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Effects of Gamma Irradiation on Seeds Germination, Plantlets Growth and *In vitro* Antimalarial Activities of *Phyllanthus odontadenius* Müll Arg

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Authors' contribution

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

Original Research Article

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ABSTRACT

Aims: To increase the production of secondary metabolites of *P. odontadenius* using gamma radiation in order to amplify those with *In vitro* antimalarial activity.
Study Design: Laboratory experiment tests; Identification of plant material, Irradiation of seeds, *In vitro* culture of seeds, *In situ* culturing plantlets, Extraction of *Phyllanthus odontadenius* aerial parts, Phytochemical screening, *In vitro* antiplasmodial tests to determine the inhibition of concentration killing 50% of parasite population (IC_{50}).
Place and Duration of Study: Department of Biotechnology and Molecular Biology, Department of Biochemistry: General Atomic Energy Commission, Regional Center of Nuclear Studies, P.O. Box. 868 Kin XI (DRC), National Institute of Biomedical Research (INRB) à Kinshasa/Gombe (RDC), UMR-MD3 Laboratory (Institute of the Biomedical Research of Army, Marseille/France. The experiments were conducted during January 2010 to June 2011.
Methodology: Seeds of *P. odontadenius* were obtained after oven drying at 45°C and they were irradiated by gamma-irradiation (^{137}Cs) at dose ranging between 0 to 300 Grays (Gy). Seeds were germinated on Murashig and Skoog medium and plantlets were transferred in the tubes or *in situ*. In addition some parameters such as height, collar diameter, number of branches and biomass from first and second generations were analyzed. Phytochemical screening was released. The *In vitro* antiplasmodial activities assays on clinical isolates of *P. falciparum* or on resistant *P. falciparum* K1 to chloroquine was determined.
Results: Results showed that gamma irradiation had negative effects on growth parameters of *P. odontadenius* in the M1 and M2 generations with greater effects observed with treatment exceeding 100 Gy. For the *In vitro* antimalarial activities from to extracts obtained with aerial materials part from directly irradiated seeds (M1), the effects observed with extracts from M1 were higher than those from M2. For M1 extracts plants, IC_{50} values were ranged between 1.0±0.22µg/ml to 6.95±0.64µg/ml and between 1±0.05µg/ml to 10.45±1.18 µg/ml for M2 extracts plants on *P. falciparum* from to clinical isolates. With *P. falciparum* K1 strain, the IC_{50} values were ranged between 0.92±0.91–4.08±1.49 µg/ml for M1 and between 3.91±0.2.69–14.11±1.31µg/ml for M2 extracts. The best activities were observed with gamma-radiation doses exceeding 150 Grays (Gy).
Conclusion: Gamma-irradiation of *P. odontadenius* seeds induced reduction of parameters which decrease linearly with increasing irradiation doses. Synthesis of secondary metabolites increase in the second generation compared to the first one with a more important synthesis in phenolic compounds. The *In vitro* antiplasmodial activity on the clinical isolates *P. falciparum* or *P. falciparum* K1 showed low antimalarial activities from M1 and M2 controls (0Gy) than that of extracts from treated plants. The high inhibitory effects of crude extracts plants from treated seeds have justified the usefulness of gamma-irradiation in the increasing production of secondary metabolite against malaria in the Word particularly in DRC.

Keywords: Phyllanthus odontadenius; gamma irradiation; secondary metabolites; malaria; antimalarial activity.

1. INTRODUCTION

Human malaria is one of the most important health problems in tropical and subtropical regions. The estimated clinical cases for WHO were 216 million in 2010 [1], approximately

40% of world's population were at risk of malaria. Nearly 655,000 died from to malaria disease, mainly children under 5, pregnant women and elderly [1,2,3]. A major obstacle to malaria control is the emergency and spread of antimalarial resistance drugs, and urgent efforts are necessary to identify new classes of antimalarial drugs.

Plants produce more than thousands of different compounds through the secondary metabolism pathways.These secondary metabolites are often the keystone in the interactions between plants and their environment as for example phytoalexins, that are involved in plant defense. The properties of these molecules are used in traditional medicine, but also in modern allopathic medicine through the use of purified or derived components obtained from chemical hemi-synthesis [4].

Plants have been used medicinally throughout history, and the two best conventional antimalarial drugs, artemisinin from *Artemisia annua (Asteraceae)* and quinine from *Cinchona sp (Rubiaceae*), are both derived from traditional medicines. Traditional medicine using plant extracts continues to provide health coverage for 80% of the World's population, especially in the developing world [5,6].

The biosynthesis of secondary metabolites is dependent on some extrinsic and intrinsic constraints [7] as some differences in quality and quantity of compounds within the same species are observed depending on regions of the world. The metabolic engineering approach is one of the pathways in the production of secondary metabolites with economic interest value. To that, the metabolic engineering approach consists to modify the plant physiology to make it produce a molecule of economic interest. However, this approach has been facing many difficulties due to the implication of secondary metabolites in many regulations, relatively unknown, contributing to maintain plant homeostasis.

In recent years rapid procedures for obtaining transgenic roots have been developed using *Agrobacterium rhizogenes*, a soil pathogen which elicits adventitious, genetically (Ri T-DNA) transformed roots [8-12]. As genetic variability is essential for any crop improvement program, the creation and management of genetic variability becomes central base to breeding. Experimentally, induced mutations provide an important source for variability. A variety of ionizing radiation forms, including X-rays, γ-rays, neutrons and ion-beams, have been used as mutagens for mutation breeding in addition to chemical mutagens [13,14].

Among chemical mutagens commonly used either are alkylating agents (N-methyl-N-nitrosoguanidine (MNNG), ethyl methane sulfonate (EMS) and sodium azide (NaN_3). Physical mutagens often act on mutagenic chromosomal and molecular physiological level [15,16].

The damages of cell at radiation were caused often through the formation of reactive oxygen species (ROS). ROS induce oxidative damage of DNA, including strand breaks, base and nucleotide modifications [14]. In general, ionizing radiation predominantly induces the formation of radical by water radiolysis, and these then attacks DNA and produce oxidative damage [17]. The formation of 7,8-dihydro-8-oxo-2'-desoxyguanosine (8-oxo-dG) represents one of the most abundant and best characterized types of oxidative damage [14,17].

Phyllanthus odontadenius Müll. Arg. (Synonym: *Phyllanthus bequaertii* Robyns & Lawalrée) is present in all the coastal countries of West Africa from Guinea-Bissau to the Congo, and from Rwanda, Kenya and Uganda to southern Africa. In Ghana, the leaves are eaten to get the hiccups. In Rwanda, the stem part extract is used to treat diarrhea and cholera. The

alcohol crude extracts of leaves and stems have used to calme diarrhea induced castor oil in mice. A water extract of the entire plant inhibited the DNA polymerase of hepadnaviruses [18],19]. [20] Reported that plant part unspecified used to treat earache in the northern Nigeria. The common names *geron-tsuntsaye*, *gero itache*, *ebógi* and *ebó zùnmaggi* are used for *P. amarus*, *P. odontadenius*, *P. pentandrus*, probably *P. urinaria* and probably also some noo-*Phyllanthus* species.

Some author published results on *Phyllanthus* species which ones by the error appellation were called *Phyllanthus niruri* that would be probably *P. odontadenius* or *P. amarus*. For that, they reported that *P. niruri* contains primarily lignans, flavonoids, alkaloids, phthalic acid and tannins [21]. It has been widely used in treating a number of traditional ailments [20], [21,22] and has demonstrated *In vitr*o antibacterial actions against *Staphylococcus*, *Micrococcus*, and *Pasteurella* bacteria as well as *In vivo* and *In vitro* anti-malaria properties.

Ion beams and γ-rays are utilized as models of high-linear energy transfer (LET) and low-LET radiation, respectively, to study the biological effects from various radiations. It is known that high-LET radiations tend to confer greater biological effects (such as cell-killing and mutagenesis) than low-LET radiations [17]. Results obtained by [14] indicate that the transversion G (guanine) to T (Thymine) was greatly induced by γ-rays exposure. [23] reported that γ-rays were the most efficient ionizing radiation for creating mutants in plants as they can induce high mutation numbers in plants.

The aim of the present study was to monitor the effects of γ-rays (^{137}Cs) on the production of active secondary metabolites in *P. odontadenius* aerials parts in order to amplify those with *In vitro* antimalarial activity.

