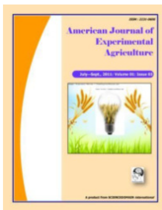

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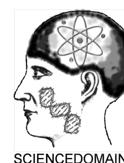

Effects of Sodium Azide (NaN_3) on Seeds Germination, Plantlets Growth and *In vitro* Antimalarial Activities of *Phyllanthus odontadenius* Müll. Arg.

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Authors' contributions

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. Authors SLN and RKN managed the analyses of the study. Author CF managed the literature Search and contributed to analysis in the field. All authors read and approved the final manuscript.

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ABSTRACT

Aims: "This study aims to investigate the production of secondary metabolites of *P. odontadenius* against malaria using some concentrations of sodium azide (NaN_3) and to choose those with high *in vitro* antimalarial activities."
Study Design: Laboratory experiment tests; Identification of plant material, Immersion of seeds in SA concentrations, *In vitro* culture of seeds, *In situ* culturing plantlets, Extraction of *Phyllanthus odontadenius* aerial parts, Phytochemical screening, *in vitro* antiplasmodial tests to determine the

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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 Kinshasa XI, Democratic Republic of the Congo (DRC). National Institute of Biomedical Research (NIBR) at Kinshasa/Gombe (DRC). The experiments were conducted firstly during August and December 2011 and secondarily during May and September 2012.
Methodology: Seeds of *P. odontadenius* were obtained after oven drying at 45℃ and they were immersed in SA at concentrations ranging firstly between 0 to 10 mM; secondarily between 0 to 20 mM. Seeds were germinated on Murashige and Skoog medium (MS) and plantlets were transferred *in situ*. In addition some parameters such as height, collar diameter, number of branches and biomass from first generation were analyzed. Phytochemical screening was released. The *in vitro* antiplasmodial activities assays on clinical isolates of *P. falciparum* was determined.
Results: Results showed that SA had positive effects on growth parameters of *P. odontadenius* in the M1 generations with greater effects observed with treatment exceeding 10 mM. For the *in vitro* antimalarial activities from to extracts obtained with aerial material parts from directly immersed seeds (M1), the effects observed with plant extracts from seeds dipped in SA were higher than those from untreated seeds. IC_{50} values were ranged between 1.04±0.02 µg/ml (10 mM) to 12.77±5.83 µg / ml (0 mM) for the first assay. And for the second test, the *in vitro* antiplasmodial activities varied between 1.47±1.07 µg/ml (10 mM) to 21.60±7.13 µg/ml (2.5 mM). The best activities were observed with SA solutions exclusive of 5 mM to 10 mM. The SA lethal doses were 4.76 mM for LD_{30} and 10.99 mM for LD_{50}.
Conclusion: Treatment of *P. odontadenius* seeds with SA induced stimulation of parameters which increase linearly with increasing concentrations of SA. Some secondary metabolites were synthesized for example alkaloids compounds compared to the untreated seeds of *P. odontadenius* with a more important synthesis in phenolic compounds. The *in vitro* antiplasmodial activities on the clinical isolates *P. falciparum* showed low antimalarial activities from M1 controls (0 mM) than that of extracts from treated plants. The high inhibitory effects (1,04±0.02 µg/mL or 1.47±1.07 µg/mL for 10 mM) of crude extracts plants from treated seeds have justified the usefulness of SA in plant breeding particularly in the increasing production of secondary metabolite against malaria in the World.

Keywords: Phyllanthus odontadenius; Sodium Azide (SA); secondary metabolites; malaria; antimalarial activity.

1. INTRODUCTION

Plants have been used in traditional medicine since a long time. About 13,000 plant species have been used as drugs throughout the world, and approximately 25% of the current materials medical are derived from plants in form of teas, extracts, or pure substances [1,2]. Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world [3]. In the Democratic Republic of Congo (DRC), among the species used in the treatment against malaria, *Phyllanthus odontadenius* is well positioned for different previous studies on this plant [4-6]. *P. odontadenius* is one of the most important medicinal plants used in different regions in the world for the treatment of various diseases such as jaundice, asthma, hepatitis, flu, dropsy, diabetes, fever causing by malaria [7-9] but its availability is drastically decreasing because of numerous harvests.

