Pharmacogn J. 2017; 9(4): 534-540
A multifaceted peer reviewed journal in the field of Pharmacy
www.jyoungpharm.org | www.phcog.net

Original Article

Antimalarial and Antioxidant Potentials of Extract and Fractions of Aerial Part of *Borreria ocymoides* DC (Rubiaceae).

Sunday Adeleke Adesegun, Celestina Ifeoma Orabueze, Herbert Alexander Babatunde Coker

ABSTRACT

Introduction: *Borreria ocymoides* (Burm F) DC is a weak, erect and decumbent herb that has several folkloric, ethno medicinal uses and is included in antimalarial preparations by some traditional healers. It is also used in treatment of ring worm, eczema and microbial infections. **Objectives:** To evaluate antimalarial activity of extract and fractions of *Borreria ocymoides* in *Plasmodium berghei* infected mice and to investigate their antioxidant activity using 1, 1-diphenyl-2-picryl-hydrazile (DPPH). **Methods:** The methanol extract of aerial part of *B. ocymoides* and the solvent fractions obtained from partition between organic solvents were assessed for antimalarial activity against chloroquine sensitive *Plasmodium berghei* NK65 infected mice using the suppressive and curative test procedures. Chloroquine (10 mg/ml) was used as positive control. The antioxidant activity was evaluated using DPPH radical scavenging ability and determination of total phenolic content. **Results:** The crude extract (250 and 500 mg kg-1) produced a dose dependent anti-plasmodial activity in the suppressive and curative tests. The chemo suppression activity was best in the ethyl acetate fraction (87.31%) and in the order ethyl acetate >dichloromethane > hexane > aqueous fraction. The DPPH radical scavenging activity of the extract increased with concentration. The antioxidant activity was less than ascorbic acid used as positive control. Oral administration up to 5 g/kg produced no noticeable deleterious effect 24 hours after dosing and up to 7 days afterwards. **Conclusion:** The results indicated that the extract has a potent anti-plasmodial activity against *Plasmodium berghei* and the activity seems to reside in the mid-polar fractions. Thus, the plant is a potential source of new antimalarial agents.

Key words: Antimalarial, Antioxidant, *Plasmodium berghei*, *Borreria ocymoides*, Solvent fractions.

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History
- Submission Date: 27-9-2016;
- Review completed: 12-12-2016;
- Accepted Date: 20-12-2016.

DOI : 10.5530/pj.2017.4.86

Article Available online
http://www.phcogj.com/v9/i4

INTRODUCTION

Malaria is an endemic infectious disease in warm climate and particularly within the less economic empowered regions of the world.[1] The World Health Organization estimates that about 41% of the total world population lives in areas with malaria risk.[2] This disease is caused by *Plasmodium* and spread out by infected female Anopheles mosquitoes called "malaria vectors" during blood meal and they bite mainly between dusk and dawn. *Plasmodium* species that can infect and cause disease in humans are *P. Falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*.[3] *Plasmodium falciparum* is the most virulent and is responsible for the majority of malaria related morbidity and mortality[4] with significant social and economic impact in developing countries. Immunity against malaria can be naturally acquired in individuals living in malaria-endemic areas after continuous exposure to the parasite, but it seems to wane in the absence of *P. falciparum* exposure and does not give a complete protection against the infection.[5-6]

In Nigeria, malaria is a major primary healthcare challenge particularly for children and pregnant women. It is responsible for 30 percent childhood mortality and 11 percent maternal mortality.[7] The problem is aggravated by the increasing resistant to available drugs and mosquito vectors to insecticides, resulting in high level of treatment failures.[8-10] Chloroquine, a low cost drug was the first line drug of choice in Nigeria before the emergency of drug resistance and subsequent changeover to WHO approved artemisinin combination therapy (ACTs).[6, 11] Clinical resistance to artemisinin and its combinations was reported in some parts of the world (Cambodia and four other countries), suggesting that some *P. Falciparum* isolates have developed the ability to grow in the presence of these antimalarial agents.[12] This strongly suggests the need for urgent research into new antimalarials[13] with improved activity. At present there are no drugs that can completely offer protection against malaria in all regions of the world.