2. MATERIALS AND METHODS

2.1 Plant Material – Mutagenesis – *In vitro* Germination

2.1.1 Plant material

The plant material used for harvesting fruits was identified by the senior Assistant research Anthony KIKUFI, Laboratory of Systematic Botany and Plant Ecology, Department of Biology (Faculty of Science). The seeds of *P. odontadenius* were used such as study material.

2.1.2 Irradiation of seeds

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 [24,25] and the treatments ranged from 0 to 300 Grays (Gy). The first generation of irradiated seeds was designed as M1.

2.1.3 *In vitro* seeds germination

The irradiated material was germinated in Petri dishes with 3 replications. M1 seeds were disinfected with 70% (v/v) ethanol for 1 min, sterilized with 0.125% (w/v) $HgCl_2$ for 3 min, and washed with sterile distilled water. They were then handled with gibberellic acid (GA_3) 200 mg/L for 4 h and finally drained before being cultivated on modified Murashige and Skoog

(MS) basal media without sucrose or growth regulators and supplemented with 0.8% agar [26,27]. The pH of the media was adjusted to 5.6 before autoclaving at 121ºC for 15 min. Cultures were incubated at 25±1ºC under fluorescent light with 16h photoperiod. Percentage of seeds or the germination rate for each dose was determined by the equation before.

$$\% \text{ of germinated seeds} = n \times 100 / N.$$

Where *n*: number of germinated seeds and *N*: the number of seeds in de Petri dish.

The reduction of emergence (%) was also determined by the relationship from Maluszynski et al. (2009) below:

Emergence reduction (%) = 100 – (Average emergence in the dose x 100)/average emergence in the control)

2.1.4 *In vitro* and in situ seedling transfer

After 2 months, M1 plantlets from Petri dishes were transferred to tubes containing MS basal media with 3% sucrose, without growth regulators supplemented with 0.8% agar for *In vitro* culture or in polyethylene bags containing 300 g of soil for *in situ* growth (Chaves, 2006). Bags were then buried in 3/4 in the ground in randomized complete block (RCB) design with 3 replications [28-31]. The plantlets placed *in situ* were watered three times a week, the odd days, with the same amount of water (20L per plot 5 dm/6dm).

2.1.5 Seedling growth and Plantlets survival

The length of vitro plants was shouted using a numeric apparatus PENTAX Optio w30 WATERPROOF and measured with Optimas6 software. The number of leaves per plant was counted manually.

The survival rate of plants was determined with following formula:

Survival rate (%) = (number of the surviving plants x 100)/number of transferred plants

The number of mutants (albina, xantha and viridis) observed following the irradiation treatment in M1 was determined and the mutation frequency (Msd) was calculated as follows [28]: Msd = [number of mutant (albina + xantha + viridis) x 100]/N with N is the total number of seedlings obtained for a dose.

Parameters such as collar diameter shoot length, number of branches for the selected M1 plants were measured after four months of culturing. The length of plants was performed using a lathe measuring 50 cm. The collar diameter was measured using Slot-foot Digital CALIPER 150 mm (6'') and the number of branches was measured manually. Fresh Biomass and dried biomass for aerial parts after plants harvest were measured using a balance DENVER APX-100.

2.2 Culturing of Second Generation Seeds

The second generation (M2) was obtained from *In vitro* culture of M1 seeds using the same methods as for irradiated seeds. After *In vitro* germination, 50 plants of each irradiation dose treatment were sowing in row and plants were watered also three times a week. The same

parameters such as diameter of collar, shoot length and branches number were determined. Fresh and dried biomass for aerial parts of M2 plants were equally measured after four months plants harvest.

2.3 *In vitro* Antimalarial Activity

Antimalarial activity assays were performed at the National Institute of Biomedical Research (NIBR) in Kinshasa/Gombe, DR Congo and at the UMR-MD3 laboratory, Institute of the Biomedical Research of the French Army - Antenna Marseille, France.

2.3.1 Effects of plant drugs on clinical isolates of *P. falciparum*

The stock solutions were 200µg/ml for M1 and 125µg/ml for M2 extracts. These solutions were prepared in 1% DMSO and diluted in two fold to have test concentration. Clinical isolates of *P. falciparum* were obtained from symptomatic malaria children (0-5 years) with high parasitaemia and who 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 (4ml) were collected in tubes containing 1% heparin, and 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 bicarbonate and 10% of pooled human serum. After homogenization, 50µl of the suspension were distributed in each well of a spot plate containing decreasing concentrations (125 to 0µg/ml) of M1 or M2 extracts [32].

Plates were then maintained at 37ºC in a humid atmosphere containing 5% CO_2. Quinine was used as control. After 48h-incubation, thin smears were made and stained with GIEMSA 5% and parasitaemia were determined with a Zeiss Primo Star microscope (GmbH/Germany) [33,34]. Inhibition of parasitaemia (percent) was calculated as following:

Inhibition (%) = (A - B/A) x 100, where A is the parasitaemia in the negative control and B, the parasitaemia in the treated plates bucket. The IC_{50} of each sample was obtained using the dose-response curves.

2.3.2 Effects of plant drugs on chloroquine-resistant strain K1

The chloroquine-resistant (IC_{50} = 1010±155nM) strain K1 (Thailand, MR4-ATCC) was tested using the isotopic micro-test method. For this the parasite was cultured according to Trager and Jensen (1976). Parasite culture was performed in RPMI 1640 medium (Gibco) supplemented with 25mM HEPES, 50mg/l hypoxanthine, 25mM $NaHCO_3$, 20mg/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]. Chloroquine was used as control. Extracts and chloroquine were placed in 96 wells microplates and serial dilutions were made in RPMI medium (final concentration ranging from 0-100µg/ml for extracts). Synchronous culture with parasitaemia of 0.8% and 1.5% final hematocrit were distributed. Parasite growth was assessed by adding 1µCi of [3H] hypoxanthine (specific activity of 14.1 Ci/mmol; Perkin-Elmer, Courtaboeuf, France) to each well at time zero. The plates were incubated for 42 h at 37ºC in an atmosphere of 10% O_2, 5% CO_2 and 85% N_2 and then frozen at - 80ºC. After thawing, the content of each well was collected onto filter plates (Filter

Mate Cell Harvester, Perkin-Elmer) and 25µl of scintillation fluid (Microscint O, Perkin-Elmer) was added to each well.

The level of parasite incorporation of radioactivity (in counts per minute) was measured with a scintillation counter (Top Count, Perkin-Elmer). Antimalarial activity 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 ICEstimator software [37].

2.4 Phytochemical Analysis

2.4.1 Preparation of crude extracts

10g of dried plant material were macerated separately with ethanol and dichloromethane (300 ml each) for 24h. Each mixture was filtered and dried at 45ºC for 72h. The aqueous extract was prepared by mixing 10g of dried plant material with 300 ml distilled water. The mixture was boiled at 100ºC for 15 min, cooled, filtered and dried at 45ºC for 72h.

2.4.2 Phytochemical screening

The chemical screening was carried out on all crude extracts. Alkaloids were detected with Draggendorff's and Mayer's reagents [38]. Saponins were detected by frotting test [38,39]. Presence of tannins was detected using Stiasny reagent and ferric chloride 2% [38,40,41]. Flavonoids were detected using Shinoda's reagent or aluminum chloride 5% [42];. Anthraquinones were detected using Börtranger's reagent [38]. Anthocyanins were identified using HCl 2N, heating and add iso-amylic alcohol (Harborne, 1998). Steroids and terpenoids were identified using Liebermann-Bouchard's reagent [38].

The presence of the different chemical groups was confirmed by Thin Layer Chromatography (TLC) performed on silica gel plates GF_{254} (Merck, Germany). Alkaloids were detected using $CHCl_3$/MetOH/NH_4OH (9:2:0.5) and EtOAc/Iso-PrOH/NH_3 (85:15:5) as mobile phases and Draggendorff's as reagent. Flavonoids were detected using *n*-BuOH/water/acetic acid (4:1:5) (Top layer) as mobile phase with NEU's reagent (1% diphenylboric acid ethanolamine complex, methanolic solution 1%). Steroids and terpenoids were detected using $CHCl_3$/MeOH (9:1) and n-Hexane/MeOH: (9:1) on mobile phases and Liebermann-Bouchard's reagent. After drying, the plate was heated at 110ºC for 10 min to intensify the spot colors. Tannins, mainly proanthocyanidins were detected using EtOAc/HOAc/HCOOH/H_2O (30:02:1.2.8) (upper phase) as mobile phase and 1% Vaniline and 5% H_2SO_4 as reagents. After spraying, drying and heating the plate, proanthocyanidins were colored in red while flavonoids appear in yellow color. Anthraquinones were identified using $CHCl_3$/MeOH (7:3) as mobile phase and Börtranger's reagent [38,43].

