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Antimalarial actions of *Lawsonia inermis, Tithonia diversifolia* and *Chromolaena odorata* in combination

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ABSTRACT

Ethnopharmacological relevance: Chromolaena odorata, Tithonia diversifolia and *Lawsonia inermis* are medicinal plants used in treating malaria in traditional medicine system. Previous studies however showed that their dichloromethane, methanol (1:1) extracts were more active against *Plasmodium* parasite than the aqueous extracts.

Aim of the study: To determine the *in vitro* and *in vivo* antiplasmodial activity of dichloromethane, methanol (1:1) extracts of *Chromolaena odorata, Tithonia diversifolia* and *Lawsonia inermis* in combination and evaluate their safety using acute limit toxicity test.

Materials and methods: Dichloromethane, methanol (1:1) extracts of *Chromolaena odorata, Tithonia diversifolia* and *Lawsonia inermis* leaves were combined at ratios 1:1, 1:3, 3:1, 1:5 and 5:1 using *in vitro* semi-automated microdilution technique against *P. falciparum* Chloroquine sensitive (D6) and Chloroquine resistant (W2) strains, with chloroquine and artemisinin as controls. The *in vivo* antiplasmodial activity of the crude extracts was carried out singly, and in combination at the different combination ratios on *Plasmodium berghei* Anka infected Swiss albino mice using Peters' 4-day suppressive test. Acute toxicity test was done in mice at 5000 mg/kg.

Results: The *in vitro* combination of *L. inermis* and *T. diversifolia* (1:1) extracts against *P. falciparum* showed the highest synergy with IC_{50} of 0.43 ± 0.02 μg/mL and 2.55 ± 0.19 μg/mL against D6 and W2 respectively; while the combination of *C. odorata* with *T. diversifolia* and *L. inermis* were antagonistic. A synergy with chemosuppression of 83.6% against *P. berghei* infected mice was observed in *L. inermis* and *T. diversifolia* (1:1) treated animals. In contrast to the *in vitro* result, combination of *C. odorata* with *T. diversifolia* and *L. inermis* showed some degrees of synergy *in vivo*. Extracts were not toxic at the concentration tested.

Conclusion: These findings rationalized the use of these plants in combination as antimalarials in traditional medicine. However, the combination of *Chromolaena odorata* with other medicinal plants should be used with caution because of its possible antagonistic effect.

1. Introduction

Malaria is a deadly disease caused by *Plasmodium* parasite and transmitted through the bites of infected female *Anopheles* mosquitoes. Despite collaborative efforts, 214 million cases of malaria and 423,000 deaths were reported in 2015 (World Health Organization, 2016). The management and control of malaria are challenged due to resistant of malaria parasites to most antimalarial drugs. Artemisinin-based combination therapy (ACT) is currently most widely used treatment regimen for malaria (World Health Organization, 2008). The combination therapy is widely advocated because it produces rapid clinical and parasitological response, and may circumvent or delay resistance (Bukirwa and Orton, 2005). Similarly, folk medicines often use combination of plants in treating series of infections.

Medicinal plants contain numerous compounds which serve as potential drug sources for human disease management (Barliana

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et al., 2014). Some of the medicinal plants that have been previously reported to possess antimalarial activities in folkloric medicine include *Lawsonia inermis*, *Chromolaena odorata* and *Tithonia diversifolia* (Afolayan et al., 2014; Elufioye and Agbedahunsi, 2004; Idowu et al., 2010; Ukpai and Amaechi, 2012). Traditionally, the use of medicinal plants usually involves a combination of more than one plant and may also include other materials.

Lawsonia inermis (L.) commonly called Henna and belongs to the family of Lythraceae is a well-known antimalarial medicinal plant (Idowu et al., 2010). Its roots, leaves, flowers and seeds are used in traditional medicinal systems for the treatment of various ailments (Chaudhary et al., 2010; Iwu, 1993). Infusion of the dried flowers of *L. inermis* is used to treat malaria in Yemen (Ali et al., 2004). The leaves are also utilized for cosmetic purposes to tint hands, feet, nails and beards (Zumrutdal and Ozaslan, 2012). *L. inermis* possesses many other reported pharmacological properties such as anticancer, antioxidant, antimicrobial, antifungal and antidiabetic (Chowdhury et al., 2014; Rajwar and Khatri, 2013; Semwal et al., 2014; Singh and Luqman, 2014; Suleiman and Mohamed, 2014).

In the same vein, *Chromolaena odorata* (L.), a perennial straggling shrub with simple leaves oppositely arranged, is from Asteraceae family and commonly called Siam weed. It is used as an antimalarial medicinal plant (Nisit et al., 2005; Ukpai and Amaechi, 2012). Other biological properties of *C. odorata* include activities against typhoid fever, cataract, diabetes and bacterial infection (Onkaramurthy et al., 2013; Vital and Rivera, 2009; Zige et al., 2013).

Furthermore, *Tithonia diversifolia* (Hemsl.) A. gray (Asteraceae), commonly called Mexican sunflower/Tree marigold, is a shrub that is distributed throughout tropical and subtropical regions of the world especially America, Asia and Africa. The antimalarial activity of *Tithonia diversifolia* has been reported (Elufioye and Agbedahunsi, 2004; Muganga et al., 2010; Oyewole et al., 2008). Other activities reported for *Tithonia diversifolia* includes antimicrobial, antiemetic, antidiarrhoel and antiamoebic properties (Ahmed and Onocha, 2013; Ezeonwumelu et al., 2012; Obafemi et al., 2006; Tona et al., 1998).

