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Improvement of Secondary Metabolites from *Phyllanthus odontadenius* against Malaria by Mutagenesis

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Authors' contributions

This work was carried out in collaboration between all authors. Author SLN performed the final protocol, read the first draft of manuscript and managed the analysis of the study. Author RKN designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches. Authors VS and FR performed the antimalarial studies by micro dilution isotopic method and ELISA assays. Authors CF and RCK read the final draft. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Majority of deaths in children aged under 5 years are due to *Plasmodium falciparum* malaria. Malaria deaths in children decreased but malaria remains a major killer of children, taking the life of a child every 2 minutes. This study aims to investigate the increasing of the *in vitro* antiplasmodial activities by mutagenesis techniques using gamma-rays (Cs-137) or sodium azide (NaN_3) as mutagens. It will allow the importance of mutagenesis use as tools for improvement of secondary metabolites against malaria parasites using chemical or physical mutagens.

Study Design: Laboratory experiment tests : identification of plant material, immersion of seeds in SA (sodium azide) solutions or irradiation by Gamma-rays (Cs-137) of *P. odontadenius* seeds for improvement of secondary metabolites against malaria parasites, *in vitro* culture of seeds followed by the *in situ* culturing of plantlets for obtaining material of study, phytochemical screening of *Phyllanthus odontadenius* aerial parts to determine the change of compounds in comparison to controls, *in vitro* antiplasmodial tests for the determination of SA concentrations or those of gamma-rays doses which killing 50% of malaria parasite populations (IC_{50}).

Place and Duration of Study: Department of Biotechnology and Molecular Biology, Department of Biochemistry: General Atomic Energy Commission, Regional Nuclear Studies Center of Kinshasa, P.O. Box. 868 Kinshasa XI, Democratic Republic of the Congo (DRC). National Institute of Biomedical Research (NIBR) at Kinshasa/Gombe (DRC). Laboratory of UMR-MD3, Aix-Marseille University, Faculty of Pharmacy/Marseille, France. The experiments were conducted from Junuary - December 2010; August and December 2011 and during May and September 2012 until July 2014.

Methodology: Aerial parts of plants M1 and M2 from Gamma-rays irradiation of *P. odontadenius* seeds or from immersion of *P. odontadenius* seeds in sodium azide solutions used as biological material for the in vitro antiplasmodial analysis. The *in vitro* antiplasmodial activities assays on clinical isolates of *P. falciparum* and on chloroquine-resistante *P. falciparum* strain K1 was determined using microscopic method, the isotopic micro-test method and using HRP2-based ELISA assay.

Results: Gamma-rays (Cs-137) increased (multiplied) the *in vitro* antiplasmodial activities from 2.48 up to 7.6 in comparison to control. Thus, the *in vitro* antiplasmodial activities were improved or exceeded from 147.57% up to 660% than those of control plant. SA had increased (multiplied) the *in vitro* antiplasmodial activities from 1.24 up to 10.15 comparing to the control plants. The antiplasmodial activities in vitro were exceeded compared to the control plants from 24.43% up to 915%.

Conclusion: Treatment of *P. odontadenius* seeds by Gamma-rays or by SA give plants high *in vitro* antiplasmodial activities. Values of *in vitro* antiplasmodial activities varried from 1.24 (147.57%) to 10.15 (915%). 125, 150 and 225 Gy of Gamma-rays (Cs-137) for physical mutagenesis and 10.15 and 17.5 mM of SA solutions for the chemical mutagenesis could be used for improving *in vitro* antiplasmodial activities against *P. falciparum* (clinical isolates of *P. falciparum* or chloroquine-resistant *P. falciparum* strain K1). Thus, plants extracts from treated seeds have justified the usefulness of mutagens in plant breeding particularly in the increasing production of secondary metabolite against malaria parasite.

Keywords: Mutagenesis; gamma rays; sodium azide; secondary metabolites; Phyllanthus odontadenius; malaria disease, antiplasmodial in vitro.

1. INTRODUCTION

Malaria is an erythrosis or erythropathy transmitted from one human to another by infected female Anopheles mosquito bites, called "malaria vectors", which bite mainly between dusk and early morning [1,2]. Malaria is responsible for major socio-economic disturbances in all subtropical and tropical countries where it is endemic [3,4,5].

Malaria is the most important parasitic disease in tropical areas. In 2016, an estimated 216 million cases of malaria occurred worldwide thus 90% for the WHO African Region, 7% for the WHO South-East Asia Region and 2% for the WHO Eastern Mediterranean Region [6].

Most deaths caused by malaria in 2015 are estimated to have occurred in the WHO African Region at 92%, at 6% for the WHO South-East Asia Region and with 2% the WHO Eastern

Mediterranean Region [7]. The vast majority of deaths (99%) are due to *Plasmodium falciparum* malaria with 70% of the global total to have occurred in children aged under 5 years. Number of malaria deaths in children is estimated to have decreased by 29% since 2010, but malaria remains a major killer of children, taking the life of a child every 2 minutes [7].

There are currently no registered vaccines against malaria or any other human parasite. An experimental vaccine against *P. falciparum*, RTS, S/AS01, is the most advanced and currently evaluated in a large clinical trial in some African countries [2]. The DRC is among the 34 of the 41 high-burden countries with the highest prevalence of malaria, which had obtained, in the past 3 years (2014 – 2016), the increases in external funding for their malaria control program [6]. The spread of muti-drug resistant *P. falciparum* such as those of ARTEMISININ [6] in addition of that of chloroquine has highlighted the urgent need to develop to find new medicinal principles with different mechanisms of action, preferably inexpensive drugs that are affordable for developing countries where malaria is prevalent.