2.4.2.1 Statistical analysis

Data were subjected to Analysis of Variance (Anova) using MSTAT-C Software (Borzouei et al. [9] and compared to the software Statistica with General Linear and LSD test (Least Significant Difference) in order to identify differences between treatments. Means of different treatments were separated with LSD at 5% level of probability.

3. RESULTS

3.1 Effects of Gamma-Irradiation (^{137}Cs) on the Growth of *P. odontadenius*

Results obtained in this work were mentioned in Figs. 1 (a-d), 2 (a-e) and 3 (a and b) and in the Tables 1 to 5.

3.1.1 Seeds Germination and Growth of *In vitro* P. odontadenius seedlings

The Table 1 exhibiting the effects of Gamma-Ray (Cs^{137}) on *In vitro* irradiated seeds germination and seedling growth, shown that rate of *In vitro* seeds germination of M1 plants decreases with increasing irradiation doses. The highest germination rate (43.85±11.17%) was observed for the control (0Gy), while the lowest germination rate (7.06±1.22%) was observed for 225Gy irradiation treatment. A small effect on *In vitro* seeds germination was observed at doses of 25, 50, 75 and 100Gy (Table 1).

The reduction of the emergence in M1 plants was higher after gamma-irradiation at 225Gy (83.90% reduction when compared with the control) (Table 1). Low reduction of emergence was found following irradiation at 25, 50 and 75Gy (19.77%, 19.73% and 16.33%, respectively). Following 100Gy irradiation treatment, the reduction of the emergence of M1 plant was above 30% (Table 1).

The size and the number of leaves seedlings from *In vitro* cultured M1 were higher than those of the control (0Gy) (Table 1). The size of leaves measured for the control plant was significantly different from that of M1 plants obtained after irradiation treatment from 150Gy to 300Gy. The number of leaves calculated for the control plant was significantly different from that of M1 plants obtained after irradiation treatment at 300Gy.

3.1.1.1 Growth of in situ P. odontadenius plants

Results on growth of *P. odontadenius* plants *in situ* were presented in Table 2 exhibiting Length, Collar diameter, seeds number per plant or seeds Weight (mg) and biomass per plants.

For plants which grew *in situ*, the size and collar diameter (14.01±5.12cm and 2.68±1.07 mm, respectively) of M1 plants obtained after seeds irradiation at 50Gy dose were significantly higher than those observed for the plant control (9.34±4.38 cm and 2.01±1.09 mm, respectively) and for seeds irradiated at 300Gy (3.60±0.28 cm and 1.10±0.14 mm, respectively).

In *P. odontadenius* control plants, the number of seeds per plant was 234±58 weighting 70.32±17.30mg. The largest number of seeds per plant was observed at 225Gy with 715±182 seeds per plant weighting 214.69±54.72 mg while the most important quantity of biomass was obtained at 50Gy (29.60±9.19 g) and the lowest at 300Gy (2.00±0g).

3.1.2 Morphological effects on *P. odontadenius* plants *in situ*

Phenotypic effects were also observed on *P. odontadenius plants* growing in the field. In particular, we observed changes on leaves of *P. odontadenius plants* whose seeds were irradiated at 50, 100, 125 and 225Gy. In particular, at dose of 100Gy, the change of plant

leaf in purple color profoundly affects the individual plant who dies (Fig.1c). These effects are reflected to the roots which would furnish themselves too specific phenotypic traits (Fig.2 cand Fig. 2d).

Color defects in the roots are appeared blackish accompanied by white roots or blackish simply for plants obtained with seeds irradiated at 100Gy (c and d). 50Gy (b) has very long roots exceeding the size of the aerial part (Fig. 2).

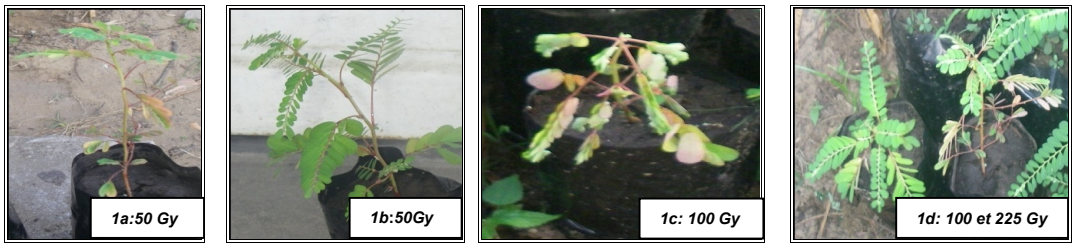

Fig. 1. Morphomogical effets of Gamma-ray on growth of *P. odontadenius* plants. 1a and 1b: plants from irradiated seeds at 50Gy, 1c and 1d: plants from 100Gy and 225Gy irradiated seeds

Fig. 2(a-e). Effects of gamma irradiation on roots and plantlets of *P. odontadenius* obtained by gamma-irradiation (Cs137). 2a: control plant; 2b: 50Gy irradiated plant; 2c and 2d: 100Gy irradiated plant and 2e: 225Gy irradiated plant

The frequency of mutations differs significantly depending on the doses used. The mutation frequency was the highest at 225Gy with 22.22%, followed by 100Gy with 13.33%. Frequencies at doses of 50 and 125Gy were respectively 8.33% and 9.09%. These percentage changes are an indication of what may be the changes in M2.

3.2 Effects of Gamma Irradiation (^{137}Cs) on *P. odontadenius* in M2

Control plants derived from seeds (T_0M_2) showed high values for the various parameters studied (size: 22.06±6.91cm, collar diameter with: 2.03±0.53 mm, number of branches: 18.58±7 and biomass per foot: 1.96±0.76g) and plants from 175Gy ($T_{175}M_2$) showed low values for the average size (10.58±4.23 cm), collar diameter (1.03±0.31 mm) and branches number (10.04±4.06).

Table 1. Effects of Gamma-Ray (Cs^{137}) on *in vitro* Irradiated Seeds Germination and seedling Growth (n=6)

Treatments Parameters	0 Gy	25 Gy	50 Gy	75 Gy	100 Gy	125 Gy	150 Gy	175 Gy	200 Gy	225 Gy	250 Gy	275 Gy	300 G)	Lsd à 5%	CV (%)
Percentage of germination (%)	43.85±11.17a	35.18±18.82ab	35.20±4.80ab	36.69±19.76ab	30.34±7.25abc	17.32±13.65cde	24.44±9.59bcd	15.24±10.82cde	10.37±4.34de	7.06±1.22e	10,73±3,69de	14.02±2.79de	12.11±3.28de	15.20	37.95%
Emergence Reduction (%)	0	19.77	19.73	16.33	30.83	60.50	44.26	65.25	76.35	83,90	75.53	68.03	72.38	-	-
lenght (cm)	6.36±2.37a	5.88±2.69a	5.09±2.33ab	5.81±1.90 a	5.78±2;22a	3.62±1.51bc	3.00±0.51 c	2.75±0.75 c	3.60±1.68bc	2,83±0,99c	2.81±0.99c	1.81±0.86c	0d	1.682	44.94%
Leaves number/plane	5.6±2.1a	4.9±1.6 abc	5.0±2.6ab	5.3±1.6 ab	5 ;2±0.9ab	5.0±1.3ab	4 ;6±1.7 abc	4.0±1.1 bcd	3.0±1.1 d	4,2±1,2abcd	3.5±1.2cd	3.0±1.1d	0e	1.43	35.68%

For the same parameter, values with identical letters were not significantly different at 5% ($p < 0.05$)

Table 2. Effects of gamma-ray (Cs^{137}) on *P. odontadenius* growth in M1 generation (n=6)