Free radicals, such as reactive oxygen species (ROS) and nitrogen oxygen species (NOS) are molecules created when the body cells use oxygen to generate energy. These free radicals at low or moderate concentrations exert beneficial effects on cellular responses and immune function but at high levels, they induce oxidative stress, a harmful process that can damage cell structures, including lipids, proteins,

Cite this Article: Adesegun SA, Orabueze CI, Coker HAB. Antimalarial and Antioxidant Potentials of Extract and Fractions of Aerial part of *Borreria ocymoides* DC (Rubiaceae). Pharmacogn J. 2017;9(4): 534-40.

and DNA.[14-15] Oxidative stress has been implicated in the development of chronic human diseases and degenerative ailments.[14, 16-17] Minimizing oxidative stress could therefore ameliorate many physical ailments and help prevent certain degenerative diseases caused by free radicals.[18] Antimalarial agents with both antimalarial and antioxidant activities will be an advantage in malarial treatment or prevention.[19]

Borreria ocymoides (Burm F) DC is a weak, erect and decumbent herb and belongs to the family Rubiaceae.[20] The plant has several folkloric and ethno medicinal uses which include dermatological use in treatment of ring worm and eczema in Nigeria, antihelmintic treatment of internal worms, treatment of microbial infections (dysentery, diarrhoea, gonorrhea), aids healing of wounds,[21] gastric ulcer protective potency[22].The plant was also mentioned by some traditional healers in treatment of malaria. The aim of the present study is to determine anti-plasmodial and antioxidant activities of methanol extract of aerial part of *Borreria ocymoides* and its solvent fractions using mice infected with chloroquine sensitive *Plasmodium berghei*.

MATERIALS AND METHODS

Plant material

The aerial parts of *B. ocymoides* was collected at Ede Oballa village, Nsukka town Anambra state, Nigeria and authenticated by a retired taxonomist of University of Nigeria, Nigeria, Mr. Ozoko and confirmed by Mr. Daramola, a curator, formerly of the University Herbarium, Department of Botany, Faculty of Science, University of Lagos Akoka. The herbarium specimen was prepared and deposited with voucher number LUH 3624.

Extract and fractions preparation

The collected plant material was cleaned, air dried under shade at room temperature and powdered by using a grinding mill (Hamburg 76 West Germany). The powdered plant material (1.3 kg) was extracted exhaustively with methanol by continuous percolation in a Soxhlet apparatus. The filtrate was concentrated using a rotary evaporator under reduced pressure (Buchi Rota vapor, Germany) at 45 rpm and 40°C to obtain dried crude extract (124 g). Extract (100 g) was suspended in 300 ml distilled water and successfully fractionated with n-hexane (3 × 300 ml), dichloromethane (3 × 300 ml) and ethyl acetate (3 × 300 ml) to yield n-hexane (23.22 g), dichloromethane(16.90 g), ethyl acetate (8.17 g), and aqueous (43.28 g) fractions respectively. The extract and fractions were kept in airtight sample bottles and were stored at 4°C for the assays.

Preliminary phytochemical screening

The phytochemical analysis of the methanol extract of *B. ocymoides* was carried out to determine the presence of phenolic compounds like tannins and flavonoids, saponnins, anthraquinone derivatives, cardiac glycosides, steroidal compounds, cyanogenetic glycosides and alkaloids using standard methods.[23-24]

Experimental animals

Swiss albino mice (20 - 22 g) of both sexes were used for the study and were bred and kept in the Laboratory Animals Center, College of Medicine, University of Lagos, Nigeria.The animals were maintained on standard rodent feed and water ad libitum under 12 h light and dark cycle at room temperature. The animals were allowed to acclimatize for 14 days preceding the experiments. All experimental protocols were in compliance with internationally accepted principles for laboratory animal use and care.[25]

Acute toxicity

The safety of the crude extract was evaluated by determining the oral acute toxicity using a modified Lorke s method.[26] The mice were starved for 24 h prior to drug administration. They were randomly divided into six groups of 10 mice per group (one control group and five treated groups). The control group received 0.2 ml of 1% tween 80, while the treated groups were given single doses of 500, 1000, 2000, 4000 and 5000 mg/kg orally, in dose volume of 0.2 ml. The mice were observed for signs of toxicity and mortality at regular intervals for 24, 48, and 72 h then subsequently daily for 7 days.[27]

Malaria parasites

The rodent parasite *Plasmodium berghei berghei* chloroquine sensitive strain (ANKA strain NK-65) was obtained from National Institute of Medical Research (NIMR), Yaba, Lagos and was maintained by continuous re-infestation in mice.