Vegetable resources have already proved their worth, as they are source of discovery of two major antimalarial drugs: quinine from *Cinchona* sp (Cinchona sp) and artemisinin from *Artemisia annua*. These two natural products have served as the basis for the hemisynthesis of many major antimalarial drugs [3]. Secondary metabolites produced by plants through the secondary metabolism pathways are often the keystone in interactions between plants and their environment. Traditional medicine using plant extracts continues to provide health coverage for over 80% of world's population, especially in developing world, but also in modern allopathic medicine through use of purified or derived components obtained from chemical hemi-synthesis [5,7,8,9].

African tropical forests are rich in plant genetic resources [10,11]. However, deforestation of many forest ecosystems for crop field needs, firewood, construction, works of art, etc. and domestication problems of most plant species lead to the reduction in biodiversity and consequently to plant protection potential of these ecosystems which are felt to be inestimable losses [12].

Plants of Phyllanthaceae family Martinov (2009) are used in DRC and in several regions of the world for treatment of several diseases [13,14, 15,16]. The *in vitro* antiplasmodial activity of *Phyllanthus* specimens varies according to the regions where the plant was harvested and according to the Harvest period, and does not allow users to use it appropriately for various virtues [16,17,18,19].

Plant biotechnology offers opportunities in plant breeding [20]. The search for new crop varieties have such as base on obtaining plant varieties with agronomic or sometimes pharmaceutical characteristics through use of mutagens [21,22]. These mutagens are likely to modify the biochemical or physiological mechanism of plant in a random or targeted way. These mutagens could also allow the accumulation of adaptation of genes under different conditions [23,24].

This study aimed to investigate improvement of secondary metabolites of *P. odontadenius* against malaria through *in vitro* antiplasmodial activity of some *Plasmodium falciparum* strains using some mutagens such as gamma rays and sodium azide (SA).

2. MATERIALS AND METHODS

2.1 Plant Materials

Plant extracts of *Phyllanthus odontadenius* from irradiated seeds by gamma rays (Cs-137) or from seeds dipped in Sodium Azide (SA) solutions and cultured in fields were used as biological materials. Physical and chemical Mutagenic techniques were used for gamma irradiation of *P. odontadenius* seeds or those of soaked seeds in SA resumption respectively in [25,26,27] and [28].

For the physical mutagenesis, seeds of *P. odontadenius* obtained from drying fruits harvested on the Kinshasa university site were irradiated with gamma rays from Cesium-137 (Cs-137) source in the Conservatome Lisa I Irradiator at the Department of Biochemistry, General Atomic Energy Commission (GAEC). The dose rate was 1.21 Gy/min [29,30].

For the chemical treatment, 100 seeds were placed in the Eppendorf microtubes (1.5 mL) and then imbibed in sterilized water for 1 h with agitation on shaker. The stock solution of sodium azide (Merck) was prepared in phosphate buffer (1 M), pH 3, filtered and stored frozen it at -20°C. Stock solution was diluted successively in water as well as in phosphate buffer of pH 3 to obtain

various concentrations for the treatment of seeds. Distilled water removed and seeds were kept under various concentrations of sodium azide for 2 h 30 of time with continuous agitation on shaker at room temperature (25°C±2). Immediately after treatment of sodium azide, seeds were washed thoroughly in distilled water to reduce residual effect of sodium azide on the seed coat during 4-5 times. A portion of seeds were submerged in deionized water for the same period of time served as control.

The first generation of irradiated seeds or dipped seeds thus as plants obtained were designed as M1.

2.2 *Plasmodium falciparum* Strains and Methods

2.2.1 Clinical isolates of *P. falciparum*

Stock extract solutions for M1 and M2 extracts were prepared in 1% DMSO and diluted in two fold to have test concentration. Clinical isolates of *P. falciparum* (CI*Pf*) were obtained from symptomatic malaria children (0-5 years) with high parasitaemia and which did not receive antimalarial treatment in the three weeks preceding the diagnosis at Maternity Hospital of the Sisters of Kindele, Mont-Ngafula, Kinshasa. Venous blood samples were collected in tubes containing 1% heparin and transported in carboglass until INRB laboratory. 4ml of venous blood were centrifuged for 5 min at 3000 rpm to separate the plasma and the erythrocytes. 1ml of erythrocytes were mixed with 9 ml of RPMI 1640 containing 25mM HEPES, 25mM sodium. In addition, antimalarial activity assays were performed by microscopic techniques adapting [31] method at the National Institute of Biomedical Research (NIBR) in Kinshasa/ Gombe, DR. Congo [27,32,33,34].

2.2.2 *Plasmodium falciparum* chloroquine-resistant strain K1

The chloroquine-resistant (IC_{50} = 1010±155nM) *P. falciparum* (CR*Pf*) strain K1 (Thailand, MR4-ATCC) was tested using firstly the isotopic micro-test method. For that, the parasite was cultured according to [31]. Parasite culture was performed in RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES, 50 mg/l hypoxanthine, 25mM $NaHCO_3$, 20 mg/l gentamicin and 10% human serum at 37ºC and in a standard gas mixture consisting in 10% O_2, 5% CO_2 and 85% N_2. Parasites were synchronized with sorbitol before use [35].

The *in vitro* susceptibility to extracts was determined by measurement of [^{3}H]-hypoxanthine incorporation into parasite nucleic acids using the method of [36,37].

