

In vivo Antiplasmodial and Insecticidal Activities of *Citrus Limon* (L.) Osbeck (Rutaceae), Leaves Extracts

Bonkian LN^{1*}, Yerbanga RS^{2,3}, Sangare I⁴, Koama B⁴, Soma A¹, Cisse M^{1,4}, Bamba S⁴, Valea I¹, Tinto H⁵, Ouedraogo JB^{2,3} and Traore M²

¹Department of Biomedical Sciences, Centre MURAZ, Bobo Dioulasso, Burkina Faso

²Institut de Recherche en Sciences de la Santé /Direction Régionale de l'Ouest, Bobo Dioulasso, Burkina Faso

³Institut des Sciences et Techniques, INSTech, Bobo Dioulasso, Burkina Faso

⁴Université Nazi BONI, Bobo Dioulasso, Burkina Faso

⁵Clinical Research Unit of Nanoro, Nanoro, Burkina Faso

***Corresponding author:** Bonkian LN, Department of Biomedical Sciences, Centre MURAZ, Bobo Dioulasso, Burkina Faso, Tel: 00226 70965393, E-mail: leabonkian@yahoo.fr

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Abstract

Citrus limon was found after an ethnobotanical survey as used in the treatment of malaria and also for protection against mosquitoes. Aim of this study was to assess the *in vivo* antiplasmodial activity on mice and the insecticidal activity on *Anopheles gambiae* of *Citrus limon* leaves extracts. The acetone extract and the decoction of *Citrus limon* dried leaves powder were used to treat mice infected with *Plasmodium berghei* ANKA. Hexane extract was tested against adult mosquitoes of *Anopheles gambiae*. The reduction of mice parasitemia was 75.8% for the acetone extract and 27.7% for the decoction at the dose of 250 mg/kg body weight. The mean of mortality rate of the hexane extract on mosquitoes was 2.6%. In conclusion, the acetone leaves extract has shown a good antiplasmodial activity on mice in opposit to the decoction. The hexanic extract has displayed a poor insecticidal activity against *Anopheles gambiae* mosquitoes.

Keywords: *Citrus limon*; *Plasmodium berghei* Anka; *Anopheles gambiae*

Introduction

The control of malaria continues to be a challenge worldwide. This situation is due to several reasons: the resistance of the parasite to antimalarial drugs [1], the resistance of the vector, the mosquito of genus *Anopheles* to insecticides [2], accessibility issues and sometimes problems with adherence to Artemisinin-Based Combination Therapy [3]. In developing countries, many people used herbal medicine as primary care. In Burkina Faso, 80% of the rural population use plants to treat themselves [4]. Surveys showed that various plants species are used to treat malaria and also against mosquitoes [5,6]. The experimental study of some of certain plants has proved their effectiveness. Alkaloids providing from *Guiera senegalensis* J.F. Gmel (*Combretaceae*), *Pavetta crassipes* (K. Schum) and *Acanthospermum hispidum* (DC) demonstrated a good antiplasmodial activities [7,8]. The good mosquito repellency activity of essential oils of two plants belonged to *Lamiaceae* family, *Hytis suaveolens* (L) and *Hyptis spicigera* (Lam) have been successfully assessed on human volunteer [5]. An ethnobotanical survey of traditional healers from the Sahelian region of Burkina Faso identified *Citrus limon* as a major part of the recipes used for curative treatment of malaria and also for preventive treatment against mosquitoes [6]. *Citrus limon*, commonly called lemon tree, is a plant belonging to the *Rutaceae* family. This is a thorny evergreen tree, reaching 3 to 6 meters of height. It is widely cultivated in tropical countries for its fruits which are used in food industry, in beverages, in pharmaceutical industry, in perfumery and cosmetics [9]. All parts of this tree are used to treat various diseases. In this study, we investigated the antiplasmodial activity of the leaves extracts of *Citrus limon* on NMRI (Naval Medical Research Institute) mice infected with *Plasmodium berghei* and tested the adulticidal activity of the plant against *Anopheles gambiae*.

Material and Methods

Plant material

Citrus limon leaves were used. They were harvested in Djibo, in the Sahelian Region of Burkina Faso at the beginning of the raining season, in July. The plant material was identified by an experienced botanical technician from "Institut de Recherche en Sciences

de la Santé/ Direction Régionale de l'Ouest, Bobo-Dioulasso" (IRSS/ DRO) , and voucher specimen CL2017 leaves have been deposited in the Unit of Pharmacognosy in the institute. The leaves collected were cleaned and dried at ambient temperature in the shade, then grounded. The powder was stored in dried containers until preparation of the extract.

Preparation of the extracts

Three kinds of extract were prepared: decoction, hexane and acetone extracts.