Inoculum preparation

Parasitized erythrocytes were obtained from a donor infected mouse by cardiac puncture. The inoculum was prepared by determining percentage parasitaemia and erythrocytes count of the donor mouse and diluting it with normal saline in proportions as indicated by the determinations.[28]

Antimalarial Analysis

Suppressive test

The Peter's 4-day suppressive test was used to determine the suppressive activity of the extract and its fractions against chloroquine sensitive *Plasmodium berghei* NK 65 infection in mice.[29] On first day (D_0), 24 mice were inoculated by intraperitoneal injection (i.p.) with 0.2 ml of inoculum of *Plasmodium berghei berghei* containing 1 x 10^7 infected erythrocytes. The animals were randomly divided into 4 groups of six mice each and three hours later drugs were administered to the mice. Groups 1-2 received 250 and 500 mg/kg/day extract respectively, group 3 was the negative control and received 10 ml/kg/day of 1% tween 80 in distilled water while groups 4 served as positive control received 10 mg/kg/day of chloroquine. The drug administration continued daily for 4 days (D_0-D_3) and on 5th day (D_4) thin blood films were prepared from tail blood obtained from each mouse then stained with Giemsa to show parasitized erythrocytes out of 500 in a random field of the microscope. The above procedure was repeated for hexane, dichloromethane, ethyl acetate and aqueous fractions respectively to evaluate their antimalarial activity (250 mg/kg).

Percentage parasitaemia was calculated as

$$\% \text{ Parasitaemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC counted}} \times 100$$

$$\% \text{ Chemosuppression} = \frac{\text{APN} - \text{APT}}{\text{APN}} \times 100$$

Where APN is average parasitaemia in negative control and APT is average parasitaemia in test group.

Curative test

The test was carried out as described by Liu & co workers[30] with modifications. Twenty four mice were infected intrapertoneally with standard inoculum of 1x10^7 (0.2ml) *Plasmodium berghei berghei* NK 65 infected erythrocytes on the first day (D_0) and left for 72 h then mice were divided into 4 groups of six mice each. Groups 1-2 received orally 250 and 500 mg/kg/day extract respectively, group 3 was the negative control and received 10 ml/kg/day of 1% tween 80 in distilled water while group 4 served as positive control 10 mg/kg/day of chloroquine. The treatment

was carried out once daily for 5 days and Giemsa's stained blood smears were prepared from the tail blood sample of each mouse collected on each day of treatment to monitor parasitaemia level. Any death that occurred during this period was noted and used to determine the mean survival time over a period of 28 days (D_o-D_{27}).

Antioxidant activity

DPPH radical scavenging test

The antioxidant activity of the extract and its fractions were determined by the free radical scavenging ability using DPPH (1, 1-diphenyl-2- picrylhydrazyl) assay.[31] About 1ml of sample solution of different concentrations (0.05-1.0 mg/ml) was mixed with 3 ml of methanol and 1 ml of 1 mM DPPH in methanol was added to make up to 5 ml. The mixtures were shaken, allowed to stand at room temperature in a dark chamber for 30 minutes and the absorbance was measured at 517 nm using spectrophotometer. Ascorbic acid was used as positive standard control. The percentage of inhibition DPPH (%) was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Abs of control} - \text{Abs of test sample}}{\text{Absorbance of control}} \times 100$$

Absorbance of control

The concentration of extract leading to 50% reduction of DPPH (IC50) was determined from the curve of % inhibitions plotted against the respective concentrations.