Secondly, the CR*Pf* strain K1 (Thailand, MR4-ATCC) (IC_{50} = 1156.50 ± 190.42 nM) was tested using homemade HRP2-based ELISA assay. The study was conducted by UMR-MD3, Aix-Marseille University/Marseille, France. *P. falciparum* strain K1 was cultured *in vitro* in RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES, 50 mg/l hypoxanthine, 25 mM NaHCO3, 20 mg/l gentamycin and 10% human serum (complete medium), at 37°C and under an atmosphere containing 5% CO2, 10% O2 and 85% N2 [31]. Parasites were cultured (0.5% parasitemia; 1.5% hematocrit) in the presence of increasing concentrations of extracts and chloroquine (used as reference drug) in 96-well plates (final volume: 200 µl/well). The revelation of parasite growth was realized using a homemade HRP2-based ELISA assay.

Antimalarial activity of the CR*Pf* strain K1 was determined as concentration of drugs inducing 50% of growth inhibition (IC_{50}) by nonlinear regression analysis from the dose-response relationship as fitted by software - ICEstimator 1.2 (http://www.antimalarialicestimator.net) [38].

2.3 Determination of the Increasing of *In vitro* Antiplasmodial Activity

The comparison of *In vitro* antiplasmodial activities from plant extracts (report) was released between control plant extracts (CPE) against treated plant extracts (TPE) by the follow relationship:

$\boldsymbol{R}_{PE}$= Average of $IC_{50\,(CPE)}$/Average of $IC_{50\,(TPE)}$

This relationship could be translate in percentage by the last formula from [39]:

$\boldsymbol{R}_{PE}$ (%) = [(Average of $IC50_{CPE}$/Average of IC_{50TPE}) x100] – 100]

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Control *In vitro* antiplasmodial activity

The averages of in vitro antiplasmodial activities from control plant extracts realized with three trials were presented in Tables 1 and 2.

Table 1. Averages of *in vitro* antiplasmodial activity from control plant extracts using gamma-rays as physical mutagen

Strain of *P. falciparum*	IC_{50} (µg/ml) in M1	IC_{50} (µg/ml) in M2
Is*Pf*	7.60±0.00	5.30±4.24
*Pf*K1	1.12±0.55	9.68±2.21

Legend: IsPf: Isolate of Plasmodium falciparum; PfK1: chloroquine-resistant Plasmodium falciparum strain K1; M1: First generation of plants from irradiated seeds; M2: Second generation of plants from irradiated seeds

Table 1 show that the IC_{50} of M1 plant extracts was high (7.60±0.0 µg/ml: low *in vitro* antiplasmodial activity) on Is*Pf* in comparison to M2 plant extracts which presented low IC_{50} (5.30±4.24 µg/ml) but high *in vitro* antiplasmodial activity. For *Pf*K1, M1 plant extracts presented low IC_{50} (1.12±0.55 µg/ml) but high *in vitro* antiplasmodial activity in comparison to M2 plant extracts which were showed high IC_{50} (9.68±2.21 µg/ml) but low *in vitro* antiplasmodial activity.

3.1.2 Increasing of *in vitro* antiplasmodial activities of plant extracts by irradiation (Cs-137) of *Phyllanthus odontadenius* seeds

1° In vitro antiplasmodial activity of Extracts from treated plants on Isolate of Plasmodium falciparum from human blood.

Fig. 1 illustrates the increasing *In vitro* antiplasmodial activity between the aqueous extracts from M1 and M2 plants obtained by gamma-irradiated seeds against control plant extracts on isolate of *Plasmodium falciparum*.

With regard to Fig. 1, it turns out that gamma radiation increased *in vitro* antiplasmodial activity up to 7.6 (Fig. 1a) at the dose of 150 Gy in M1. This *in vitro* antiplasmodial activity is enhanced up to 660% (Fig. 1b) than of the control. In M2, it increased up to 5.3 (Fig. 1c) times compared to the control; this *in vitro* antiplasmodial activity was improved by 430% (Fig. 1d) compared to the control. 125 and 150 Gy are the two doses of gamma irradiation that showed very high values compared to the control.

2° In vitro antiplasmodial activity of extracts from treated plants on CRPf strain K1.

Fig. 2 illustrates the increasing *In vitro* antiplasmodial activity between the aqueous extracts from M1 and M2 plants obtained by gamma-irradiated seeds against control plant extracts on the CR*Pf* strain K1.

In contrast to the effect of *Phyllanthus odontadenius* extracts from treated plants on *P. falciparum* isolates in both M1 and M2, these extracts did not show an increase in antiplasmodial activity *in vitro* on CR*Pf* strain K1 in M1 (Fig. 2). It is in M2 where there is an increase of *in vitro* antiplasmodial activity from doses from150 to 225 Gy compared to the control. Plant extracts at 225 Gy increased the *in vitro* antiplasmodial activity up to 2.48 (Fig. 2c) compared with the control and improves this activity by 147.57% (Fig. 2d).

3.1.3 Increasing of *In Vitro* Antiplasmodial activities through chemical mutagenesis by Immersed seeds of *Phyllanthus odontadenius* in Sodium Azide (NaN_3) solutions

1° In vitro antiplasmodial activity of Extracts from treated plants (M1 and M2) on Isolate of Plasmodium falciparum from human blood.