Malaria is the most important parasitic disease in tropical areas. The estimated clinical cases for WHO were 216 million in 2010, 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 [10-12]. 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. In the last decades resistance to several antimalarials drugs became widely disseminated, while the cost of effective treatment is prohibitive for the large majority of the populations in these areas. It continues to cause morbidity and mortality on a large scale in tropical countries. There is an urgent need for new chemotherapeutic compounds, which are easy to administer and store, and which are of low cost [13,14].

Mutations are the tools used by the geneticists to study the nature and function of genes which are the building blocks and basis of plant growth and development, thereby producing raw materials for genetic improvement of economic crops [15]. It is known that various chemicals have positive or negative effects on living organisms. Chemical mutagen generally produce induced mutations which lead to base pair substitution especially GC► AT (guanine:cytosine to adenine:tymine) resulting in amino acid changes, which change the function of proteins but do not abolish their functions as deletions or frame shift mutations mostly [15,16]. These chemomutagens induce a broad variation of morphological and yield structure parameters in comparison to normal plants.

Sodium azide (NaN_3), which has been demonstrated to have these effects, is a mutagen and it has proved to be one of the most powerful mutagens in crop plants. It is a common bactericide, pesticide and industrial nitrogen gas generator if known to be highly mutagenic in several organisms, including plants and animals [17,18]. The mutagenicity created by NaN_3 is mediated through the production of an organic metabolite of azide compound, presumably azidoalanine (N_3-CH_2-$CH(NH)_2$-COOH). The production of this metabolite was found to be dependent on the enzyme O-acetylserine sulfhydrylase (E.C.4.2.99.8.) [16]. In order to understand that sodium azide is mutagenic mechanism used for the improvement economic characters to many studies in rice, wheat, Barley and Sorghum [19].

In this report, we studied the mutagenic effects of sodium azide on growth and yield traits of *Phyllanthus odontadenius*. Secondarily, to evaluate the effects of sodium azide on the production of active secondary metabolites in *P. odontadenius* aerials parts against *Plasmodium falciparum*. For that, concentrations of sodium azide presented high *in vitro* antimalarial activities were determined.

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 Immersion of seeds in SA solutions

Seeds of *P. odontadenius* used in this work were obtained by drying plant fruits which harvested on the Kinshasa university landscape. After seeds selecting and counted by binocularly microscopic Lens Cole Palmer, VERNON HILLS, HILLINOIS 60061, 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 1 M phosphate buffer, pH 3, filtered and stored frozen it, at -20℃. Stock solution was diluted successively in water as well as in phosphate buffer of pH 3 to obtain various concentrations (0.5 mM,1.5 mM, 2.5 mM, 3.5 mM, 4.5 mM, 5 mM and 10 mM) and (2.5 mM, 5 mM, 7.5 mM, 10 mM, 12.5 mM, 15 mM, 17.5 mM and 20 mM) 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℃± 2). Immediately after treatment of sodium azide, the seeds were washed thoroughly in distilled water to reduce the residual effect of the mutagen on the seed coat during 4-5 times to remove excess sodium azide. A portion of seeds were submerged in deionized water for the same period of time served as control.

2.1.3 *In vitro* seeds germination

Seeds were disinfected with 70% (v/v) ethanol for 1 min, sterilized with 0.125% (w/v) HgCl2 for 3 min, and washed with sterile distilled water. They were then handled with gibberellic acid (GA3) 200 mg/L for 4 h and finally drained before being cultivated on modified Murashige and Skoog (MS) basal medium without sucrose or growth regulators and supplemented with 0.8% agar [20,21]. The pH of the medium was adjusted to 5.6 before autoclaving at 121℃ for 15 min. Cultures were incubated at 25±1℃ under fluorescent light with 16 h photoperiod. Percentage of germinated 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 [19] below:

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

2.1.4 *In situ* seedling transfer

Plantlets growths *in vitro* were transferred in polyethylene bags containing 300 g of soil for *in situ* growth [22]. Bags were then buried in 3/4 in the ground in randomized complete block (RCB) design with 3 replications [19, 23-25]. The plantlets placed *in situ* were watered three times a week, the odd days, with the same amount of water (20 L per plot 5 dm/6 dm). 6 plants from each replicate were used for the measurement of plant growth.