- Hexane extract was prepared by macerating 100g of leaves powder in 1000 ml of hexane for 24 hours. The extract was filtered and the solvent evaporated in a rotary evaporator, then the extract was stored in dark bottles at 4 °C until use.
- Acetone extract was performed using the mark resulting from extraction with hexane; 100 g of mark was macerated in 1000ml of acetone for 24 hours. The extract was filtered and dried in a rotary evaporator, then extract was stored in a dark bottle at 4 °C until use.
- For the decoction, 100g of powder were boiled into 1000 ml of distilled water for 30 minutes. Then extract was dried by lyophilization and stored at 4 °C in dark bottles until use.

In vivo antiplasmodial activity

The evaluation of the antiplasmodial activity was performed according to Peter's 4-days suppressive test [10].

- Animals used for the *in vivo* test were NMRI (Naval Medical Research Institute) female mice from 7 to 8 weeks old and weighing between 25 and 30 g. Mice were bred in "Centre International pour la Recherche-développement pour l'Élevage en zone Sub-humide" (CIRDES). They were then transferred to IRSS/DRO where experiments were conducted. The mice were acclimatized during 2 days at 25 °C ± 2 °C with a 12 hours photoperiod and fed with standard food and water. At the end of the acclimation period, the mice were weighed and marked to differentiate them. They were divided into group of six mice, each mouse has been identified by indelible painting on different parts of his body and kept per cage. 8 groups of 6 mice (48 mice in total) were used for the test: 4 groups for the acetone extract and 4 groups for the decoction. Each mouse was inoculated intraperitoneally with 10⁷ *Plasmodium berghei* infected red blood cells two hours before starting the treatment with the extracts.
- Parasites used were chloroquine sensitive *Plasmodium berghei* Anka strain (MRA-311, MR4, ATCC Manassas Virginia).
- Preparation and administration of the treatment: an extemporaneous preparation of the treatment doses were performed just before administration. Solvent used to dilute the extract was administrated to the control group. Acetone extract was weighed and diluted into a solution of 5% of tween 80 using distilled water. For the decoction, only distilled water was used. The doses of treatment administrated to each group of mice were 100, 250 and 500mg/kg body weight once at the same time every day during 4 days for both extracts.

The following day after the last treatment, thin blood smears were made with the blood drawn from the tail of each mouse. Slides were stained with 10% Giemsa and read 3 times under an optical microscope at 100X magnification and the parasitemia calculated. The percentage of parasitemia was estimated using the following formula:

$$\% \text{Parasitemia} = \frac{\text{(Number of infected Reds blood cells)}}{\text{Total Number of Reds blood cells}} \times 100$$

Activity of the extract was determined by calculating the percentage of reduction of the parasitemia of treated group compared to the negative control group as follow:

$$\% \text{ reduction} = \frac{(\% \text{ parasitemia of Negative control} - \% \text{ parasitemia of Treated group})}{\% \text{ parasitemia of Negative control}} \times 100$$

Insecticidal anti-mostiquo activity

The test was performed in the insectarium of IRSS / Centre MURAZ in Bobo Dioulasso (Burkina Faso).

- Mosquitoes used were females of 3days old "Kisumu" reference strains of *Anopheles gambiae* 100% susceptible to all chemical class of insecticides maintained in the insectarium of IRSS / Centre MURAZ.
- The product tested was the hexane extract of the leaves of *Citrus limon*. A solution of 50% mass / volume of extract prepared by dilution of the extract in a mixture of acetone (1volume) and silicone (1/2 volume) were prepared for the impregnation of Wattman paper (15cm*12cm). Each paper was impregnated with 2 ml of the solution and dried at room temperature. The control paper was impregnated with 2ml of solvent. The papers were then wrapped in aluminum foil and kept in the fridge until use.
- The modified harmonized tube test procedure of World health organization [11] was used for the assessment of insecticidal activity. Three (3) groups of 25 adult mosquitoes were exposed 1hour to 3 papers impregnated with extract and placed in 3 tubes. For control group, 25 mosquitoes were exposed to the control paper placed in a tube. During the exposure the knock down was determined every 5 minutes. After 1 hour of exposure, the mosquitoes were transferred into observation tubes and fed with 5% glucose juice for 24 hours. The laboratory temperature was 25 ± 2 °C and the relative humidity was 80 ± 10%. The mortality rate was evaluated after 24 hours and the results were interpreted according to the WHO criteria.

Data analysis

All data were entered and analyzed with Excel 2013 software and Epi Info 6.04.

► The *In vivo* antiplasmodial activity results were expressed in mean parasitemia \pm standard deviation and the reduction of parasitemia was calculated and expressed in percentage (%). The student test was used to compare the results and a p value < 0.05 was considered as statistically significant. The *in vivo* antiplasmodial activity was classified as:

- Moderate, when the percentage of reduction of parasitemia was $\geq 50\%$ at the dose of 500 mg extract/kg body weight.
- Good when the percentage of reduction of parasitemia was $\geq 50\%$ at the dose 250 mg extract/kg body weight
- Very good when the percentage of reduction of parasitemia was $\geq 50\%$ at the dose of 100 mg extract/kg body weight [12].