Table 2. Averages of *in vitro* antiplasmodial activity from control plant extracts using Sodium Azide as chemical mutagen

Strains / IC_{50}(µg/ml)	Is*Pf*			*Pf*K1 strain		
	M1	M2.1	M2.2	M1	M2.1	M2.2
Values of *in vitro* antiplasmodial	14.91±3.85	41.53±3.67	20.95±8.30	1.38±0.12	3.82±0.43	11.98±0.98

Legend: IsPf: Isolate of Plasmodium falciparum; PfK1: Plasmodium falciparum chloroquine-resistant strain K1; M1: First generation of plants from immersed seeds in Sodium Azide solutions; M2.1: Second generation of plants from immersed seeds in Sodium Azide solutions field I; M2.2: The second generation of plants from immersed seeds in Sodium Azide solutions field II

Table 2 show that M1 plant extracts presented low IC_{50} (14.91±3.85 µg/ml) but high *in vitro* antiplasmodial activity in comparison to M2 plant extracts which presented respectively 41.53±3.67 µg/ml (M2.1) and 20.95±8.30 µg/ml (M2.2) as IC_{50} on Is*Pf*. On *Pf*K1 strain, M1 plant extracts showed low IC_{50} (1.38±0.12 µg/ml) but high *in vitro* antiplasmodial activity in comparison to M2 plant extracts (IC_{50} = 3.82±0.43 µg/ml for M2.1 and IC_{50} = 11.98±0.98 µg/ml for M2.2) which presented low *in vitro* antiplasmodial activity.

Fig. 3 illustrates the increasing *in vitro* antiplasmodial activity between the aqueous extracts from M1 and M2 plants obtained by immersion of *P. odontadenius* seeds in SA solutions against control plant extracts on isolate of *Plasmodium falciparum*.

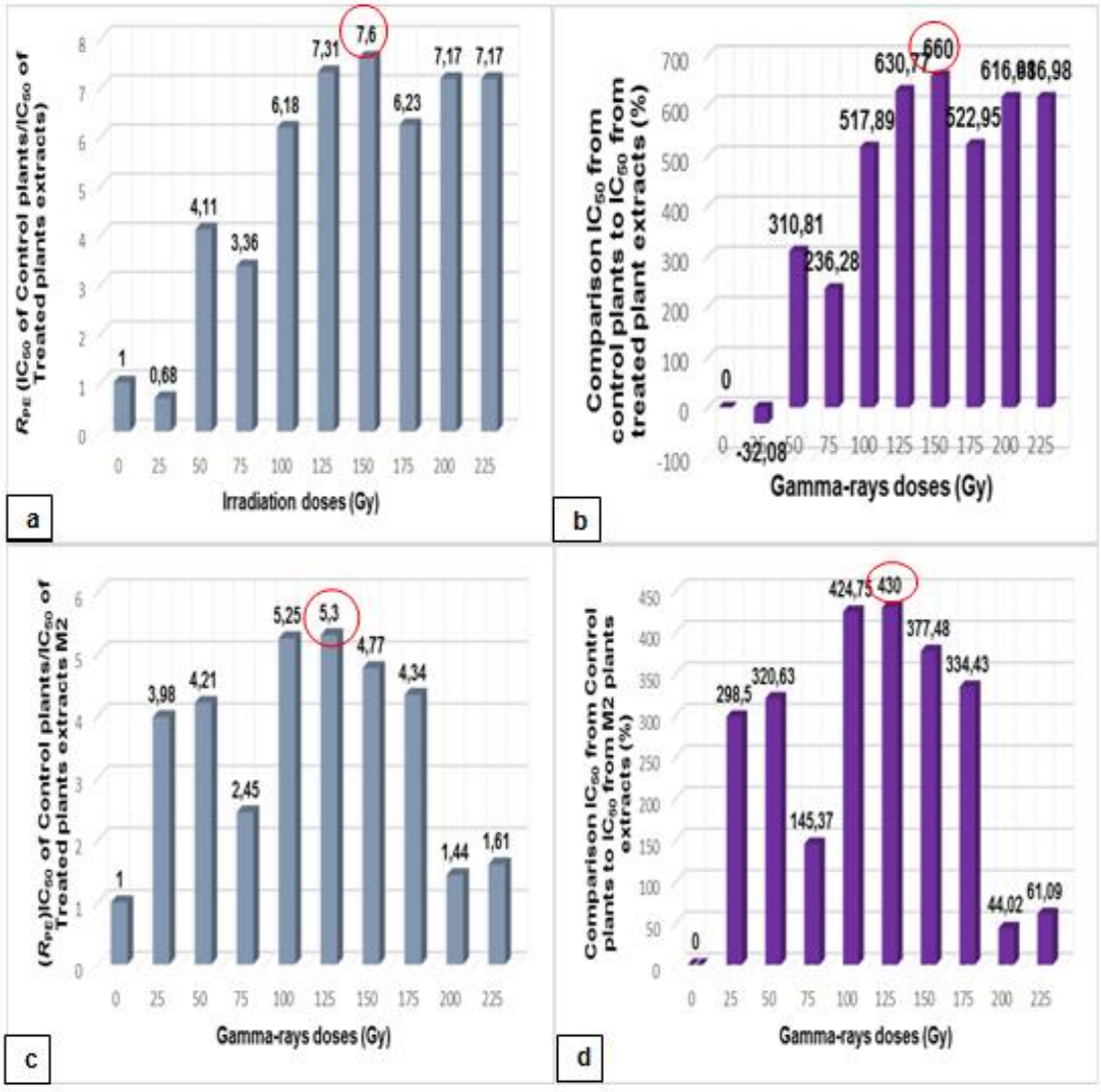

Fig. 1. *In vitro* antimalarial activity of *P. odontadenius* plant extracts obtained from gamma-irradiated seeds (Cs-137) on clinical isolates of *P. falciparum*. a: ratio between IC_{50} values (µg/ml) obtained from irradiated-plant extracts with that of control plant extracts in M1; b: The increasing of *in vitro* antimalarial activity in % compared to control in M1; c: ratio between IC_{50} values (µg/ml) obtained from irradiated-plant extracts with that of control plant extracts in M2; d: The increasing of *in vitro* antimalarial activity in % compared to control in M2