According to the ethnobotanical survey conducted in Omu-Aran, Kwara State, Nigeria by our team, the leaves of *Lawsonia inermis*, *Chromolaena odorata* and *Tithonia diversifolia* are traditionally used singly or in combination for treating malaria. However, the antimalarial activity of these plants in combination has not been elucidated. Hence, this study investigated the antiplasmodial activity of the combination of *L. inermis*, *T. diversifolia* and *C. odorata* using *in vitro* and *in vivo* methods.

2. Materials and methods

2.1. Collection and identification of plant materials

The leaves of *Chromolaena odorata* (L.) R.M. King & H. Rob commonly called Siam weed, *Tithonia diversifolia* (Hemsl.) A. Gray also referred to as Tree Marigold and *Lawsonia inermis* (L.) commonly called Henna, were collected in Eleyin around Omu-Aran, Kwara State, Nigeria. Authentication and identification of the plants were done at Forest Herbarium, Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State, where voucher specimens were deposited. Each plant was assigned a voucher specimen number; *C. odorata* (FNI 108427); *T. diversifolia* (FNI 108428) and *L. inermis* (FNI 108429).

2.2. Extraction of plant materials

The leaves of *Chromolaena odorata, Tithonia diversifolia* and *Lawsonia inermis* were air-dried at room temperature (25 °C) for 2 weeks, after which they were ground to fine powders. The extracts were obtained by cold maceration of 250 g of each plant material in 1000 mL of dichloromethane and methanol (1:1) mixture at room temperature for 24 h. The extracts were filtered and concentrated using rotary evaporator.

2.3. In vitro antiplasmodial bioassay

2.3.1. Parasites

Two strains of *Plasmodium falciparum*: the Sierra Leonean CQ-sensitive (D6) and the Indochinese CQ-resistant (W2); obtained from Malarial laboratory, Centre for Biotechnology and Research Development, Kenya Medical Research Institute, Nairobi, Kenya, were used in the study. Parasite cultivation was carried out using the procedure described by Trager and Jensen (1976).

2.3.2. Test sample reconstitutions

Each extract was dissolved in RPMI diluted DMSO ($<1\%$) to initial concentration of 100 μg/mL (SigmaChemical Co., St Louis, MO, USA). Chloroquine (1 μg/mL) and artemisinin (1 ng/mL) were used as reference drugs. Combined drug solutions were dispensed into 96-well micro-titre plates to give five combinations in ratios of 1:1, 1:3, 3:1, 1:5, and 5:1 (*Lawsonia inermis*: *Tithonia diversifolia*; *Lawsonia inemis: Chromolaena* and *Tithonia diversifolia: Chromolaena odorata*) (Akala et al., 2010). Each combined drug ratio (50 μL) was added in duplicate into wells of row A. Two-fold serial dilutions were done by adding 25 μL of RPMI to row B to H and transferring 25 μL of the drug with a multi-channel micropipette from row A down to row G (last 25 μL from G wells were discarded). Row H wells were exempted since they served as controls (wells without drugs). Thus, rows A, B, C, D, E, F and G wells had concentrations of 100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL, 3.125 μg/mL and 1.5625 μg/mL respectively. Consequently, row A had 100% drug concentration while those of G had 1.5625% of the test samples. The plates were then covered and kept at 4 °C.

2.3.3. In vitro antiplasmodial drug assessment

The semi automated micro-dilution technique was adapted in assessing *in vitro* antiplasmodial activity (Desjardins et al., 1979; Le Bras and Deloron, 1983). This technique measures the ability of the extracts to inhibit the incorporation of [G-^{3}H] hypoxanthine (Amersham International, Burkinghamshire, UK) into the malaria parasite. The test culture which was at ring stage (trophozoite), with percentage parasitaemia (%P) of 4% or more and growth rate (GR) of 3% or more was used for sensitivity tests. The test culture to be added to the wells of predosed plates was adjusted to 0.4% and haematocrit (hct) adjusted to 1.5% with 50% RBC. Culture Medium with Serum (CMS), which consists of RPMI (10.4 g/L), HEPES (5.9 g/L), human serum (15 mL), $NaHCO_3$ (5%) and Gentamycin (10 μg/mL), was added into 25 cm^2 (50 mL) culture flask and the appropriate volume of 50% RBC added. This was flushed with a gas mixture of 3% CO_2, 5% O_2 and 92% N_2 (BOC®, Kenya) and incubated at 37 °C for 5 min, after which 200 μL of the test culture was dispensed into the predosed plate wells exempting wells H_9 to H_{12} where unparasitized red blood cells (UPRBC) and CMS were added to serve as negative control while H_1-H_8 contained parasitized red blood cells with no drug (positive control).

2.3.4. Incubation of the plates, harvesting of cells and scintillation counting

The covered microtitre plates were agitated gently and placed in a gas tight box, which had damp cotton gauze to maintain humid atmosphere in the chamber. The gas box was covered, flushed with 3% CO_2, 5% O_2 and 92% N_2 (BOC®, Kenya) and