Treatments Parameters	0 Gy	25 Gy	50 Gy	75 Gy	100 Gy	125 Gy	150 Gy	175 Gy	200 Gy	225 Gy	250 Gy	275 Gy	300 Gy	Lsd à 5%	CV (%)
Lenght (cm) for 4 months	9.34±4.38bc	10.96±4.56b	14.01±5.12 a	8.84±3.76bc	9.31±3.65bc	8.55±3.60c	7.86±3.39c	8.80±4.28 bc	9.01±4.33bc	9.90±4,22bc	7.94±3.42c	7.61±3.42c	3.60±0.28d	4.741	15.18%
Collar diameter (mm) for 4 months	2.01±1.09abc	2.23±1.24ab	2.68±1.07a	1.87±0 .70bc	1.95±0.87abc	1.75±0.69bcd	1.83±0.75bcd	2.00±1.08 abc	1.83±0.75bcd	1.93±0.83abc	1.92±0.65abc	1.39±0.61cd	1.10±0.14d	0.782	23.33%
Seeds Weight (mg) (Number/plts)	70.32±17.30f (234±58)	166.1±24.60b (554±62)	174.3±48.32b (581±161)	115.2±25.78de (384±86)	8107±2042ef (270±68)	167.5±37.33 b (558±124)	159.9±13.14bc (533±44)	125.4±35.14 cd (418±117)	137.0±16.37bcd (457±55)	214.6±54.72a (715±182)	26.75±7.78g (89±26)	82.00±20.78ef (273±69)	29.60±0g (99±0)	39.51	22.33%
Biomass (g)	21.00±5.17b	11.17±1.64d	29.60± 8.19a	3.43±0.7f	10.63±2.68d	9.26±2.06de	10.17 ±0.84d	10.52±3.14d	13.40±1.60cd	17.00±4.34bc	4.42±1.29ef	10.87±2.76d	2.00±0 f	4.943	28.22%

For the same parameter, values with identical letters were not significantly different at 5% ($p<0.05$)

Table 3. Effects of gamma-ray (Cs^{137}) on *P. odontadenius* growth in M2 generation (n=30)

Treatments Parameters	0 Gy	25 Gy	50 Gy	75 Gy	100 Gy	125 Gy	150 Gy	175 Gy	200 Gy	225 Gy	Lsd à 5%	CV (%)
Lenght (cm)	22.06±6.91a	15.59±6.13b	12.60±4.65bc	12.58±4.50bc	12.78±6.04bc	13.28±6.45bc	11.83±5.76c	10.58±4.23c	11.62±7.99c	12.44±7.53bc	3.633	42.78%
Collar Diameter (mm)	2.03±0.53a	1.71±0.38b	1.44±0.39cd	1.59±0.39bc	1.23±0.47de	1.33±0.39d	1.33±0;44 d	1.03±0.31e	1.23±0.56 de	1.25±0.45de	0.2979	38.98%
Number of branchs	18.58±7.16a	14.08±3.60bc	12.04±5.33bcd	14.27±4.68b	12.31±4.61bcd	10.27±3.32d	11.31±4.32cd	10.04±4.06d	10.08±5.09d	10.31±4.35d	2.810	36.33%
Biomass (g)	1.96±0.76a	1.48±0.63b	1.16±0.73bcd	1.37±0.61bc	1.07±0.77cde	0.97±0.58def	0.89±0.56def	0.73±0.62ef	0.68±0.73f	0.85±0.69def	0.389	55.64%

For the same parameter, values with identical letters were not significantly different at 5% (p<0.05)

Table 4 . Phytochemical Screening of *P. odontadenius* crude extracts from M1 plants

Chemical groups	Treatment Doses (Gy)											
	0	25	50	75	100	125	150	175	200	225	250	275
Alkaloids	–	–	–	–	–	–	–	–	–	–	–	–
Saponins	–	+	+	+	+	–	+	–	–	–	+	–
Tannins	+	+	+	+	+	+	+	+	+	+	+	+
Anthocyanins	+	+	+	+	–	+	–	+	+	+	+	+
Free Quinones	–	–	+	–	–	–	–	–	+	–	+	+
Flavonoids	+	–	–	–	–	+	–	+	–	+	–	+
Terpenoids and Steroids	+	+	–	–	–	–	–	+	+	+	–	+
Anthraquinones	–	–	–	–	–	–	–	–	–	–	–	–
Polyphenols	+	+	+	+	+	+	+	+	+	+	–	+

+ : found; –: not found

Table 5. Phytochemical screening of *P. odontadenius* crude extracts from M2 Plants

Chemical group	Doses treatments (Gy)											
	0	25	50	75	100	125	150	175	200	225	250	275
Alkaloids	—	+	+	+	–	–	–	–	–	–	–	–
Saponins	—	—	—	—	—	—	—	–	–	—	—	—
Tannins	+	+	+	+	+	+	+	+	+	+	+	+
Anthocyanins	+	+	+	+	+	—	—	—	—	+	+	+
Free Quinones	—	+	+	—	+	+	+	+	+	—	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
Terpenoids and Steroids	+	+	+	—	+	+	+	+	+	+	+	+

3.3 *In vitro* Antimalarial Activities of Aqueous Extracts from P. Odontadenius

Antimalarial activities of *P. odontadenius* crude extracts obtained after different irradiation treatment are reported in Figs. 3a and 3b.

The results on the clinical isolates *P. falciparum* showed higher values of antimalarial activity from M1 and M2 controls (0 Gy) than that of extracts from treated plants. The *in vitro* antiplasmodial activities of *P. odontadenius* on *P. falciparum* isolates presented the IC_{50} values ranged between 6.95±0.64 µg/ml for M1 control (0 Gy) *versus* 10.45±1.18 µg/ml for M2 control (0 Gy).

The antimalarial activity of crude extracts from M1 plants and those from M2 plants samples obtained after irradiation was greater than the antimalarial activity of M1 and M2 controls. For crude aqueous extracts from M1 plants, values of IC_{50} ranged between 1.06±0.12µg/ml (100Gy) to 2.96±0.25µg/ml (75Gy) (Fig. 3a and Table 6). For crude extracts from M2 plants (Fig. 3b and Table 6), values ranged between 1.00±0.22µg/ml (200Gy) to 5.96±0.91 µg/ml (75Gy). The lowest antimalarial activity was thus observed for M1 and M2 crude extracts obtained after seeds irradiation at 75Gy.

The same results were obtained on the *P. falciparum* K1 strain. For crude extracts from M1 plants, the IC_{50} were ranged from 1.12±0.33µg/ml (M1 control: 0 Gy) to 4.08±1.49µg/ml (100 Gy). For crude extracts from M2 plants, the IC_{50} were ranged between 3.91±2.69µg/ml (225 Gy) and 14.11±1.31µg/ml (75Gy). Antimalarial activity of control (IC_{50}=9.68±2.21 µg/ml) did not differ significantly from the other except that of 75 Gy obtained in the second generation (IC_{50} = 14.11±1.31µg/ml) ($P>0.05$).

Regarding the Table 6, crude extracts obtained from *P. odontadenius* M1 showed high activity against both the *P. falciparum* K1 strain and *P. falciparum* isolates with IC_{50}<5µg/ml (1.12±0.55 µg/ml - 4.08±1.49 µg/ml) or $5 < IC_{50} < 15$ µg/ml. Plant extracts of *P. odontadenius* M2 obtained with a single dose of irradiation (225 Gy) showed high antimalarial activity, IC_{50} = 3.91±2.69 µg/ml. All doses gave promising antimalarial activities ($5 < IC_{50} <$15µg/ml) on *P. falciparum* K1 whereas with *P. falciparum* isolates, control and 75Gy extracts gave promising antimalarial activities with IC_{50} values 10.45±1.18µg/ml and 5.96±0.91 µg/ml, respectively.