2.1.5 Seedling growth

Parameters such as collar diameter, shoot length, number of branches for the selected M1 plants were measured after four months of sowing. 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 Phytochemical Analysis

2.2.1 Preparation of crude extracts

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

2.2.2 Phytochemical screening

The chemical screening was carried out on all crude extracts. Alkaloids were detected with Draggendorff's and Mayer's reagents [26]. Saponins were detected by frotting test [26,27]. Presence of tannins was detected using Stiasny reagent and ferric chloride 2% [26,28,29]. Flavonoids were detected using Shinoda's reagent or aluminum chloride 5% [30]. Anthraquinones were detected using Börtranger's reagent. Anthocyanins were identified using HCl 2N, heating and add iso-amylic alcohol. Steroids and terpenoids were identified using Liebermann-Bouchard's reagent [26].

The presence of the different chemical groups was confirmed by Thin Layer Chromatography (TLC) performed on silica gel plates GF254 (Merck, Germany). Alkaloids were detected using CHCl3/MetOH/NH4OH (9:2:0.5) and EtOAc/Iso-PrOH/NH3 (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 CHCl3/MeOH (9:1) and n-Hexane/MeOH: (9:1) on mobile phases and Liebermann-Bouchard's reagent. After drying, the plate was heated at 110℃ for 10 min intensify the spot colors. Tannins, mainly proanthocyanidins were detected using EtOAc/HOAc/HCOOH/H2O (30:02:1.2.8) (upper phase) as mobile phase and 1% Vaniline and 5% H2SO4 as reagents. After spraying, drying and heating the plate, proanthocyanidins were colored in red while flavonoids appear in yellow color. Anthraquinones were identified using CHCl3/MeOH (7:3) as mobile phase and Börtranger's reagent [26,31].

2.3 *In vitro* Antimalarial Activity

Antimalarial activity assays were performed at the National Institute of Biomedical Research (NIBR) in Kinshasa/Gombe, DR. Congo.

The stock solutions were 20 mg/ml extracts. These solutions were prepared in 1% DMSO (Dimethyl sulfoxid, Merck KGaA) and diluted in two fold to have test concentrations. 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. Maternity Hospital of the Sisters of Kindele (Mont-Ngafula in Kinshasa) was our site for blood samples. Venous blood samples (4 ml) were collected in tubes containing 1% heparin, and centrifuged for 5 min at 3000 rpm to separate plasma and erythrocytes. 1 ml of erythrocytes was mixed with 9 ml of RPMI 1640 containing 25 mM HEPES, 25 mM sodium bicarbonate and 10% of pooled human serum. After homogenization, 50 µl of the suspension

were distributed in each well of a spot plate containing decreasing concentrations of extracts [32]. Plates were then maintained at 37°C in a humid atmosphere containing 5% CO2. Quinine was used as control. After 48 h of 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:

$$\text{Inhibition (\%)} = (A - B/A) \times 100$$

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.

2.4 Statistical Analysis

Data were subjected to Analysis of Variance (ANOVA) using MSTAT-C Software [35] and compared to the software Statistica software 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 Sodium Azide (NaN_3) on the Growth of *P. odontadenius*

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

In view of Table 1, it is clear that with the exception of the rate of *P. odontadenius* seeds germination the witness showed high value is 16.33±0.58%, all other parameters showed weak values comparing to the control. The high values are those of 10 mM. However the values of the witness not differ significantly from that at 10 mM (11.0±1.32%). With the exception of germination where the control does not differ significantly from 10 mM, the values of other parameters (size, collar diameter, number of branches and the fresh biomass of plant) with higher values than the control significantly different to those of the latter to a confidence level of 5%.

Table 2 shows that the control plants exhibit lower values for most cases compared to plants from seeds soaked in 20 mM of sodium azide solution. Lowest values were found in most plants whose seeds were soaked in 7.5 mM of sodium azide solution. When control values compared with those of 10 mM, the latter has higher values than the control for all measured parameters. With the exception of germination 11.33±2.67% for 5 mM and 6.67±2.0% for 10 mM in Table 2, the measured values of other parameters in plants whose seeds were soaked in 5 mM and 10 mM does not differ.

In view of Tables 3 and 4, it is apparent that the alkaloids have been found present in all extracts plants from treated seeds (Table 3) and they were only present in extracts plants from seeds treated at 5 mM; 7.5 mM; 10 mM; 17.5 mM and 20 mM concentrations of SA. They are absent in the controls in Tables 3 and 4 and then in the extracts plants from seeds treated with SA at 2.5 mM; 12.5 mM and 15 mM concentrations (see Table 4). Saponins are absent everywhere, while flavonoids are found everywhere such as tannins in the two tables (3 and 4). Anthocyanins are present in the control extracts as in the treated extracts except at concentrations of 1.5 mM and 2.5 mM in Table 3 and 15 mM in Table 4. Steroids and terpenoids are absent in the control extracts as extracts plant from seeds treated at 2.5 mM and 3.5 mM concentrations of SA (Table 3). They are present in the control as treated extracts in Table 4 where the anthraquinones and free quinones are absent. However, the free quinones are absent in controls and in 4.5 mM and 5 mM. Anthraquinones are present in the control and in 1.5 and 10 mM concentrations of SA (Table 3).

