Parasitaemia was assessed by preparing smears of the blood samples collected from tail vein. The smears were air dried, fixed in methanol and stained with Giemsa solution (Giemsa powder 3 g/500 mL of 1:1 solution of methanol and glycerin). The slides were then washed in running water, air dried and observed under microscope for the infection. The percent infection was calculated by number of infected erythrocytes to that of total erythrocyte count.

Culturing of normal RBCs and infected RBCs

The mice were anaesthetized with diethyl ether. Blood was collected in citrate saline (2:1) by incising jugular vein under sterile conditions.

Culture medium

RPMI (Roswell Park Memorial Institute) 1640 (Gibco, USA) was used as the culture medium supplemented with penicillin (100 $\mu g.mL^{-1}$), streptomycin (100 $\mu g.mL^{-1}$), gentamicin (50 $\mu g.mL^{-1}$), sodium bicarbonate (5%, *m/v*) and HEPES (*N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid (0.6% *m/v*). The medium was filtered through 0.22-µm membrane filter (MDI, India) using syringe filter assembly. The incomplete medium thus prepared was transferred to an autoclaved vial and fetal calf serum (10% *v/v*) was finally added to completely constitute the medium.

Preparation of normal red blood cells

Normal RBCs collected from uninfected mice were centrifuged at 1500 rpm for 10 min. The upper layer containing plasma and buffy coat of leucocytes was aspirated and sediment containing the RBCs was washed twice with incomplete medium. A 50 % suspension was made by diluting the RBCs with equal volume of incomplete medium.

Preparation of infected red blood cells

Blood collected from *P. berghei* infected mice in citrate saline (2:1) was centrifuged at 1500 rpm for 10 min. Plasma and buffy coat of leucocytes was removed through aspiration. The uppermost layer of the sediment comprising of trophozoites and shizonts was aspirated and transferred to a sterile vial. It was washed twice with incomplete medium and suspended in known volume of incomplete medium. The number of infected cells per mL of medium were counted using haemocytometer. *P. berghei* infected RBCs were mixed with 50% suspension of uninfected normal RBCs in such a proportion that percent parasitaemia at 0 h was about 1-2%.

Preparation of test samples

The test samples were dissolved in minimum amount of solvents and diluted with RPMI to get different doses. The suspended samples were then incubated with infected blood (1% parasitaemia) for 20 h. Normal control, which contained infected blood in complete medium, was also performed along with the test compounds.

Assay of antimalarial activity

A suspension (0.9 mL) of *P. berghei* infected red blood cells (0.1% parasitaemia, 3-5% cell hematocrit) was added to 24 wells tissue culture plate containing different doses of test substances and control (chloroquine phosphate, 0.039 to 0.39 mM). Microtitre plates were incubated for 20 h at 37 °C in a sealed candle jar. The assay was terminated by aspirating the medium. The smears were prepared, air dried, fixed in methanol (2 min) and stained with Giemsa solution (30 min). The stained smears were washed under running water, air dried and observed under microscope. The number of infected RBCs per 2000 RBCs was counted. The results were expressed as percent invasion inhibition. Antimalarial studies

The *in-vitro* assay of methanolic, chloroform, ethyl acetate, n-butanol and pure isolates were carried out using *P. berghei* test model. All the test samples were tested at

different doses. The antimalarial activity was calculated by percent invasion inhibition of parasitaemia.

Statistical analysis

Statistical analysis was carried out using the one-way and two-way ANOVA test. All statistical calculations were performed with the GRAPH PAD Prism 4.01 software.

RESULTS AND DISCUSSION

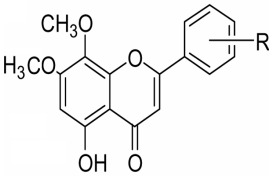
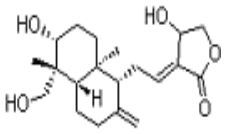
Extraction, fractionation and characterization of active constituents