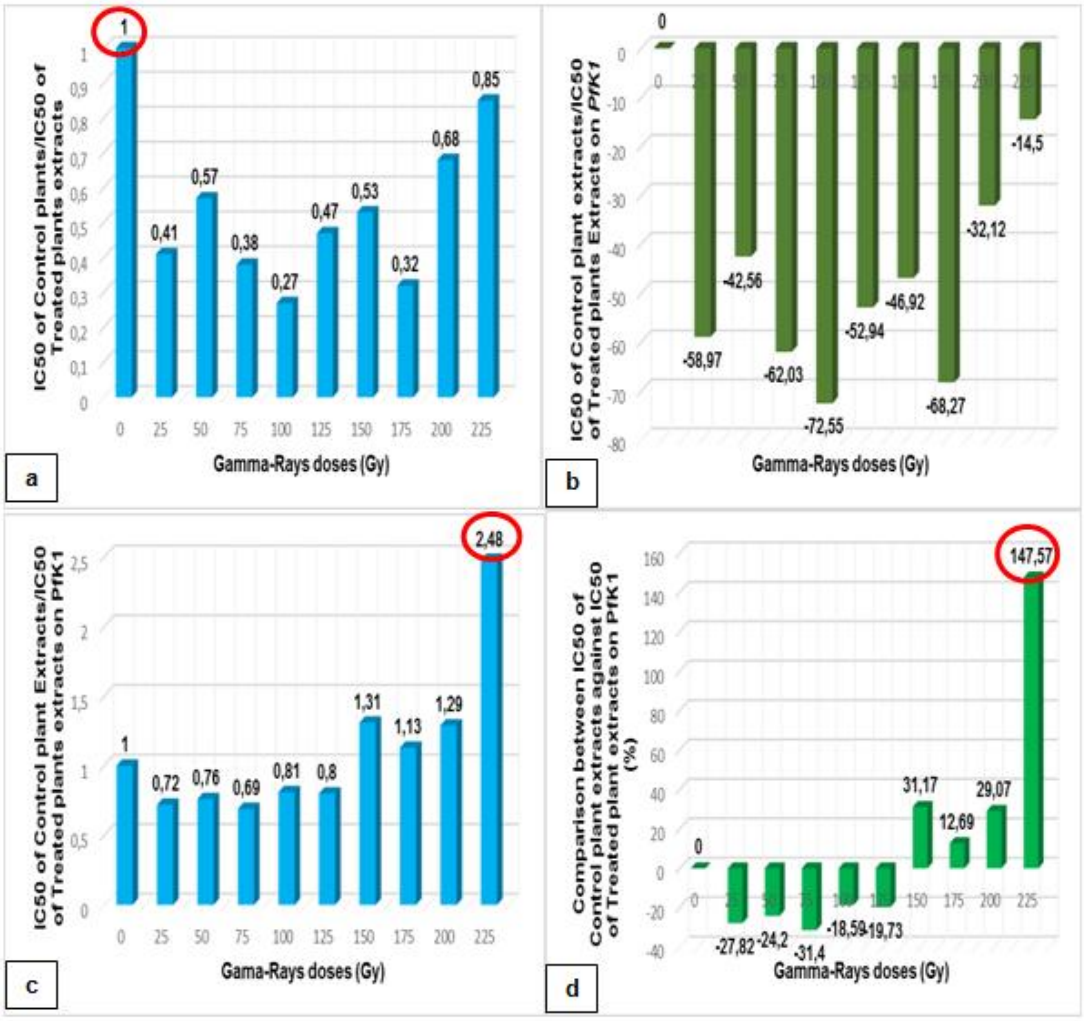

Fig. 2. Increasing of *in vitro* antimalarial activity from plant extracts of *P. odontadenius* obtained by gamma-irradiation seeds (Cs-137) on chloroquine-resistant *Plasmodium plasmodium* strain K1. a: Comparison of IC_{50} (µg/ml) between treated plant extracts and control plant extracts in M1; b: Improvement of *in vitro* antimalarial activity in % compared to control in M1; c: Comparison of IC_{50} (µg/ml) between treated plant extracts and control plant extracts in M2; d: Improvement of *in vitro* antimalarial activity in % compared to control in M2

With regard to Fig. 3, it turns out that SA increased *in vitro* antiplasmodial activity up to 10.15 (Fig. 3a) at the concentration of 10 mM in M1. This *in vitro* antiplasmodial activity is enhanced up to 915% (Fig. 3b) than of the control. In M2.1, it increased up to 6.39 times at the concentration of SA of 17.5 mM (Fig. 3c); this *in vitro* antiplasmodial activity was improved by 538.92% (Fig. 3d) compared to the control. Regarding M2.2, the *in vitro* antiplasmodial activity increased up to 9.11 (Fig. 3e) and SA enhanced up to 810.87% (Fig. 3f) the *in vitro* antiplasmodial activity than of the control. The concentration of 10 mM of SA showed in general the best *in vitro* antiplasmodial activities compared to all excepted 17.5 mM of SA in M2.1 and M2.2.

2° In vitro antiplasmodial activity of extracts from treated plants on Chloroquine-Resistant Plasmodium falciparum strain K1.

Fig. 4 illustrates the increasing of *in vitro* antiplasmodial activity between the aqueous extracts from M1 and M2 plants obtained by immersion of *P. odontadenius* seeds in SA solutions against control plant extracts on the CR*Pf* strain K1.