3.2 Sodium Azide Chemosensibility of *P. odontadenius*

The Sodium Azide (NaN_3) chemosensibility of *P. odontadenius* was determined by seeds germination or by length of plantlets. Results obtained were showed in Figs. 1 (a-d).

In view of Fig. 1, it is apparent 4 linear regression equations as $y = -3.210\ x + 85.27$ for Fig.1a; $y = -0.579\ x + 132.2$ for Fig.1b; $y = 7.448 + 78.17\ x$ for Fig.1c and finally the equation: $y = 0.163\ x + 3.438$ for Fig.1d.

Lethal doses 50 (LD_{50}) and 30 (LD_{30}) calculated from these equations of linear regression are shown in Table 5.

Table 1. Effects of sodium azide (NaN_3) on seeds germination and plant growth of *Phyllanthus odontadenius*

mM of NaN_3 Parameters	0	0.5	1.5	2.5	3.5	4.5	5	10	CV (%)	Lsd
Rate (%) of seeds germination	16.33±0.58a	15.00±0.77a	11.0±0.29 a	14±0.5 a	9.33±0.36 a	15±0.44 a	5.33±0.32 a	11±1.32 a	29.10	12.446
Emergence reduction (%)	0	37.51	79.97	39.98	79.97	92.50	87.47	69.99	_	_
Size (cm) of plants	**16.02±2.56 b**	18.21±4.22 ab	16.6±4.36 b	17.32±2.92 b	17.65±3.30 b	18.30±3.51 ab	18.50±3.21 ab	**20.16±3.89 a**	21.39	14.66
Collar diameter (mm) of plants	**1.56±0.27 bc**	1.59±0.17 bc	1.60±0.24 bc	1.60±0.30 c	1.61±0.30 bc	1.72±0.25 ab	1.68±0.24 ab	**1.87±0.22 a**	15.87	0.067
Number of branches	12±1.96 bcd	12.05±2.96 d	12.40±2.0 abcd	**11.85±1.35 cd**	12.85±1.84 a	12.95±174 abc	13.6±243 ab	**13.8±2.20 a**	18.14	5.313
Biomass of aerial part (g)/plant	1.63±0.52 b	**1.45±0.46 ab**	1.75±0.56 ab	1.66±0.59 ab	1.79±0.51 ab	1.81±0.63 ab	1.81±0.50 ab	**2.08±0.61 a**	32.72	0.334

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

Table 2. Effects of sodium azide (NaN_3) on Seeds germination and plant growth of *Phyllanthus odontadenius*

mM of NaN3 Parameters	0	2.5	5	7.5	10	12.5	15	17.5	20	CV (%)	Lsd
Rate (%) of seeds germination	5.33±089 cde	7.33±1.11 bc	**11.33±2.67 a**	**4.33±10 e**	6.67±2.0 bc	7.0±15 bc	5.67±3.78 cd	4.67±0.83 de	8.33±0.92 b	15.60	2.208
Emergence reduction (%)	0	-37.52	-112.57	18.76	-25.14	-31.33	-6.38	12.38	-56.29	-	-
Size (cm) of plants	11.83±2.55 ab	10.87±2.9 ab	14.31±2.54 a	**8.18±2.18 b**	12,6±2.35 ab	**15.22±0.82 a**	11.73±1,56 ab	14.05±2.26 a	14.47±0.55 a	12.36	5.465
Collar diameter (mm) of plants	1.15±0.21 cde	1.05±0.27 de	1.60±0.31abc	**0.85±0.25 e**	1.38±0.15 bcde	1.82±0.06 ab	1.47±0.26 abcd	1.61±0.34 abc	**1.95±0.04 a**	10.7	0.5328
Number of branches	10.53±1.33 cde	9.6±1.85 de	13.33±1.47 abc	**7.43±1.68 e**	11.3±2.31 bcd	14.37±0.71 ab	10.93±0.95 bcde	12.3±2.78 abcd	**15.5±1.21 a**	8.86	3.642
Biomass (g)/plant	2.03±1.1a	**1.72±0.95 a**	2.79±1.40 a	2.05±0.60 a	2.81±1.30 a	2.57±1.56 a	2.24±0.58 a	2.62±0.81 a	**3.05±0.22 a**	28.85	2.464