The whole plant of *A. paniculata* was macerated with methanol at room temperature (25 ^{0}C) to protect thermo-labile active constituents. The methanolic extract was suspended in water and sequentially partitioned with chloroform, ethyl acetate and n-butanol to separate compounds of different polarity. However, only chloroform extract was used to isolate its constituents using column chromatography (Table 1). The column chromatography of chloroform soluble portion of methanolic extract yielded 30 fractions (F001 to F030). The pure isolates were obtained from chloroform extract, which were duly characterized and identified. The crystalline isolate was obtained from the fraction F011 eluted in chloroform. It was re-crystallized from a mixture of chloroform and methanol. The yellow crystalline isolate was characterized for melting point, UV absorption and other spectral techniques as discuss (Table 2). The single spot on TLC, which was brightened on spraying with 25% lead acetate indicating the presence of flavone ring. FTIR data also supported the TLC results and provided complementary evidences for the flavone due to the presence of hydroxyl group, C=O stretch of γ-pyrone ring, asymmetrical C-O-C stretch and symmetrical C-O-C stretch, respectively. Further, the flavone ring was confirmed by ^{1}H NMR spectra showed 6H sharp singlet at δ 3.95 ppm corresponding to two methoxy groups. A singlet at δ 6.67 for 1H was ascribed to H-3. The singlet at δ 6.44 showed the presence of C-6 proton. The hydroxy and methoxy groups contributed to up field shift of aromatic proton indicating unsubstituted flavone nucleus appeared at δ 6.7-7.1 ppm. The multiplet at δ 7.75 (J=2.0 Hz) and at δ 7.95 (J=7.5, 2.4 Hz) represented other protons of flavone nucleus. A broad singlet at δ 12.56 ppm was assigned to hydroxy protons at 5' position. Therefore, structure of the isolated compound was proposed to be 5-hydroxy-7,8-dimethoxyflavone based upon its NMR data and further confirmation was done by mass spectroscopy.

TABLE 1: COLUMN CHROMATOGRAPHY OF CHLOROFORM SOLUBLE PORTION OF METHANOLIC EXTRACT

Fraction code	Fraction number	Eluent	Mass of the eluate (g)
F001	1-2	Chloroform	0.40
F002	3-4	Chloroform	0.12
F003	5	Chloroform	0.21
F004	6	Chloroform	0.03
F005	7-10	Chloroform	0.05
F006	11-16	Chloroform	0.11
F007	17-20	Chloroform	0.32
F008	21-23	Chloroform	0.10
F009	24-28	Chloroform	0.30
F010	29-30	Chloroform	0.22
F011	31-32	Chloroform	0.10
F012	33-35	Chloroform	0.61
F013	36-37	Chloroform	0.19
F014	38-42	Chloroform: Methanol (99:1)	0.07
F015	43-47	Chloroform: Methanol (99:1)	0.10
F016	48-54	Chloroform: Methanol (99:1)	0.68
F017	55-60	Chloroform: Methanol (99:1)	0.72
F018	61-65	Chloroform: Methanol (99:1)	0.40
F019	66-69	Chloroform: Methanol (99:1)	0.07
F020	70-72	Chloroform: Methanol (99:1)	0.03
F021	73-74	Chloroform: Methanol (98:2)	0.02
F022	75-76	Chloroform: Methanol (98:2)	0.02
F023	77-80	Chloroform: Methanol (96:4)	0.75
F024	81-83	Chloroform: Methanol (96:4)	1.40
F025	84-86	Chloroform: Methanol (96:4)	0.97
F026	87-88	Chloroform: Methanol (96:4)	0.60
F027	89-90	Chloroform: Methanol (96:4)	0.16
F028	91-92	Chloroform: Methanol (96:4)	0.07
F029	93-96	Chloroform: Methanol (96:4)	0.02
F030	97-100	Chloroform: Methanol (96:4)	0.01

TABLE 2: CHARACTERIZATION AND IDENTIFICATION OF ACTIVE CONSTITUENTS PRESENT IN *ANDROGRAPHIS PANICULATA* EXTRACTS BY VARIOUS SPECTROSCOPY TECHNIQUES

Chemical structure	Spectroscopy techniques
 R=H 5-hydroxy 7,8-dimethoxy flavone	Yield: 7.2 mg (7.2%) M.p.: 182-184 ^{0}C TLC: R_f - 0.9 (chloroform: acetone; 99.5::0. 5) UV λ_{max}^{MeOH}: 274, 211 nm FTIR peaks (cm^{-1}): 3440 (-OH group), 1660 (C=O stretch of γ-pyrone ring), 1210 (asymmetrical C-O-C stretch), 1020 (symmetrical C-O-C stretch) ^{1}H NMR peaks (δ ppm): 3.95 (s, 6H), 6.44 (s, 1H), 6.67(s, 1H), 7.55 (m, 3H, J=1.99 Hz), 7.95 (m, 2 H, J=7.47, 2.43 Hz) 12.56 (s, 1H) Mass spectrum: M^+ = 298, m/z –255, 139
 Andrographolide	Yield: 121 mg (2.4%) M.p.: 228-229 ^{0}C TLC: R_f: -0.48 (chloroform: methanol; 9:1) UV λ_{max}^{MeOH}: 223.2 nm FTIR peaks (cm^{-1}): 3400(-OH group), 2900 (assymetrical C-H stretching) 2850 (symmetrical alkane C-H stretching), 1680 (C=O stretch), 1210 (asymmetrical C-O-C stretch), 1020 (symmetrical C-O-C stretch; α, β-unsaturated δ-lactone ring) 900 (C-H out of plane bending; exocyclic methylene group) ^{1}H NMR peaks (δ ppm): 0.67 (s, 3H), 1.75 (s, 3H), 2.35 (d, 1H, J=6.7 Hz), 2.52 (t, 1H), 3.21 (d, 1 H), 3.96 (d, 1H) 4.07 (dd, 1H), 4.37 (dd, 1H, J=10.0, 6.0 Hz), 4.63 (s, 1H), 4.83 (s, 1H), 4.91 (d, 1 H), 6.65 (dd, 1H)