Determination of Total Phenolic Content

The total phenolic content of the extract was measured according to the Folin-Ciocalteu method.[32] The extract solution (0.25% w/v; 1 ml) was mixed with 5 ml of 1 in 10 aqueous solution of Folin-Ciocalteu reagent and 5 min later 4 ml of 7.5% w/v sodium carbonate was added then the mixture was incubated at room temperature for 1 h. The absorbance was measured at 760 nm with a spectrophotometer. Gallic acid solutions with concentration ranging from 5-200 µg/ml were used for calibration. A dose response linear regression was generated by using the gallic acid standard absorbance and the level in the extract was expressed as gallic acid equivalent (mg of GAE/g of extract). The procedure was done in triplicate and results were expressed as mean ± SEM.

Statistical analysis:

Results were expressed as mean ± S.E.M. The significance of difference was determined using one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Preliminary phytochemical analysis

The phytochemical screening of the crude extract of *B. ocymoides* indicated the presence of phenolic compounds like flavonoidsand tannins, steroidal compounds and saponins while alkaloids, cyanogenetic glycosides and anthraquinones werenot detected.

Acute toxicity

There was no mortality recorded in the mice upon oral administration even at doses as high as 5,000 mg/kg. This indicates that the experimental doses used are relatively safe.

Suppressive activity

The results of the study showed that the extract displayed schizonticidal activity at the studied doses. Percentage inhibition analysis indicated that the extract produced a significant ($p<0.05$) dose-dependent decrease in parasitaemia at 250 and 500 mg kg^{-1} compared to the negative control group (Table 1). The hexane, dichloromethane and ethyl acetate fractions

Table 1: Suppressive effect of extract of *B. ocymoides* against *P. berghei* infected Swiss albino mice

Drug	Dose (mg/kg)	Mean Parasitaemia	% Chemosuppression
1% Tween 80		3.13 ± 0.11	-
Chloroquine	10	0.56 ± 0.05	82.11*
B. ocymoides	250	1.50 ± 0.00	52.08*
	500	0.96 ± 0.00	69.33*

Parasitaemia expressed as mean ± SEM, n = 5, results are expressed as the percent suppression of parasitaemia with reference to non-treated mice, *significant difference from control at $p < 0.05$

Table 2: Suppressive effect of various fractions of *B. ocymoides* against *P. berghei* infection in mice

Drug/Fraction	Dose (mg/kg)	% Parasitaemia	%Parasite Chemosuppression
1% tween 80		38.68 ± 7.03	
Chloroquine	10	1.76 ± 0.08	95.45*
Hexane	250	27.91 ± 1.69	27.84*
Dichloromethane	250	22.36 ± 0.55	42.19*
Ethylacetate	250	4.91 ± 1.63	87.31*
Aqueous	250	33.18 ± 2.52	14.22*

Parasitaemia expressed as mean ± SEM, n = 5, results are expressed as the percent suppression of parasitaemia with reference to non-treated mice, *significant difference from control at $p < 0.05$

also reduced parasitaemia level significantly ($p < 0.05$) compared to the negative control. The order of chemo suppression of the fractions was ethyl acetate > dichloromethane > hexane > aqueous (Table 2).

Curative activity:

The result of the curative test showed extract of *B. ocymoides* demonstrated a dose- dependent reduction in mean parasitaemia ($p < 0.05$) in mice by 76.2 and 84.6% at 250 and 500 mg/kg respectively like chloroquine treated groups while the negative control group showed a daily increase in parasitaemia (Table 3). Parasitaemia reduction was also observed for chloroquine, the standard drug, where reduction started from D_1, (3.11%) to D_5, where total clearance was recorded (100%). Death did not occur in the chloroquine treated groups but occurred at 250 and 500 mg/kg of extract at 40 and 20% respectively. The survival was 0, 60, 80 and 100% for negative control, extract 250, 500 mg/kg and chloroquine group respectively.

Antioxidant activity

The extract of *B. ocymoides* exhibited a concentration dependent DPPH radical scavenging activity which was significantly less ($P > 0.05$) than ascorbic acid used as positive control with IC_{50} value 1.85 and 0.05 respectively (Figure 1). The total phenolic content of methanol extract of *B. ocymoides* was calculated as 48.0 ± 2.49 mg GAE/g of extract.