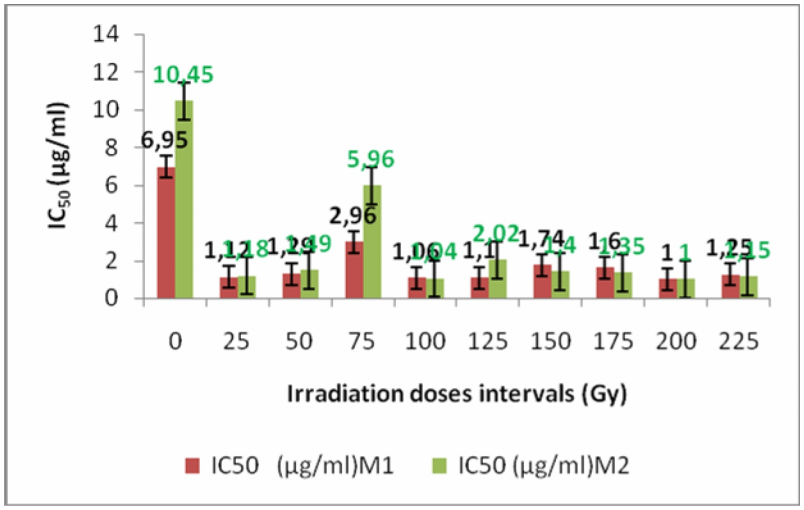

Fig. 3a. *In vitro* activities of *P. odontadenius* on the isolates of *P. falciparum*

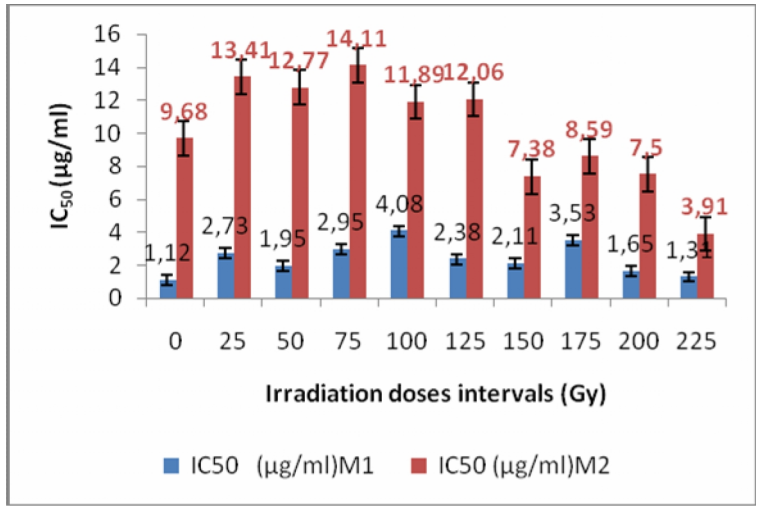

Fig. 3b. In vitro antiplasmodial activity on the P. falciparum K1 strain

Tests druds: significa from normal control, P<0.05; Mean±SEM=Mean values±Standard error of means of three experiments

Table 6. IC_{50} (µg/ml) of extracts *from P. odontadenius plants obtained by gamma-rays* (Cs^{137}) *irradiation seeds on Plasmodiem falciparum isolats and P. falciparum* K1 strain

	High (IC_{50} < 5 µg/ml)				Promising (5 < IC_{50} < 15 µg/ml)				Moderate (15 < IC_{50} <50 g/ml)		Inactive IC_{50} > 50 µg/ml	
Doses (Gy)	**M1-PfK1**	**M1-Pfls**	**M2-PfK1**	**M2-Pfls**	**M1 Pfk1**	**M1-Pfls**	**M2 Pfk1**	**M2-Pfls**	**M1**	**M2**	**M1**	**M2**
0	1.12±0.55c	-		-	-	6.95±0.64a	9.68±2.21abc	10.45±1.18a	-	-	-	-
25	2.73±0.24abc	1.12±0.25d		1.18±0.08c	-	-	13.41±2.45ab	-	-	-	-	-
50	1.95±0.06abc	1.29±0.39cd		1.48±0.12c	-	-	12.77±1.31ab	-	-	-	-	-
75	2.95±0.50abc	2.96±0.25b		-	-	-	14.11±1.31a	5.96±0.91b	-	-	-	-
100	4.08±1.49a	1.06±0.12d		1.04±0.44c	-	-	11.89±2.98ab	-	-	-	-	-
125	2.38±0.13abc	1.01±0.31d		2.02±0.21c	-	-	12.06±1.44ab	-	-	-	-	-
150	2.11±2.11abc	1.74±0.5c		1.4±0.1c	-	-	7.38±2.14bc	-	-	-	-	-
175	3.53±0.23ab	1.59±0.25cd		1.35±0.1c	-	-	8.59±1.68abc	-	-	-	-	-
200	1.65±0.58bc	1.00±0.22d		1.00±0.05c	-	-	7.5±3.25bc	-	-	-	-	-
225	1.31±0.19bc	1.25±0.2cd	3.91±2.c	1.15±0.05c	-	-	-	-	-	-	-	-
250	2.78±0.50abc	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
275	0.92±0.91c	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lsd	2.26	0.566		1.10			6.449					
Cv (%)	0.2807	13.44		19.45			0.1812					

Legen: M1-PfK1: M1 Extracts on Plasmodium falciparum K1; M1-Pfls: M1 Extracts on P. falciparum isolats; M2-PfK1 : M2 Extracts on Plasmodium falciparum K1; M2-Pfls: M2 Extracts on P. falciparum isolates; ND: no determined

3.4 Phytochemical analysis of *P. odontadenius*

Phytochemical analysis indicates that different types or chemical groups of secondary metabolites are more present in the second generation than in the first one (Tables 4 and 5).

Except for tannins, anthocyanins and polyphenols that were detected in M1 and M2 plants control and the absence of anthraquinones in M1 and M2 control plants and those obtained after irradiation of seeds in any samples, the presence of other major chemical groups in both M1 and M2 generations varied depending of irradiation treatment. Alkaloids were not detected in M1 plants but were detected in M2 to plants from irradiated seeds at 25, 50 and 75Gy. By contrast, saponins were found only in M1 plants obtained after irradiation at 25, 50, 75, 100, 150 and 250Gy. Finally, compounds like free quinones, flavonoids, terpenes and steroids were more found in M2 than in M1 (Tables 4 and 5).

4. DISCUSSION

On isolates *P. falciparum*, all extracts M1 from irradiated seeds have higher antimalarial activity against *P. falciparum* with the exception of those of control with 6.95µg/ml who exceeded 5µg/ml and has moderate antiplasmodiale activity [32]. In M2, except the control (0Gy) and 75Gy those have IC_{50} exceeding 5µg/ml with10.45±1.18 and 5.96±0.91µg/ml and were presented moderate antimalarial activity, all extracts from irradiated seeds presented higher antimalarial activities against *P. falciparum* with their IC_{50} low than 5µg/ml.

Absence or presence of one or another group of secondary metabolites in samples from M2 seeds could be explained by changes at the molecular level that would be induced by the energy of radiation given during the irradiation of the seeds. Some genes are sensitive and others were no sensitive. Certain genes would be sensible and the others never because of the sequence reparations on DNA damages or the presence of redundant genes can be explain this phenomena [44-46]. It knows that punctual mutation request two respective cycles of replication for their definitive fixation. Their effectiveness was possible in the second cycle of replication in the moment that the incorrect pair of DNA produce definitive change in the sister molecules [47].

From the above, the results obtained in this work confirm the hypothesis that secondary metabolites are stimulated by both abiotic (irradiation and various chemical compounds) and biotic elicitors groups (glycoproteins, molecules derived from microorganisms and polysaccharides) [23]. The gamma rays interact with atoms and molecules to create free radicals which are capable of altering the major components of plant cells. These radicals affect morphology, anatomy, biochemistry and physiology of the plant according to the irradiation dose.

From 100Gy dose, there is presumption of probable and obvious mutations or changes because emergencies reduction from 100Gy were upper 30% [28]. Our results are consistent with those of [39] who obtained a high frequency of mutations with durum wheat (*Cicer arietinum* L.) to 100Gy for Sofu cultivar.

Studies have shown that parameters such as the rate of seed germination, shoot length, root and seeding are affected by irradiation and chemical mutagen. For example, [48] showed that the rate of seed germination and plant height (stem or root) decreased when seeds of rice (*Oryza sativa* L.) were treated with gamma radiation and sodium chloride (NaCl). These

observations were confirmed by [49] on *Jatropha curcas* L. using mutagens (EMS and gamma rays). [50] Reported that sodium azide (NaN_3) affects the rate of seed germination, shoot length, root and delays germination. All the parameters decrease with increasing doses of irradiation or with increasing chemical mutagen concentrations. Then, as the survival of mature plants depends on the nature and extent of chromosomal damage [51], the increasing frequency of chromosomal damage associated with increased doses may be responsible for poor germination and reduced size and survival of plants.

Doses causing an emergency reduction of seeds or a reduction in size of plants by over 30% compared to the control are considered to be high for a large-scale program for mutation of plants with high probability to obtain mutants [28].

Survival rate of plants is significantly reduced and the lowest rate (10%) was observed at 300Gy irradiation doses. Witness presented 100% survival of plants followed those of 125Gy and 175Gy 46.67% to 46.15%.