The molecular ion peak appeared at M^+ 298, which was equivalent to molecular mass of 5-hydroxy-7, 8-dimethoxyflavone. Other m/z peaks appeared at 255 and 139. This fragmentation pattern was similar to that of flavones, which further confirmed the structure of isolated compound. The product isolated was characterized for melting point, UV absorption maxima and spectral techniques similar to 5-hydroxy-7,8-dimethoxyflavone as shown in Table 2. The compound brightened on spraying with 60% H_2SO_4 followed by heating. The UV spectrum of the compound exhibited absorption maximum at 223.2 nm, characteristic of α, β-unsaturated ketone structure. FTIR spectrum assured the presence of hydroxyl, asymmetrical and symmetrical alkane. C-H stretching, C=O stretching, C-O-C stretching and symmetrical C-O-C stretching confirmed the presence of α, β-unsaturated δ-lactone and C-H out of plane bending verified the exo-cyclic methylene group. The compound was identified as andrographolide from its spectroscopic data.

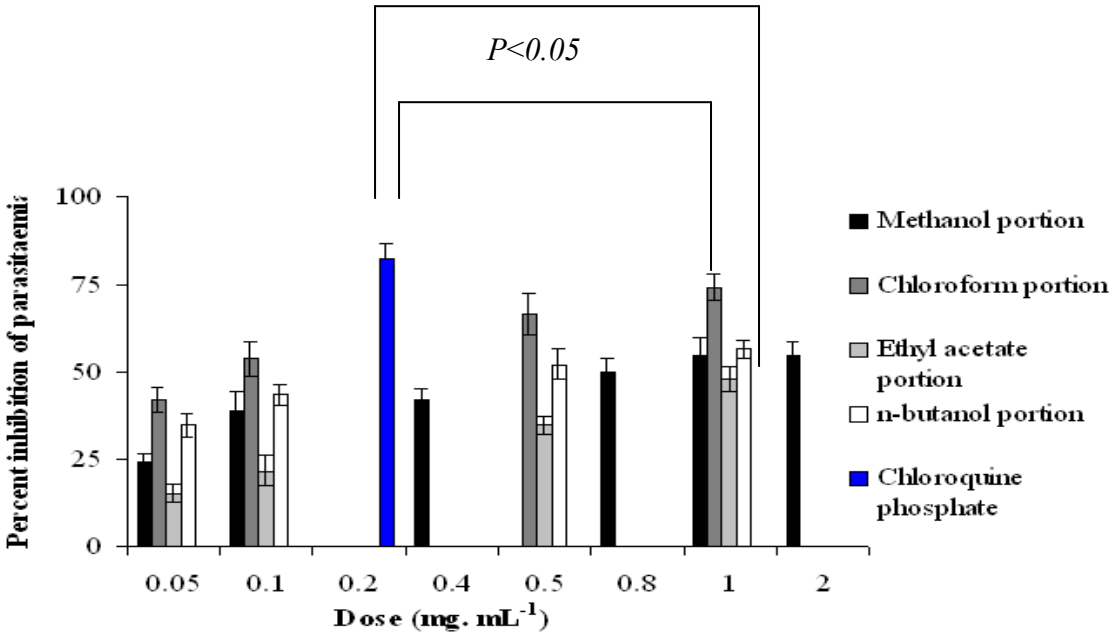
Antimalarial activity

The *in-vitro* antimalarial activity of methanolic extract and chloroform, ethyl acetate, n-butanol soluble fractions of methanolic extract as well as two pure isolates, 5-hydroxy-7, 8-dimethoxyflavone and andrographolide was determined against *P. berghei* parasite infection. All the test samples were tested at different doses. Results indicated that chloroform soluble fraction of methanolic extract significantly ($P<0.05$) inhibits parasitaemia (74 ± 3.8) % at 1 mg. mL^{-1} in comparison of methanolic extract (54.9 ± 4.8) %, n-butanol (56.5 ± 2.8) % and ethyl acetate (47.8 ± 3.6) % soluble fractions of methanolic extract, respectively. However, it was significantly ($P < 0.05$) lower from chloroquine phosphate (82.6 ± 3.8) % inhibition at 0.39 mM (Figure 1 A). On the other hand, active constituents isolated from chloroform portion (F011) and methanolic extract have also shown promising results. Andrographolide, isolated from methanolic extract showed significantly ($P < 0.05$) better inhibition (53.9 ± 3.1)% against parasitaemia than 5-hydroxy -7,8-dimethoxyflavone (15.4 ± 2.9)% but it was also significantly lower than that of chloroquine phosphate (61.5 ± 3.1 at 0.039 mM)% (Fig. 1 B). The lower antimalarial activity of 5-hydroxy-7,8-dimethoxyflavone might be attributed to the hydroxyl group at 5' position. It was proposed that 1, 2-dihydroxy-6, 8-dimethoxy