DISCUSSION

In many parts of the world where malaria is endemic, herbal preparations are used to treat the disease. Plants produce phytochemicals which have protective or preventive properties against human diseases. Antimalarial activities of many medicinal plants have been reported[33-35]. In this study anti-malarial activity of the extract and fractions of *B. ocymoides* were tested using *in vivo* anti-plasmodial effect against chloroquine-sensitive *Plasmodium berghei* NK 65-infected mice. The results obtained showed that the extract elicited significant ($P < 0.05$) dose dependent suppressive effect against early *Plasmodium* infection (suppressive effect) and established infection (curative effect) (Table 1 & 3). The extract fractions also exhibited different degrees of parasitaemia clearance in the 4-day suppressive schizontocidal activity test. The ethyl acetate and the dichloromethane fractions were found to possess higher blood schizontocidal activity (87.31 and 42.19% respectively) than the hexane and the aqueous fractions (27.8 and 14.22% respectively) (Table 2). This suggests the possible localization of the active ingredients in these two fractions, particularly the ethyl acetate fraction. The anti-plasmodial effect may be due to one of the active compounds of the extract or a combined effect

Table 3: Curative effect of crude extract of *B. ocymoides* on parasitaemia in mice

Drug	Dose (mg/kg)	Parasitaemia	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
1% Tween 80		% Parasitaemia	17.03 ± 0.71	19.21 ± 0.42	23.69 ± 0.30	30.21 ± 0.22	39.25 ± 0.10
		% Cure	0	0	0	0	0
Crude extract	250	% Parasitaemia	16.68 ± 2.13	15.71 ± 0.76	12.98 ± 0.83	11.59 ± 1.63	9.34 ± 0.55
		% Cure	2.06	18.23	45.21	61.64	76.20
	500	% Parasitaemia	15.85 ± 0.43	13.58 ± 1.20	10.58 ± 1.22	8.23 ± 1.14	6.04 ± 2.03
		% Cure	6.93	29.31	55.34	72.76	84.61
Chloroquine	10	% Parasitaemia	16.5 ± 0.10	14.13 ± 0.07	9.51 ± 0.26	3.40 ± 0.03	0.00
		% Cure	3.11	26.44	59.85	88.75	100

Parasitaemia expressed as mean ± SEM, n = 5, results are expressed as the percent suppression of parasitaemia with reference to non-treated mice.

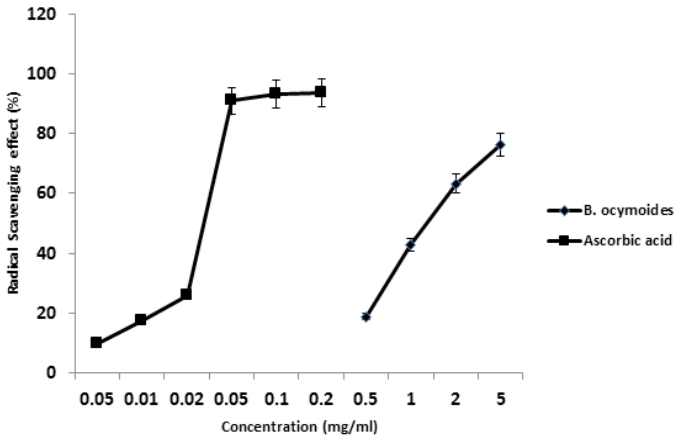

Figure 1: Radical Scavenging Effects of *B. ocymoides* and Ascorbic acid on DPPH Radical

of many. Previous studies on individual effects of *Cinchona* alkaloids and synergistic effect between the various alkaloids has shown improved activity over quinine against resistant *P. falciparum in vitro*,[36] particularly when quinine is combined with cinchonine.[37] The synergistic effects of the active compounds has numerous benefit since it could lead to enhanced efficacy, decreased dosage or increased level of target inhibition, reduced or delayed development of drug resistance and reduction of toxic effects.[38]

The Rane's test evaluates the curative ability of compounds on established infection and is commonly used for antimalarial drug screening. In this test, extract of *B. ocymoides* has better parasitaemia clearance than in the suppressive test. In the 4-day suppressive study, the percentage parasitaemia was lower (69. 33%) at the dose of 500 mg/kg than the reduction recorded in the curative test (84. 61%) at the same dose. This lower activity may probably be due to specificity of the crude extract on established infection. The mean survival time of the infected mice was prolonged as 20% death was observed at higher dose of the extract increased while 100% mice-mortality occurred in the untreated control.