Effects of gamma irradiation varied according to whether they could be positive, increasing an existing character for such dose, or negative, decrease or disappear to a character that existed for such another dose. [52] Show that the best plant survival of chickpea (*Cicer arietinum* L.) was observed at 180Gy. [53] Observed that Survival of carrot plants from seed in their study was reduced by >50% at the 10- and 20-krad doses. The decrease in survival of irradiated plant material resulting in a physiological imbalance at various events affecting vital cellular macromolecules. [54] Noted that the lower survival rate of plants after gamma irradiation is attributed to the destruction of auxin.

In contrast to the stimulation of the size observed with increasing doses by [55] for two varieties of chickpea (*Cicer arietinum* L.), *P. odontadenius* showed in this study, a decrease in size *in vitro* and *in situ*, in the second generation, when the plants are subjected to a continuous shade. Average plant height of *P. odontadenius* showed some higher values regardless of the growth rates *in situ*. Our results are consistent with those obtained by [56] in the analysis of the effects of sodium azide (NaN_3) on growth and yield of *Eruca sativa* L. for the 30th and 45th day. Comparing our results with those obtained by the same authors in the 90th day, they do not correspond to those found with *E. sativa*.

In vitro control plantlets showed higher mean values for the rate of seed germination, rate of seedling survival and for the seedling average number of leaves than of all average rates of third parameters for plantlets from irradiated seeds. Parameters of *P. odontadenius* plant transferred *in situ* showed high average values of height, number of seeds per plant, collar diameter and biomass average for plants from irradiated seeds indiscriminately compared to control plants. Many authors agree that observed characteristics are also dependent on environmental factors [30,57] because the phenotype is the sum of factors genetic and environmental.

The effects of size and collar diameter have been reported previously by [58] on the primary and mutagenic effects of gamma irradiation on *A. thaliana* seeds. These observations confirm the theory that the first generation generally have a higher rate of heritable traits than the following for which the expression of these traits results from genetic and external conditions [59,30,57].

However, the quantity of biomass per plant and fertility tend to decrease as the radiation dose increases. The decrease in biomass could be explained by disturbances in the

synthesis of chlorophyll, reliable molecular index for the assessment of genetic effects and gas exchange at the plant [60,61,54] explains that the abnormal leaf growth (biomass) is due to disruption of pigments (e.g., chlorophyll), the chromosome aberration and inhibition of mitotic division.

These disturbances are responsible for some of the negative effects expected by the treated plant material to gamma radiation [62-64]. They include changes in cell structure and metabolism of the plant, for example, dilatation of thylakoid membranes, and alteration of photosynthesis or modulation antioxidant systems and accumulation of phenolic compounds [65].

Effects of mutation were more pronounced in M1 than of M2 generation comparing to the control. Results obtained by [66] assessment of changes in oil yield and oleic acid after gamma irradiation of Guizote (*Guizotia abyssinica* (L.f.) Cass) reported that M1 plants show considerable changes in their different characters. They showed that the composition of the seed oil guizote is 34.60% for the control, from 43.50 to 47.60% for M1 and 39.70 to 44.70% for M2. It is 28.10% for the control, from 43.72 to 58.21% for M1 and 43.10 to 53.34% for the M2 composition of oleic acid.

[67] Confirm that seeds M1 of beans (*Cyamopsis tetragonoloba* (L.) Taub) produced more lethal and effects of pollen sterility while in M2, it are more thoroughly, the presence of various viable chlorophyll mutations. However, mutagenic effects increase both M1 in M2 with increasing doses of mutagenic [13]. [68] Show that sodium azide (NaN_3) gives more pronounced effects at 0.5mM concentration than 0.1mM only when the rice (*Oryza sativa* L.) is soaked in sodium azide. These results confirm the hypothesis that effects of irradiation are more pronounced in the first generation compared to the second [67,66].

[69] Give four main groups of secondary metabolites in plants including terpenoids for the first group, phenolic compounds for the second group, Saponins, cardiotonic heterosids and cyanogenic heterosids and glucosinolates for the third group and alkaloids the fourth. These four groups are found in varying amounts in plants of *P. odontadenius* derived from irradiated seeds.

Flavonoids and other phenolic compounds (tannins, free quinones, anthocyanins and polyphenols) were prominently reported previously. [70] Reported that phenolic compounds have antioxidant activities, allowing them to protect plants against damage caused by radiations. Flavonoid synthesis can be explained by the activity of phenylalanine amino-lyase (PAL), which, as a response to radiation (gamma and UV) in alleviating the damage caused by it [23].

With the presence of flavonoids in all samples of M2, our results confirm those of Lois (1994) cited by [23] which showed that irradiation of the Arabet plant (*Arabidopsis thaliana* of the Brassicacaceae family) increased the level of flavonoids which are accumulated in the aerial parts of the plant. Besides flavonoids, our results show that the level of alkaloids, free quinones, terpenes and steroids and anthocyanins increased.

Presence of alkaloids in M2 and their absence to M1 generation could be explained by the effect of gamma radiation on cellular proteins that release amino acids such as lysine, phenylalanine, tyrosine, tryptophan and ornithine which are the starting point for the biosynthesis of alkaloids [69]. It could be the same even for terpenoids and steroids derived from acetyl-CoA pathway, this latter come from to a disorder of the Embden-Meyerof way.

Our results on antimalarial activity obtained in this work, M1 and M2 appear low values of IC_{50} in all, to say that high antimalarial activities, compared to those obtained by Soh et al. (2009) on the antimalarial activity of *P. niruri* (certainly *P. odontadenius* or *P. amarus*) collected at three different sites in the Democratic Republic of Congo (DRC). The best antimalarial activity was the one with the aqueous extracts from stems of Kisantu's *P. niruri* with IC_{50} value of 11± 2µg/ml, whereas we obtained IC_{50} <11 except for doses of 25, 50, 75, 100 and 125Gy in M2.

In vitro antimalarial activities from crude extracts of *P. odontadenius* with high doses of gamma rays can be explained by the increase in total phenolics and specific compounds such as coumarin, *p*-coumaric acid and salicylic acid unlike what [71] found on the biosynthesis of phenolic compounds in seedlings cilantro gamma irradiated at low doses.

Control M1 crude extract showed good antiplasmodial activity on *P. falciparum* chloroquino-resistant exceeding all samples. This exceptional antiplasmodial activity can be explained by original compounds found in *P. odontadenius* or by phenolic compounds produced during storage which give place on enzymatique reactions [72,73]. It known that effects of mutation were more pronounced in M1 than of M2 generation comparing to the control [66].

5. CONCLUSION

The investigations carried out Gamma-irradiation of *P. odontadenius* seeds induced reduction of parameters such as percentage of seeds germination (43.85±11.17% to 7.06±1.22%), sizes (14.01±5.1cm to 3.60±0.28cm; 22.06±6.91cm to 10.58±4.23cm), collar diameter (2.68±1.07mm to 1.10±0.14mm; 2.03±0.53 mm to 1.03±0.31 mm) and biomass (29.60±8.19g to 2.00±0g; 1.96±0.76g to 0.68±0.73g) which decrease linearly with increasing irradiation doses.

Synthesis of secondary metabolites increase in the second generation compared to the first one with a more important synthesis in phenolic compounds. The *In vitro* antiplasmodial activity on the clinical isolates *P. falciparum* (6.95±0.64 µg/ml to 1.00±0.22 µg/ml; 10.45±1.18µg/ml to 1.00±0.05µg/ml) or *P. falciparum* K1 (4.08±1.49µg/ml to 0.92±0.91 µg/ml; 9.68±2.21µg/ml to 3.91±2.69µg/ml) showed low antimalarial activities from M1 and M2 controls (0 Gy) than that of extracts from treated plants.

The highest *In vitro* antiplasmodial activity was observed at 100Gy while for M2 crude-extract, the best antimalarial activities were observed at 50, 175, 200 and 225Gy. The high inhibitory effects of crude extracts plants from treated seeds have justified the usefulness of gamma-irradiation in the increasing production of secondary metabolite against malaria in the Word particularly in DRC.