xanthone possesses a stronger antiplasmodial activity against *P. falciparum* than 1, 4, 8-hydroxyl groups and reported that hydroxyl group at 2' position is more potent than 1, 4, 8-hydroxyl groups [9]. Hence, andrographolide possesses stronger antimalarial activity than 5-hydroxy-7, 8-dimethoxyflavone.

P<0.05

A



B

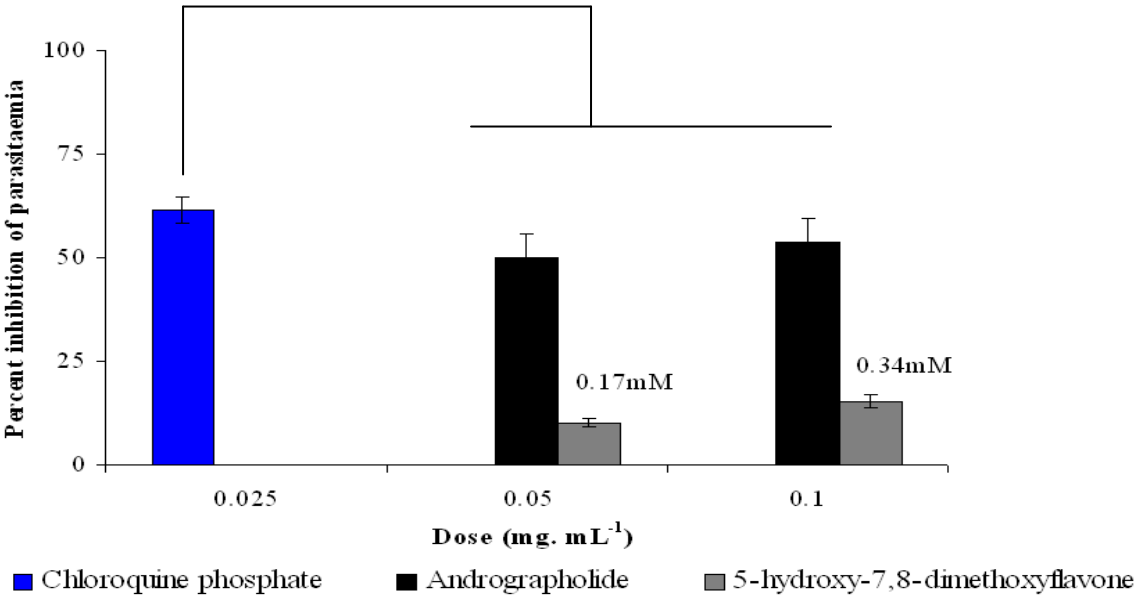


Figure 1

In-vitro antimalarial evaluation of (A) *Andrographis paniculata* extracts compared with chloroquine phosphate. Chloroform soluble fraction of methanolic extract significantly (*P<0.05*) inhibits parasitaemia (74 ± 3.8) % at 1 mg. mL^{-1} in comparison of methanolic extract (54.9 ± 4.8) %, n-butanol (56.5 ± 2.8) % and ethyl acetate (47.8 ± 3.6) % soluble fractions of methanolic extract, respectively. However, it was significantly (*P < 0.05*) lower from chloroquine phosphate (82.6 ± 3.8) % inhibition at 0.39 mM (mean ± SD, *n* = 3) (B) Isolated pure compounds of *Andrographis paniculata* compared with chloroquine phosphate (mean ± SD, *n* = 3). Andrographolide, isolated from methanolic extract showed significantly (*P<0.05*) higher inhibition (53.9 ± 3.1)% against parasitaemia than 5-hydroxy-7,8-dimethoxyflavone (15.4 ± 2.9) %. Moreover, it was also significantly lower than that of chloroquine phosphate (61.5 ± 3.1% at 0.039 mM)

CONCLUSION

The *in vitro* antimalarial activity of methanolic, chloroform, ethyl acetate and n-butanol extracts and two pure isolates 5-hydroxy-7, 8-dimethoxyflavone and andrographolide were determined using *P. berghei* strain. Although the results showed a significant percent inhibition in all the test samples, the chloroform soluble portion of methanolic extract exhibited maximum percent inhibition and andrographolide proved better among the isolated compounds. Therefore, andrographolide, an active constituent of *A. paniculata* warrants thorough *in-vitro* and *in-vivo* study for scale up the technology.

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