The study on acute toxicity revealed absence of mortality even up to the dose of 5000 mg/kg body weight of extract administered orally, which indicates the safety of the extract at the various test doses.

The preliminary phytochemical analysis of the crude extract of *B. ocymoides* showed the presence of phenolics, steroidal compounds and saponins. Phenolic compounds like flavonoïds and tannins are good antioxidant substances which have been reported to have biological activities and prevent or control oxidative stress related disorders.[39] The crude extract tested negative to alkaloids.This finding is contrary to report of Conserva and Ferreira *et al*. in 2012 that alkaloids were present in species of *Borreria*.[40] Previous studies indicated that many phenolic and steroidal compounds possessed anti-plasmodial activities.[41-43]

Malarial pathogenesis was reported to be associated with free radicals formation and decrease of antioxidant level.[17, 44-45] This suggests that an increase in the body antioxidant can initiate the decrease in parasite number and severe infection at the long run.[17, 46] Oxidative stress has been implicated in the pathophysiology of malaria.[47] The efficacy of antioxidants is usually associated with their ability to inhibit oxidative damage by scavenging free radicals.[48] DPPH is a very useful reagent for investigating free radical scavenging ability of compounds. *B. ocymoides* extract demonstrated good antioxidant activity by reducing the purple colored DPPH radical solution to yellow colored diphenylhydrazine due to the presence of hydrogen-donating antioxidants.[49] The extract also has phenolic compounds that are antioxidant agents which also act as free radical terminators.[50] The Folin-Ciocalteau reagent method used measures the level of oxidizable or phenolic compounds in the extract.[51] Phenolic compounds are considered to be the most important antioxidative plant components and the antioxidant activity of plant extracts correlates with the content of their phenolic compounds.[52-53] The phenolic compounds in the extract may be involved in the antimalarial activity exhibited by the *B. ocymoides* crude extract. The antimalarial activity of phenolic compounds was reported to be due to elevation of red blood cell oxidation, inhibition of parasite's protein synthesis and also by counteracting the oxidative damage induced by the malaria parasite.[47] Plants and compounds with antioxidant activity may ameliorate the progress of malarial infection and possibly prevent the development of cerebral complications and other malaria related complications.[54] However, the challenge here is that some antimalarial drugs generate the free radicals to produce their effect suggesting that their activity may be reduced in the presence of antioxidants. The anti-plasmodial activity observed in this study is consistent with the traditional use of the *B. ocymoides* as herbal medications against malaria in Nigeria.

CONCLUSION

This study indicates that methanol extract and solvent fractions of *B. ocymoides* have good anti-plasmodial and antioxidant activities. The extract appeared to be superior in curative parasitaemia reduction than suppressive activity. The findings suggested that the phytochemicals responsible for antimalarial activity of the plant are polar in nature with antioxidant properties. Future studies on the plant regarding antimalarial activity should be conducted to isolate compounds responsible.

ACKNOWLEDGEMENT

The authors are grateful to University of Lagos and Tertiary Education Tax Fund (TETFUND), Nigeria for financial support CRC/TETFUND/No.2011/18. We also appreciate Mr. D. Ota of Department of Physiology, College of Medicine, University of Lagos, Nigeria for his assistance in animal studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this research paper.

ABBREVIATIONS USED

DPPH: 1,1-diphenyl-2-picrylhydrazyl; **GAE:** Gallic acid equivalent; **ANOVA:** Analysis of variance.

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SUMMARY

- *Borreria ocymoides* plant is a widely used in traditional medicine in Nigeria to treat ring and internal worms, microbial infections, wounds, ulcer and malaria.
- Attempt has been made to establish anti-plasmodial and antioxidant activities of the plant
- Extract of *B. ocymoides* exhibited potent anti-plasmodial activity against *Plasmodium berghei* and the activity seems to reside more in the mid-polar fractions.

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Cite this Article: Adesegun SA, Orabueze CI, Coker HAB. Antimalarial and Antioxidant Potentials of Extract and Fractions of Aerial part of *Borreria ocymoides* DC (Rubiaceae). Pharmacogn J. 2017;9(4): 534-40.