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

Table 3. Phytochemical screening of *P. odontadenius* crude extracts from M1 plants

Chemical groups analyzed	Treatments								Natural plant
	0	0,5	1,5	2,5	3,5	4,5	5	10	N
Alkaloids	–	+	+	+	+	+	+	+	–
Saponins	–	–	–	–	–	–	–	–	–
Anthocyanins	+	+	–	–	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+
Free quinones	–	+	+	+	+	–	–	+	–
Anthraquinones	+	–	+	–	–	–	–	+	+
Steroids and terpenoids	–	+	+	–	–	+	+	+	–
Flavonoids	+	+	+	+	+	+	+	+	+

Legend: N: Harvested natural plant; —: Absence; +: Presence

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

Chemical groups analyzed	Concentrations (mM) of sodium azide (NaN_3)								
	0	2 ,5	5	7,5	10	12,5	15	17,5	20
Alkaloids	–	–	+	+	+	–	–	+	+
Saponins	–	–	–	–	–	–	–	–	–
Anthocyanes	+	+	+	+	+	+	–	+	+
Tannins	+	+	+	+	+	+	+	+	+
Free quinones	–	–	–	–	–	–	–	–	–
Anthraquinones	–	–	–	–	–	–	–	–	–
Terpoids and steroids	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+
Polyphenols	+	+	+	+	+	+	+	+	+

*Legend: **+** : presence ; **–** : absence ; **+/–** : Trace*

Table 5. LD_{30} and LD_{50} from linear regression equation of seeds germination and plantlets length

Equation LD	y = -3.210x + 85.27 (0 – 10 mM)	y = -0.579x + 132,2 (0 – 20 mM)	y = 7.448x + 78.17 (0 – 10 mM)	y = 0.163x + 3.438 (0 – 20 mM)
30	4,76 Mm	107,43 mM	-1,10 mM	408,36 mM
50	10,99 mM	141,97 mM	-3,78 mM	285,66 mM

In view of Table 5, it appears that the parameter showed a stimulation effect of sodium azide (NaN_3) gives values of DL_{30} and DL_{50} that far exceed the concentrations used for testing. Only the germination of soaked seeds at concentrations varying from sodium azide 0 - 10 mM, which showed values of DL_{30} and DL_{50} which only deviates DL_{50} but also not too relevant concentrations.

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

The Figs. 2 (a – b) showed that low values of IC_{50} (µg/mL) were those obtained with plants from seeds immerged in 5 and 10 mM solutions (1.09±0.13 and 1,04±0.02 µg/mL)(Fig. 2a). It's the same for plants obtained by immersion of seeds to 0 – 20 mM ; the low values were those at 5 to 10 mM (4.32±0.38 µg/mL for 5 mM ; 1.90±1.75 µg/mL for 7.5 mM ; 1.47±1.07 µg/mL for 10 mM). These concentrations (5 – 10 mM) have high antiplasmodial activities which were showed in Fig. 2a and Fig. 2b. than all concentrations of sodium azide.

Comparing Fig.1a the IC_{50} according with LD_{50} (Fig. 2a and Fig. 2b), concentrations of 5 to 10 mM were promising for plant breeding with sodium azide for treatment of malaria disease.