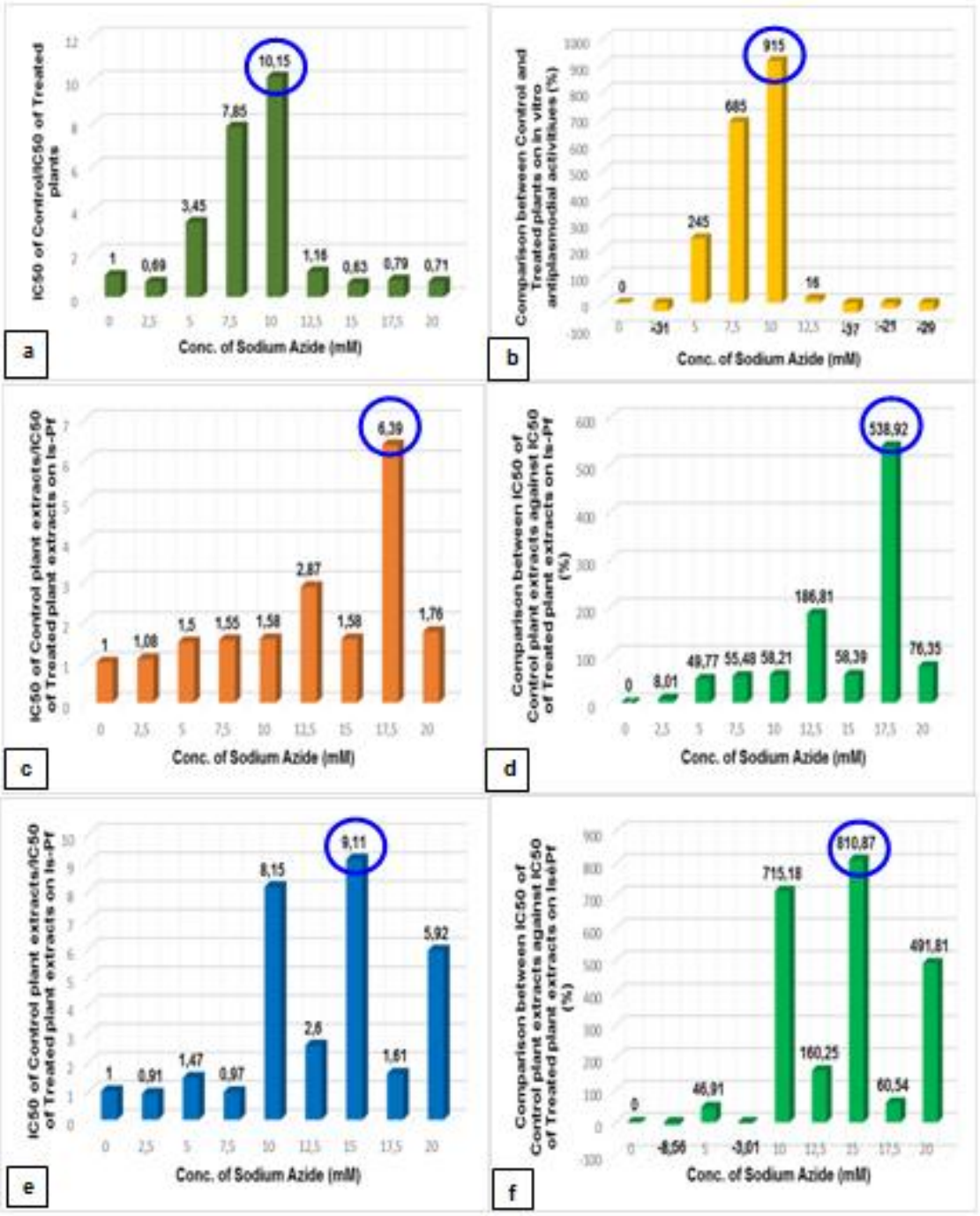

Fig. 3. *In vitro* antimalarial activity from plant extracts of *P. odontadenius* obtained by immersion of seeds in SA solutions on isolates of *P. plasmodium*. a: Comparison of IC_{50} (µg/ml) between treated plant extracts and control plant extracts in M1; b: Improvement of *in vitro* antimalarial activity in % compared to control in M1; c: Comparison of IC_{50} (µg/ml) between treated plant extracts and control plant extracts in M2.1; d: Improvement of *in vitro* antimalarial activity in % compared to control in M2.1; e: Comparison of IC_{50} (µg/ml) between treated plant extracts and control plant extracts in M2.2; 5.3f: : Improvement of *in vitro* antimalarial activity in % compared to control in M2.2