We need to test, however, whether the plant extracts from these doses could also provide high antimalarial activities and lower toxicity on human cells. These studies are essential for plant breeding of *P. odontadenius* in the antimalarial activities improving.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. WHO, world malaria report 2011. Report. Consulted in March; 2011. Availble:www.WHO.int/malaria/world
2. Orhan I, Sener, B, Atici T, Brun R, Perozzo P, Tasdemir D. Turkish freshwater and marine macrophyte extracts show *In vitro* antiprotozoal activity and inhibit FabI, a key enzymes of *Plasmodium falciparum* fatty acid biosynthesis. Phytomedicine. 2006;13:388 – 393. Available Online at www.sciencedirect.com.
3. Guédé NZ, N'guessan K, Dibié TE, Grellier P. Ethnopharmacological study of plants used to treat malaria, in traditional medicine, by Bete Populations of Issia (Côte d'Ivoire); Journal of Pharmacology Science and Research. 2010;2(4):216–227. Available Online: www.jpsr.pharmainfo.in.
4. Gravot A. DeP450s study involved labiosynthèse Ruta graveolens in furocoumarins. PhD thesis, National Polytechnic Institute of Lorraine (INPL), Nancy-Colmar; 2002. French.
5. Subramanian SS, Nagarajan S, Salochana N. Flavonoids of some Euphorbiaceaous plants. Phytochemistry. 1971;10:2548-2549.
6. Willcox M, Bodeker G. Traditional herbal medicines for malaria. Br. Med. J. 2004;329:1156-1159.
7. Tourte Y. Genetic Engineering and Biotechnology: Concepts and Methods. Applications Agronomiques. 2ème Ed. Dunod, Paris; 2002.
8. Chabaud M, Boisson-Dernier A, Zhang J, Taylor CG, Yu O, Barker DG. Agrobacterium rhizogenes-mediated root transformation. Medicago truncatula handbook. 2006;1–8.
9. Hu ZB, Du M. Hairy root and its application in plant Genetic Enginnering. Journ of Integrative Plant Biology. 2006;48(2):121-127.
10. Häkkinen ST, Moyano E, Cusido' RM, Palazon J, Pinol TM, Caldentey KMO. Enhanced secretion of tropane alkaloids in *Nicotiana tabacum* hairy roots expressing heterologous hyoscyamine-6β-hydroxylase. Journ of experimental Botany. 2005;56(420):2611-2618.
11. KimY, Wyslouzil BE, Weathers PJ. Secondary metabolism of hairy root cultures in bioreactors. *In vitro* Cell. Dev. Biol. – Plant. 2002;38:1–38.
12. Bajaj YPS, Ishimaru K. Genetic transformation of Medecinal Plants. Transgenic Medicinal Plants, Edited by YPS Bajaj, Biotechnology in Agriculture and Forestery, Springer-Verlag, Berlin Heidelberg. 1999;45:2-28.
13. Makeen K, Babu GS. Mutagenic effectiveness and efficiency of gamma rays, sodium azide and their synergistic effects in urd bean *(Vigna mungo L.).* World Journal of Agricultural Sciences. 2010;6 (2):234-237.
14. Nakagawa M, Takahashi S, Narumi I, Sakamoto A. Role of AtPolζ, At Rev1 and AtPolη in ray-induced mutagenesis. Plant Signaling & Behavior. 2011;6(5):728–731. Landes Bioscience.

15. Zrÿd JP. Plant cell cultures, tissues and plant organs. Theoretical and practical uses. Presses Polytechnic Romandes, Tec & Doc-Lavoisier Lauzanne; 1988. French.
16. Muthusamy A, Vasanth K, Jayabalan N. Induced high yielding mutants in cotton (*Gossypium hirsuthum* L.), Mutation Breeding Newsletter and Reviews; 2005.
17. Yoshihara R, Nozawa S, Hase Y, Narumi I, Hidema J, Sakamoto AN. Mutational effects of γ-Rays and carbon ion beams on Arabidopsis seedlings. J Journal of Radiation Research. 2013;1–7.
18. Oudhia P. *Phyllanthus fraternus* G. L. Webster. In: Schmelzer GH, Gurib-Fakim A. (Editeurs). Prota 11(1): Medicinal plants/Plantes médicinales 1. [CD-Rom]. PROTA, Wageningen, Pays Bas; 2008.
19. Fischer E. Taxonomic results of the Bryotrop expedition to Zaïre and Rwanda. 8. *Riccia vulcanicola* E. Fischer (Subgenus Ricciella, Section Carvenosae), a new species from Virunga volcanoes, Rwanda. Tropical Biology. 1993;8:69–74. Available: http://tropical-bryology.org/Articles/open/VOL8/firicc.pdf.
20. Unander DW, Grady LW, Blunmber BS. Uses and bioassays in *Phyllantus* (*Euphorbiaceae*): A compilation. II. The subgenus *Phyllantus?* Journal of Ethnopharmacology. Elsevier publishers, Ireland. 1991;34:97-133.
21. Ishimari K, Yoshimatsu K, Yamakawa T, Kamada H, Shimomomura K. Genetic Transformation of *Phyllanthus nirui* L. (*P. amarus*). Biotechnology in Agriculture and Forestry. 1999;45:237-248.
22. Luyindula N, Tona L, Lukembila S, Tsakala M, Mesia K, Musuamba CT, et al. *In vitro* antiplasmodial activity of callus extracts from fresh apical stems of *Phyllantus niruri* L (Euphorbiaceous); Part 1. Pharm. Biol. 2004;42(7):1-7.
23. Moghaddam SS, Jaafar H, Ibrahim R, Rahmar A, Aziz MA, Philip E. Effects of Acute Gamma Irradiation on Physiological Traits and Flavonoids Accumulayion of *Centella asiatica*. Molecules. 2011;16:4994–5007.
24. Javed MA, Khatri A, Khan IA, Ahmad M, Siddiqui MA, Arain AG. Utilization of gamma irradiation for the genetic improvement of oriental mustard *(Brassica juncea Coss).* Pak. J. Bot. 2000;32(1):77–83.
25. Mahmoud AA, Al-Twaty N. Effect of Gamma Irradiation and Sodium azide on some Economic Traits in Tomato. Saudi Journal of Biological Sciences. 2006;13(1):44–49.
26. Zao J, Liang A, Zhu Z, Tang Y. Regeneration of Chinese cabbage transgenic plants expressing antibacterial peptide gene and cowpea trypsin inhibitor gene. Euphytica. 2006;150:397–406.
27. Jimenez M, Alvarenga S, Alan E. Establishment del protocole de microprogacion para la planta médicinal *Phyllanthus niruri* L. (*Euphorbiacée*), Technologia en Marcha. 2007;20(2):32–40. (Spanish)
28. Maluszynski M, Szarejko I, Bhatia CR, Nichterlein K, Lagoda PJL. Methodologies for generating varibility. Part 4: Mutation techniques. pp. 159 – 194; in Plant breeding and farmer participation edited by Ceccarelli S, Guimarães EP, Weltzien E,FAO Rome; 2009.
29. Rohrmoser K. Manual on field trials under the Coopération Technique. CTA & GTZ, Eschborn; 1986. French
30. Khan MR, Qureshi AS, Hussain SA, Ibrahim M. Genetic variability induced by gamma irradiation and its modulation with gibberelline acid in M2 generation of Chickpea *(Cicer arietinum L.*). Pak. J. Bot. 2005;37(2):285–292.
31. Nouri H, Rahimi A, Pouzesh H. Investigation the effect of gamma rays on the amount of fat percentage and protein in Pinto bean of Khomein varieties (*Pinto beans* V. Khomein). Annals of Biological Research. 2012;3(6):2785–2789. Available Online: www.scholarsresearchlibrary.com.