► Interpretation of the insecticidal anti-mosquito activity

The mortality rate of the mosquitoes was calculated as following:

- Mortality in the control tube: $C = (\text{number of dead mosquitoes}) \times 100 / (\text{number of mosquitoes tested})$ in the control tube.
- Mortality in exposure tubes: $E = (\text{number of dead mosquitoes}) \times 100 / (\text{number of mosquitoes tested})$ in the exposure tubes.
- If the mortality rate in the control tube is between 5 and 20%, the mortality rate in exposure; E, is corrected by the Abbott formula:
- Corrected mortality rate: $E' = [(E-C) / (100-C)] \times 100$ where E is the mortality following exposure (uncorrected) and C is the mortality of the control group both expressed in %. The interpretation of the anti-mosquito tests results is as follows:

A mortality rate between 98-100% indicates susceptibility.

A mortality of less than 98% is suggestive of the existence of resistance and further investigation was needed.

The entomological parameters were compared by using the chi-square test and a p value < 0.05 was considered as statistically significant.

Ethical considerations

The ethic review board of "Centre MURAZ" approved the study protocol (ref: A 005-2014/CE-CM). Animals were used with regard to the « International Organization for Animal Care » guidelines (Directive 86/609/EEC).

Results

In vivo antiplasmodial activity

The results of the antiplasmodial activity are presented in Table 1.

Doses (mg/kg)	Acetone extract			Decoction		
	Mean of parasitemia (%)	% reduction	IC95	Mean of parasitemia (%)	% reduction	IC95
Control	39.7 \pm 1.5	0	8.5	27.7 \pm 6.9	0	4.3
100	20 \pm 5.4	49.6	4.2	33.2 \pm 2	NA	7.4
250	9.6 \pm 4.2	75.8	14.3	20 \pm 12.8	27.7	6.6
500	18.4 \pm 3.4	53.6	11.3	23.8 \pm 13.6	14	8.5

Table 1: *In vivo* antiplasmodial activity of *Citrus limon* leaves extracts

The mean value of the parasitemia for animals treated with the acetone extract was different from that of the control group ($p < 0.05$). The percentage reduction of parasitemia was similar at the dose of 100 and 500 mg/kg body weight ($p = 0.58$). A good activity was obtained at 250 mg/kg body weight. This activity was significantly higher than those displayed at 100 ($p = 0.005$) and 500 ($p = 0.003$) mg/kg body weight.

No difference was seen between the activity of the control and the tested groups ($p > 0.05$) with the decocted extract, the extract was unable to inhibit the growth of *Plasmodium berghei* in mice.

Insecticidal activity

There was no death of mosquito in the control tube: $C = 0\%$. The mean mortality of mosquitoes exposed to the extract was 2.6% at 50% of extract. The results are presented in Table 2.

Experiment series	Number tested	% Mortality
Control	25	0
1	25	0
2	25	0
3	25	8
Total	100	8

Table 2: Mosquito mortality rates in the exposure tube

Discussion

The study evaluated the *in vivo* antiplasmodial and anti-mosquito activities of *Citrus limon* leaves extracts. Extract has been used to treat mice infected with *Plasmodium berghei* Anka, a chloroquine sensitive rodent malaria parasite. Reference drug was not used in the study because the aims of the study were to assess the activity of the extract on the parasite development. The experiment on mice showed that the acetone leaves extract of *Citrus limon* had a good activity against *Plasmodium berghei* Anka (75.8% of reduction in parasitemia) while the leaves decoction was inactive. The results showed that acetone, compared to water, is a good solvent for extracting secondary metabolites from plants. However, the decoction is the form commonly used by traditional healers per os and by bath [6]. The decoction is heated to 100 °C that conducts to the evaporation of the essential oil; this may partly justify the inactivity of the decoction of *Citrus limon* leaves. The major components of leaf oil were limonene, linalool, pinene, γ -Terpinene, sabinene, geranial, 1,8-cineole, neral, linalyl acetate, ocimene, R-terpineol and a few sesquiterpenes [9]. Additional phytochemical analysis may be needed to identify the active element of the acetone extract. Studies assert the use of this plant in the treatment of malaria [13] but to our knowledge, this study is the first one that investigated the *in vivo* antimalarial activity of the leaves extract of *Citrus limon* on mice.

The assessment of the insecticidal activity of *Citrus limon* leaves extract showed a resistance of *Anopheles gambiae*. Previous studies had shown that *Anopheles gambiae* was resistant to *Citrus limon* fruits peels' essential oils [14]. On the other hand, the strong larvicidal activity of *Citrus limon* seeds extracts on *Aedes albopictus* has been demonstrated [15]. The population of the Sahelian region of Burkina Faso used the zests by fumigation against mosquitoes [6].

The efficacy and toxicity of fumigated zests can be evaluated in a real-life situation.

Conclusion

Citrus limon is a promising plant for research on antimalarial drug. A bio guided fractionating must be performed to identify the compounds responsible of the antiplasmodial activity of the extracts. The hexane leaves extract show no insecticidal activity. However research with different extracts on different stages of the mosquito could yield interesting results.

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