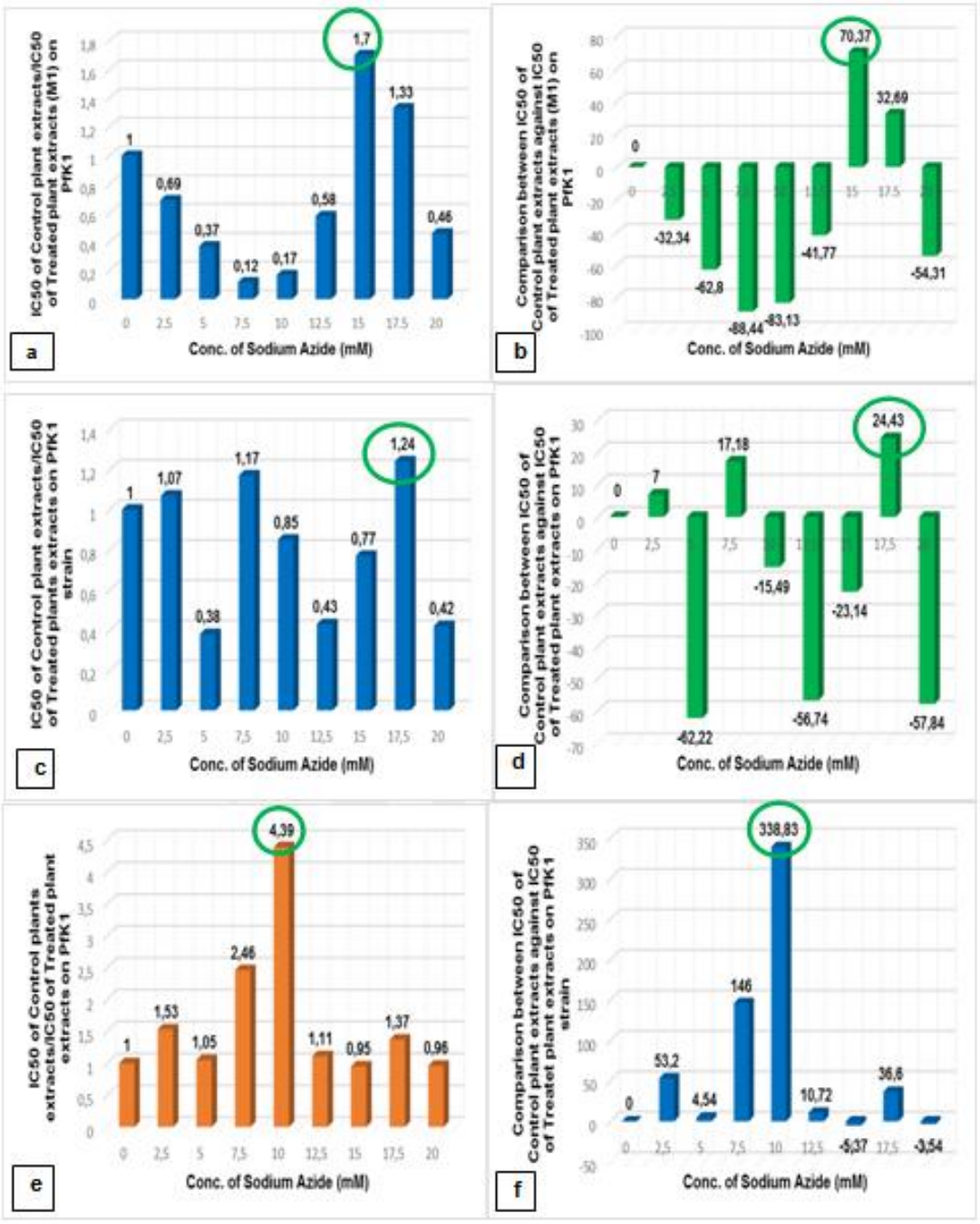

Fig. 4. Increasing of *in vitro* antimalarial activity from plant extracts of *P. odontadenius* obtained by immersion of seeds in SA solutions on chloroquine-resistante *P. plasmodium* strain K1. a: Comparison of IC50 (µg/ml) between treated plant extracts and control plant extracts in M1; b: Improvement of *in vitro* antimalarial activity in % compared to control in M1; c: Comparison of IC50 (µg/ml) between treated plant extracts and control plant extracts in M2.1; d: Improvement of *in vitro* antimalarial activity in % compared to control in M2.1; e: Comparison of IC50 (µg/ml) between treated plant extracts and control plant extracts in M2.2; f: : Improvement of in vitro antimalarial activity in % compared to control in M2.2

With regard to Fig. 4, it turns out that SA increased *in vitro* antiplasmodial activity up to 1.7 at the concentration of 15 mM in M1 on CR*Pf* strain K1 (Fig. 4a). This *in vitro* antiplasmodial activity is enhanced up to 70.37% (Fig. 4b) than of the control. In M2.1, it increased up to 1.24 times at the concentration of SA of 17.5 mM (Fig. 4c); this *in vitro* antiplasmodial activity was improved by 24.43% (Fig. 4d) compared to the control. Regarding M2.2, the *in vitro* antiplasmodial activity increased up to 4.39 times at the concentration of 10 mM of SA and thus the SA enhanced up to 338.83% the *in vitro* antiplasmodial activity than of the control (Fig. 4e and 4f). The concentration of 10 mM (M2.2) of SA showed the best *in vitro* antiplasmodial activity against all concentrations of SA.

3.2 Discussion

3.2.1 Control *in vitro* antiplasmodial activities

Regarding values of the control *in vitro* antiplasmodial activities, they differ greatly from one to another. *In vitro* antiplasmodial activity of M1 presented high value than those of M2, except for the M1 value on the isolate *P. falciparum*. This difference could be explained by difference of plant material due by composition of plant because secondary metabolites in plants differ from one species to another and take an essential part in the plant metabolism and development [40,41,42]. *Phyllanthus* species contain alkaloids, polyphenols, flavonoids, tannins, tepenes and steroids which could be explain the *in vitro* antiplasmodial activities showed in the control plant extracts [15,19,42] and [43]. *Phyllanthus* species also contain sapogenin steroids which could explain their *in vitro* antimalarial activity [44,45].

Considering control plant extracts results obtained on isolates *P. falciparum* and on CR*Pf* strain K1, it appears that according to the criteria of the WHO classification of in vitro antiplasmodial activities of extracts or drugs [32, 46], the plant extracts on strains of *P. falciparum* behaved differently. The control plant extracts presented high *in vitro* antiplasmodial activity (IC_{50}<5 µg/ml) with respective values : 1.12±0.55 µg/ml on *Pf*K1 for gamma-rays in M1, 1.38±0.12 µg/ml on *Pf*K1 for SA in M1 and 3.82±0.43 µg/ml on *Pf*K1 for SA in M2.1. They presented promising *in vitro* antiplasmodial activities (5<IC_{50}<15 µg/ml) with the follow values : 5.30±4.24 µg/ml, 7.60±0.00 µg/ml µg/ml, 9.68±2.21 µg/ml, 11.98±0.98 µg/ml and 14.91±3.85 µg/ml (Tables 1 and 2). 20±8.30 µg/ml and 41.53±3.67 µg/ml were the moderate *in vitro* antiplasmodial activities (15<IC_{50}<50 µg/ml). These results may be again explain the use of *Phyllanthus* species specially *P. odontadenius* on treatment of malaria disease [16,19] and [26].