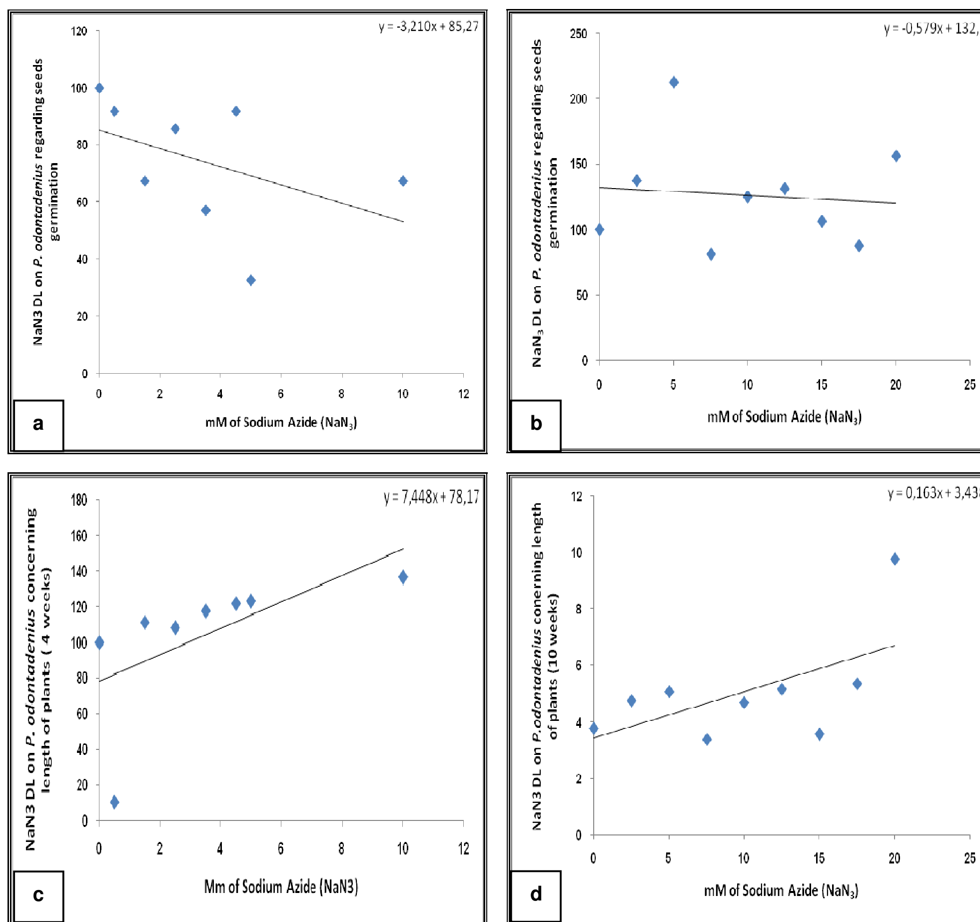

Fig. 1. Determination of Sodium Azide (NaN_3) chemosensibility on *P. odontadenius*; a : by seeds germination using 0 – 10 mM of NaN_3; b: by seeds germination using 0 – 20 mM of NaN_3; c: by length of M1 plants after 4 weeds (0 – 10 mM); d: by length of M1 plants after 10 weeds (0 – 20 mM)

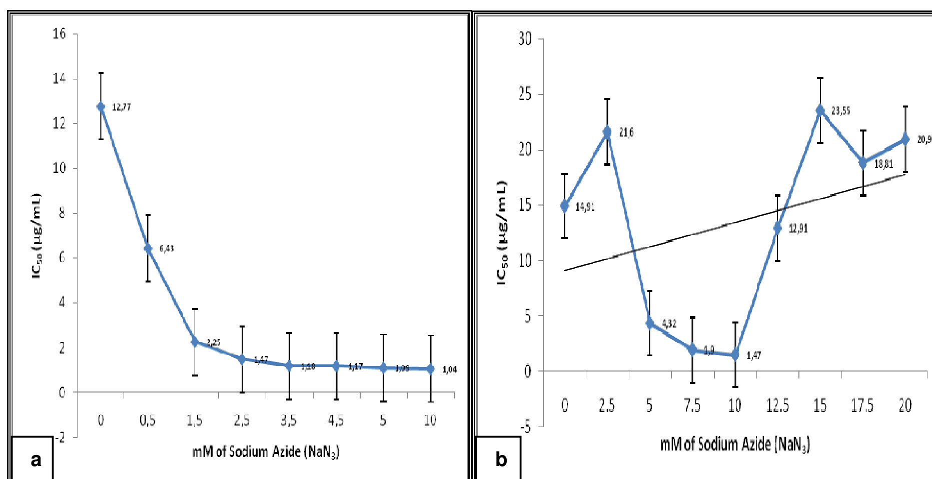

Fig. 2. Effects of aqueous crude extracts of *P. odontadenius* M1 plants from seeds treatment with NaN_3 solutions (a: IC_{50} of M1 plants from seeds treated with 0 – 10 mM NaN_3 solutions; b: IC_{50} values of M1 plants from 0 – 20 mM NaN_3 solutions)

4. DISCUSSION

Sodium azide (NaN_3) is powerful chemical mutagen, safer and more efficient, which increases the yield and quality of field crop against harmful pathogens [16]. This mutagen is limited to only a few plant species [36,19]. [16] reported in addition that sodium azide (NaN_3) affects the rate of seed germination, shoot and root length, and seed germination delay. They reported further that chemical mutagens induce a large variation to the parameters of morphological structure and performance compared to plants whose seeds were not treated with NaN_3. In general, all these parameters decrease with increasing doses of irradiation or chemical mutagen. [37] confirm a reduction germination rate and height with the seeds of *Jatropha curcas* L. where mutagens (EMS and gamma rays) are used to obtain the interesting traits.