32. Lekana-Douki JB, Oyegue Liabagui SL, Bongui JB, Zatra R, Lebibi J, Toure-Ndouo FS. *In vitro* antiplasmodial activity of crude extracts of *Tetrapleura tetraptera* and *Copaifera religiosa*. BioMed central Research Notes. 2011;4:506. Available: http://www.biomedcentral.com/1756-0500/4/506.
33. Kroqstad DJ, Gluzman IY, Kyle DE, Oduola AM, Martin SK, Milhous WK, et al. Efflux of chloroquine from *Plasmodium falciparum*: Mechanism of chloroquine resistance. Science. 1987;238(4831):1283-5.
34. Ljungström I, Perlmann H, Schlichtherle M, Scherf A, Wahlgren M. Methods in malaria research. MR4/ATCC, Manassas, Virginia; 2004.
35. Lambros C, Vanderberg JP. Synchronizarion of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. 1979;65:418-420.
36. Desjardins RE, Canfield J, Haynes D, Chulay JD. Quantitative assessment of antimalarial activity in vitro by a semi-automatic microdilution technique. Antimicrobial Agents and Chemotherapy. 1979;16:710-718.
37. Woodrow CJ, Dahlström S, Cooksey R, Flegg JA, Le Nagard H, Mentré F, et al. High-Throughput Analysis of Antimalarial Susceptibility Data by the World Wide Antimalarial Resistance Network (WWARN) *In vitro* Analysis and Reporting Tool. J. Antimicrob Agents Chemother. 2013;57:3121-30.
38. Harborne JB. Phytochemical Methods. Chapman and Hall. London; 1998.
39. Leconte O, Bonfils JP, Bauvaira Y. Protective effect of iridals from saponin injury in *Candida albicans* cells. Phytochemistry. 1997;44:575-579.
40. Hagerman AE. Radial diffusion assay: Method for determining tanins in plant extracts. J. Chem. Ecol. 1988;13:437-449.
41. Peng S, Jay-Allemand C. Use of antioxidants in extraction of tannins from valnut plats. J. Chem. Ecol. 1991;17:887-896.
42. Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavonoids. Spring-Verlag, New York; 1970.
43. Wagner H, Blat S, Zgainski EM. Plant drug analysis. A thin layer chromatography. Atlas translated by Th. A. Scott. Spring-Verlag, Berlin Heidelberg, New York. 1984;145–146.
44. Etienne J, Clauser E, Housset C, Roingearg P. Biochimie génétique –Biologie Moléculaire. 9éme Ed., Elsevier Masson, Issy-les-Moulineaux, France; 2006.
45. Cain ML, Damman H, Lue RL, Yoon CK. Découvrir la biologie. 1èr Ed., de Boeck, Nouveaux Horizons, 3éme tirage 2010, Varese, Italie; 2006. French.
46. Bernot A: Analysis of genomes, transcriptomes and Proteomes. 3rd Ed, Dunod, Paris, France; 2001. French.
47. Maftah A, Petit JM, Julien R. Mini Manual Molecular Biology. Dunod, Paris, France; 2007. French
48. Dehpour AA, Gholampour M, Rahdary P, Jafari Talubaghi MR, Hamdi SMM. Effect of gamma irradiation and salt stress on germination, callus, protein and proline in rice (*Oryza sativa* L.). Iranian Journal of Plant Physiology. 2011;1(4):251 – 256.
49. Dhakshanamoorthy D, Selvaraj R, Chidambaram A. Physical and chemical mutagenesis in *Jatropha curcas* L. to induce variability in seed germination, growth and yield traits. Rom. J. Biol. - Plant Biol. 2010;55(2):113–125.
50. Khan S, Al-Qurainy F, Anwar F. Sodium azide: A chemical mutagen for enhancement of agronomic traits of crop plants. Environmental & We an International Journal of Science & Technology. 2009;4:1–21. Available online at www.ewijst.org.
51. Borzouei A, Kafi M, Khazaei H, Naseriyan B, Majdabadi A. Effects of gamma radiation on generation and physiological aspects of wheat (*Triticum aestivum* L.) seedlings. Pak. J. Bot. 2010;42(4):2281–2290.

52. Akhar FK, Baghrei A, Moshtaghi N, Nezami A. The effect of gamma radiation on freezing tolerance of chickpea (*Cicer aretinum* L) at *In vitro* Culture. J. Biol. Environ. Sci. 2011;5(14):63-70.
53. Al-Safadi B. and Simon P.W. Gamma irradiation-induced variation in carrots (*Daucus carota* L.). J. AMER. SOC. HORT. SCI. 1996;121(4):599–603.
54. Ramesh HL, Yogananda VN, Munirajappa Effect of Gamma radiation on morphological and Growth parameters of mulberry variety Ms. International Journal of Science and Nature. 2012;3(2):447–452.
55. Shah TM, Mirza JI, Haq MA, Atta BM. Radiosensitivity of various Chickpea genotypes in M1 Generation I-Laboratory Studies. Pak. J. Bot. 2008;40(2):649–665.
56. Al-Qurainy F. Effects of sodium azide on growth and yield traits of *Eruca sativa* (L.). World Applied Sciences Journal. 2009;7(2):220-226. ISSN 1818–4952, IDOSI Publications.
57. Available: Http://www.thecanadianencyclopedia.com/: Sélection des plantes.
58. Bounias M. Effets primaires et mutagènes de l'irradiation gamma des graines d'*Arabidopsis*; 1973.
Available:www.arabidopsis.org/ais/1973/BOUNI-1973-aabba.html-).
59. Sakin MA, Sengar O. The effects of different doses of Gamma Ray and EMS on formation of Chlorophyll Mutations in Durum Wheat *(Triticum durum Desf.).* Tarim Bilimleri Dergisi. 2002;8(1):15–21.
60. Bhosale UP, Hallale BV. Gamma radiation induced mutations in black gram *(Vigna mungo* (L.) Hepper*).* Asian Journal of Plant Science and Research. 2011;1(2):96–100. Available Online: www.pelagiaresearchlibrary.com.
61. Devi SA, Mullainathan L. Effect of gamma rays and Ethyl methane sulphonate (EMS) in M3 generation of Blackgram (*Vigna mungo* L. Hepper). Afr. J. Biotechnl. 2012;11(15):3548–3552.
62. Radfar M, Aharizad S, Mohammadi SA, Zaefizadeh M. The effects of gamma irradiation and PEG on germintion of two Varieties of Rapessed. Scholars Research Library, Annals if Biological Research. 2012;3(4):1828 – 1832. Available Online at www.scholarsresearchlibrary.com.
63. Sarduie-Nasab S, Sharifi-Sirchi GR, Torabi-Sirchi MH. Assessment of dissimilar gamma irradiations on barley (*Hordeum vulgare* spp.). Journal of Plant Breeding and Crop Science. (2010): 2(4):059–063.
Available Online: http://www.academicjournals.org/jpbcs.
64. Sakin MA. The use of induced Micro-Mutations for quantitative Characters after EMS and Gamma Ray Treatments in Durum Wheat Breeding. Pakistan Journal of Applied Sciences. 2002;2(12):1102–1107.
65. Kovács E. and Keresztes À. Effect of gamma and UV-B/C radiation an plant cells. Micron. 2002;33:199–210. Pergamon.
66. Shabnam AA, Jaimini D, Sarkar C, Phulara P, Pawar SE. Evaluation of gamma rays-induced changes in oil yield and oleic acid content of niger, Guizotia abyssinica (L.f.) Cass. Current Science. 2011;101(25):490–491.
67. Bhosle SS, Kothekar VS. Mutagenic efficiency and effectiveness in clusterbean (*Cyamopsis tetragonoloba* (L.)TAUB.). Journal of Phytology. 2010;2(6):21–27. Available Online: www.journal-physiology.com.
68. Ando A. Montalván R. Gamma-ray radiation and sodium azide (NaN3) mutagenic efficiency in rice. Crop Breeding and Applied Biotechnology. 2001;1(4):339-346.
69. Hopkins WG. Plant Physiology. Translation of the 2nd Ed By Serge American Rambour. Scientific Lichens Charles-Marie Evrard. De Boeck, Brussels; 2003. French.

70. Zhang R, Kang KA, Kang SS, Park JW, Hyun, JW. Morin (2', 3, 4', 5, 7-Pentahydroxyflavone) Protected cells against Ɣ-Radiation-Induced Oxidative stress. Basin & Clinical Pharmacology & Toxicology, Nordic Pharmacologycal Society. 2010;1-10.
71. El-Hamid AA. Biosynthesis of Phenolic compounds and Water soluble vitamins in Culantro (*Eryngium foetidum L*.) Plantlets as affected by low doses of Gamma Irradiation. Analele Universitatii din Oradea – Fascicula Biologie, Tom XVII. 2010;2:356–361.
72. Macheix JJ, Fleuriet A, Jay-Allemand C. Phenolic Compounds plants: An example of secondary metabolites of economic importance, Ed. Polytechnic and university romandes presses, Lausanne, Italie; 2005. French.
73. Cimanga RK, Tona L, Luyindula N, Mesia K, Lusakibanza M, Musuamba CT, et al. *In vitro* antiplasmodial activity of callus culture extracts and fractions from fresh apical stems of *Phyllantus niruri* L. (*Euphorbiaceae*): Part 2. Journal of Ethnopharmacology. 2004;95:399-404.

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