3.2.2 Increasing of *in vitro* antiplasmodial activities

The *in vitro* antiplasmodial activities were increased greatly with the isolates of *P. falciparum* than that of CR*Pf* strain K1. These results showed the capacity of resistance for the strain K1. This effect of the same extracts on CR*Pf* strain K1 and on isolates of *P. falciparum* revealed that parasites do not request the same compounds. The difference on phytochemical compounds in *P. odontadenius* plants was already proved by some works of [26,27] and [28]. The difference on phytochemical composition in M1 and M2 plants could explain the difference on *in vitro* antiplasmodial activity of plant extracts from irradiated seeds or immersed in SA solutions.

For physical mutagenesis by gamma rays, the *in vitro* antiplasmodial activities against isolates *P. falciparum* in M1 increased up to 7.6 (150 Gy) in comparison to control plant extracts and up to 5.3 (125 Gy) comparing to the control plant extracts in M2. These values exceeded in the *in vitro* antiplasmodial activities of control plant extracts respectively to 660% and 430%. For a breeding program of any plant species, the determination of LD30 and LD50 is recommended [47,48]. For *P. odontadenius*, the gamma-rays values of DL30 and LD50 from gamma-rays obtained are respectively 79,05 Gy and 155, 10 Gy [49].

On CR*Pf* strain K1, the M1 treated plant extracts showed low effect against the parasite, only in M2 where the *in vitro* antiplasmodial activity increased up to 2.48 (225 Gy) times in comparison to control plant extracts; those value of the *in vitro* antiplasmodial activity exceeded up to 147.57% than of control. In comparison to clinical *P. falciparum*, CR*Pf* strain K1 not followed the letal doses (79.05 Gy and 155.10 Gy). This in vitro antiplasmodial activity on CR*Pf* strain K1 at 225 Gy could be explained by metabolism of others compounds differed to those produced at 125 and 150 Gy.

For chemical mutagenesis by SA, the *in vitro* antiplasmodial activities against parasites in M1

increased up to 10.15 (10 mM) and 1.7 (15 mM) respectively for the isolates *P. falciparum* and CR*Pf* strain K1. SA improved thus respectively the *in vitro* antiplasomodial activities to 915% and 70.37% in comparison to control plant extracts In M2.1, *in vitro* antiplasmodial activities increased up to 6.39 (17.5 mM) and 1.24 (17.5 mM) and improved respectively to 538.92% and 24.43% in comparison to control plant extracts. In M2.2, the *in vitro* antiplasmodial activities of treated plant extracts on parasite, isolates of *P. falciparum* and CR*Pf* strain K1, increased respectively up to 9.11 (15 mM) and 4.39 (10 mM) in comparison of the control plant extracts. These values showed an improvement of *in vitro* antiplasmodial activity respectively up 810.87% and 338.83% in comparison to the control plant extracts. For the breeding program of *P. odontadenius* species by SA, values of LD30 and LD50 were respectively 4.76 mM and 10.99 mM [27]. Regarding results, plant extracts from 10 mM in M1 on the clinical isolate of *P. falciparum* and in M2.2 on CR*Pf* followed values of LD30 and LD50. Others values obtained for example at 15 and 17.5 mM could be explained in the effects of mutagens on change of secondary metabolites [21,22].

These different values could be explained the use of mutagens in plant breeding for improvement of antimalarial drugs or the improvement of secondary metabolites against malaria parasite by mutagenesis using physical or chemical mutagenesis. Concrete aims of using plant genetic resources (PGR) in crop improvement are : to develop cultivars that are specifically adapted to abiotic or biotic stresses, to assure sustainable production in high-yielding environments through reduced application of agrochemicals and increased nutrient and water efficiency ; and to open production alternatives for farmers through development of industrial, energy or pharmaceutical crops [50].

4. CONCLUSION

In this study, we proved that it's possible to improve the *in vitro* antimalarial activities using physical and chemical mutagenesis techniques. Our work illustrated an increasing *in vitro* antiplasmodial activity plant extracts treated by irradiation of *P. odontadenius* seeds or immersion of *P. odontadenius* seeds in SA solutions.

Plant extracts from treated seeds by gamma rays have significant effects on in the in vitro antiplasmodial activities than control plant extracts respectively with 660% in M1 and 430% in M2. For SA, the *in vitro* antiplasmodial activities of plant extracts from immersed seeds in sodium azide exceeded those of control plant extracts to 915% (M1), 538.92% (M2.1) and 810.87% (M2.2).

125 and 150 Gy are the doses of Gamma-rays used for improving the *in vitro* antiplasmodial activities against isolates of *P. falciparum* and 225 Gy could be used as dose for improvement of *in vitro* antiplasmodial activity against the strain K1 of *P. falciparum* resistant to chloroquine. 10, 15 and 17.5 mM were the various concentrations of SA used for improving *in vitro* antiplasmodial activities against isolates of *P. falciparum* or chloroquine-resistant *P. falciparum* strain K1.

CONSENT

It is not applicable. We do not use patient or laboratory animals during our work.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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