The results obtained in this study show that *P. odontadenius* is likely to be improved by sodium azide (NaN_3). It has been found that, contrary to the effects of chemical mutagens reported by the authors above; including reduced height, collar diameter, number of branches and fresh biomass; SA stimulated most of these parameters. If seed germination was affected when they were dipped in solutions ranging from 0-10 mM as confirmed [37], the other parameters indicate that their values have been stimulated at high concentrations than low concentrations of SA (0 - 2.5 mM or 0 - 7.5 mM) most surprisingly affected plant growth. [15] reported that sodium azide (SA) causes reversible inhibitory effects and thus. The toxicity of SA and most of its physiological effects can be traced to its reversible inhibitory effect on enzymes containing a coordinated divalent ion, such as those of cellular respiration.

Height, collar diameter and number of branches per plant would be subject to the expression of related genes and disruption at the molecular level that affects the gene or groups of genes automatically lead to the same effects on these three parameters. These genes could be those controlling the synthesis of growth regulators such as auxins and cytokinins [38,39].

Results on length, collar diameter and number of branches of *P. odontadenius* plant confirm also the hypothesis by the fact that at the same concentration, when the length showed a high or low value, automatically the other two parameters, collar diameter and number of branches have also the same effects. Otherwise the observed value of a parameter not showed significant difference at the 5% confidence values between low or high values of other parameters.

The decrease in biomass could be attributed to disturbances in the synthesis of chlorophyll, reliable molecular index for the assessment of genetic effects and gas exchange at the plant [40,41]. [42] showed that the fresh and dry biomass of *Arabidopsis thaliana* seedlings subjected to low doses of gamma irradiation were not changed significantly.

The results obtained on the fresh biomass of *P. odontadenius* plants from seeds treated with NaN_3 corroborate [42]. Results on fresh biomass show that they have little varied and showed no significant difference in confidence level of 5%.

It is known that besides conventional primary metabolites (carbohydrates, proteins, lipids and nucleic acids), plants often accumulate some compounds so-called "secondary metabolites". These molecules are not directly involved in plant development but rather involved in relations with biotic or abiotic stress. On the one hand, they increase the efficiency of breeding and secondarily, they represent an important source of molecules used by humans in areas as diverse as pharmacology or food. Secondary metabolites are present in all plant species but differ from one species to another and play an essential role in plant metabolism and development [43,44].

Indeed, [45] showed that the amount of increase in total flavonoids of *Centella asiatica* when stems of this plant suffered acute radiation from 0 to120 Gy for 5 days compared with the control. Many other authors have worked to highlight the role that play secondary metabolites in protecting plants against stress due to ionizing radiation [42, 46 and 49].

[50] had reported that to *Phyllanthus* they have described as *Phyllanthus niruri* plant contained alkaloids, polyphenols, flavonoids, tannins, terpenes and / steroids but didn't contain anthraquinones and saponins. The chemical analysis of large groups of *P. odontadenius* plants from seeds treated with sodium azide (NaN_3) reveals the presence of alkaloids in concentrations of 0.5 mM ; 1.5 mM ; 2.5 mM ; 3.5 mM ; 4.5 mM ; 5 mM and 10 mM (Table 3) and in

concentrations of 5 mM ; 7.5 mM ; 10 mM ; 17.5 mM and 20 mM (Table 4). This presence could be explained by a possible stimulation of the biosynthesis of the alkaloids which were synthesized in most cases from to a small number of amino-acids such as tyrosine, tryptophan, ornithine, arginine and lysine [43].

[51] reported that *Phyllanthus emblica* L. contains alkaloids and saponins that are not found in *P. odontadenius*. However, flavonoids, phenols, triterpenoids and tannins are present both in *P. odontadenius* and in *P. emblica*. Quinones are absent in the two species of *Phyllanthus*.

Considering the results obtained in this work, it appears that according to the criteria of the WHO classification of *in vitro* antiplasmodial activities of extracts or drugs [52] ; [32] , extracts from two tests behaved differently. In the first trial (Table 3), the control (0 mM) extracts with 12.77±5.83 µg/ml value showed moderate *in vitro* antiplasmodial activity (IC_{50} between 10-50 µg/ml). Extracts of plants from treated seeds at 0.5 to 10 mM with their respective values of 6.43±6.13 µg/ml (0.5 mM), 2.25±0.16 µg/ml (1.5 mM), 1.47±0.14 µg/ml (2.5 mM), 1.18±0.068 µg/ml (3.5 mM), 1.17±0.06 µg/ml (4.5 mM) 1.09±0.13 µg/ml (5 mM) and 1.0 ±0.02 µg/ml (10 mM) showed good *in vitro* antiplasmodial activities (IC_{50} <10 mcg / ml). In the second experiment; extracts from control plants (0 mM), 2.5 mM, 12.5 mM, 15 mM, 17.5 mM and 20 mM exhibited moderate *in vitro* antiplasmodial activities (values between 10 to 50 µg/ml) with respective IC_{50} values of 14.91±3.85 µg/ml, 21.6±7.13 µg/ml; 12.91±6.06 µg/ml; 23.55±10.73 µg/ml; 18.81±3.37 µg/ml and 20.95±8.30 µg/ml [52]. And extracts of plants from seeds treated with concentrations of 5 mM; 7.5 mM and 10 mM of SA exhibited good *in vitro* antiplasmodial activity (IC_{50} <10 µg/ml) with respective values of 4.32±0.38 µg/ml; 1.90±1.75 µg/ml and 1.47±1.07 µg/ml .

The *in vitro* antiplasmodial activity of plants aqueous extracts from seed treated firstly from 0.5 mM to 10 mM and secondly from 5 mM to 10 mM of SA (NaN_3) could be explained by the presence of alkaloids synthesized in plants which disclosed in some concentrations of SA. [52] report that among the agents contain antimalarial or antiplasmodial activities from plants exist further alkaloids, terpenes and related compounds, flavonoids, chromones, xanthones, and anthraquinones and various related compounds and others such as S- isogeranyle and isovaleric acids. These compounds were found in all the different aqueous extracts of plants grown from seeds treated with NaN_3.

5. CONCLUSION

The main objective of this study was to improve the plant *P. odontadenius* by chemical mutagenesis in order to a possible fight against parasite of malaria. The effects of sodium azide (NaN_3) on *P. odontadenius* plants from seeds soaked in different solutions of sodium azide (NaN_3) show that this has more mutagenic stimulatory effects than inhibitors effects opposite to *P. odontadenius*.

This study showed that the length of plants ranged from 16.02±2.56 cm (0 mM) to 20.16±3.89 cm (10 mM); collar diameter from 1.56±0.27 mm (0 mM) to 1.87±0.22 mm (10 mM); the number of branches from 11.85±1.35 (2.5 mM) to 13.80±2.20 (10 mM) and fresh biomass from 1.45±0.46 mg (0.5 mM) to 2.08±0.16 mg (10 mM).

The *in vitro* antiplasmodial activity of aqueous extracts of plants from seeds soaked in different solutions of sodium azide (NaN_3) shows that concentrations between 5-10 mM with respective IC_{50} of 1.09±0.13 µg/mL (5 mM) and 1.04±0.02 µg/mL (10 mM) or 4.32±0.38 µg/mL (5 mM) 1.90±1.75 µg/mL (7.5 mM) and 1.47±1.07 µg/mL (10 mM) are those that deserve to be used in the improvement program of *P. odontadenius* secondary metabolites against *Plasmodium falciparum*.

Studies of the next generation or M2 are necessary to verify the heritable effects for to choose possible concentrations of plant breeding. The analysis of active principles involved in the death of trophozoites would be also necessary to justify the molecular effects of sodium azide in *P. odontadenius*.

However, it is necessary to know whether the plant extracts from these doses showed high antiplasmodial activity and also which one would present less toxicity on human cells. These studies are essential in the selection of plants with *P. odontadenius* antiplasmodial activity enhanced by sodium azide. Furthermore, the study of the fractions or molecules involved in improving the in *vitro* antiplasmodial activity by sodium azide is indispensable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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