European Journal of Medicinal Plants
18(3): 1-10, 2017; Article no.EJMP.30181
ISSN: 2231-0894, NLM ID: 101583475

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Effects of Sodium Azide (NaN_3) on Growth of *Phyllanthus odontadenius* M2 and Evaluation of *in vitro* Antiplasmodial Activity by Elisa HRP2

Rufin Kikakedimau Nakweti[1,2*], Véronique Sinou[3], Sébastien Luyindula Ndiku[1], François Sabot[4] and Claudine Franche[4]

[1]Division of Life Sciences, Department of Biotechnology and Molecular Biology, General Atomic Energy Commission, Regional Center of Nuclear Studies of Kinshasa (GAEC/RCNS-K.), P.O.Box 868 KIN.XI, Democratic Republic of the Congo.
[2]Institut Supérieur Pédagogique de Kenge (ISP/Kenge), Kenge 1, Province of Kwango, P.O.Box: 4728 Kin II, Democratic Republic of the Congo.
[3]Faculty of Pharmacy, UMR-MD3, Aix-Marseille University, Marseille, France.
[4]Institut de Recherche Pour le Développement (IRD), UMR-DIADE, Montpellier, France.

Authors' contributions

This work was carried out in collaboration between all authors. Author RKN designed the study, performed the statistical analysis, wrote the protocol for sodium azide (SA) treatment and wrote the first draft of manuscript. Author VS designed the study of antimalarial activities by ELISA HRP2, wrote the protocol on antimalarial activities on parasite strain K1. Author SLN managed the analyses of the study and author CF managed the literature searches and corrected French version of manuscript. Author FS corrected the first version of manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2017/30181
Editor(s):
(1) Shanfa Lu, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, China.
(2) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
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(3) T. Pullaiah, Sri Krishnadevaraya University, India.
(4) Imoru Joshua Oloruntobi, Obafemi Awolowo University Ile-Ife, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/18483

Original Research Article

Received 22nd October 2016
Accepted 2nd January 2017
Published 3rd April 2017

ABSTRACT

Aims: "This work was to increase the production of secondary metabolites of *P. odontadenius* M2 using sodium azide (SA) in order to amplify those with *in vitro* antimalarial activity using *Plasmodium falciparum* strain K1".

**Corresponding author: E-mail: frankciakika@gmail.com;*

Study Design: Laboratory experiment tests; Immersion of seeds in SA concentrations; *In situ* culturing seeds, Harvesting plants M1; seeds M2 collected; Sowing of M2 seeds in to fields; Extraction of *P. odontadenius* aerial parts; Phytochemical screening, *In vitro* antiplasmodial testes to determine the inhibition of concentrations killing 50% of Plasmodium falciparum strain K1.
Place and Duration of Study: Department of Biology and Molecular Biology; General Atomic Energy Commission, Regional Center of Nuclear Studies, P. O. Box.: 868 Kinshasa XI, Democratic Republic of the Congo (DRC). The *in vitro* antiplasmodial activities conducted at the Faculty of Pharmacy, UMR-MD3, Aix-Marseille University, Marseille, France. The experiments were conducted firstly during August and December 2011; secondarily during May and September 2012 until July 2014.
Methodology: Seeds M2 from of *Phyllanthus odontadenius* M1 plants were obtained after oven drying at 45°C and they were immersed in SA at concentrations ranging between 0 to 20 mM. They sow in two fields (M2.1 and M2.2) and plants harvested after four months. In addition, some parameters such as height, collar diameter, number of branches and biomass from second generation (M2) were analysed. Phytochemical screening was released. The *in vitro* antiplasmodial activities assays on Plasmodium falciparum strain K1 was determined by ELISA HRP2.
Results: Results obtained showed that SA generally reduced the growth parameters such as heigth and colar diameter that reduce directly fresh biomass with the lowest reduction being -11.42% (10 mM SA) and the higher -22.37% (17.5 mM SA). Alkaloids were absents in the all samples. Tannins were present only in the extracts treated with 10 mM SA and anthocyanins, flavonoids and steroids/terpenoids were observed in all samples. For the *in vitro* antimalarial activities, better activities were observed for M2 extracts obtained from plants treated with 7.5 mM (3.26±0.05 µg/ml and 4.87±1.57 µg/ml) and 10 mM (4.52±0.12 µg/ml and 2.73±1.67 µg/ml) SA.
Conclusion: In conclusion, SA has, in general, negative effects on growth parameters of *P. odontadenius* and enhances the *in vitro* antimalarial activity of *P. odontadeius* extracts.

Keywords: Phyllanthus odontadenius; sodium azide; second generation (M2); ELISA HRP2; antimalarial activity.

1. INTRODUCTION

Malaria is a disease of tropical origin. It is transmitted to humans through the bites of infected female *Anopheles mosquitoes*, called "malaria vectors", which bite mainly between from dawn to dusk [1,2]. Malaria is responsible for important socio-economic problems in all subtropical and tropical countries where it is endemic [3,4,5]. In Saharan sub-Africa, malaria is a major public health problem because it remains one of the dominant pathologies and life-threatening.

Two biggest killers of our time, Malaria and AIDS, are two infectious diseases that each year causes millions of deaths worldwide. The former caused by a parasite and the later caused by a virus, their effects on humans are very similar: they constantly thwart the defense mechanisms of the host and easily develop resistance to all active molecules that we oppose them. Their incidence and mortality inflict on poorest people and for that research on new drugs has become one of priorities [6].

The research for new therapeutic targets through the ethno-pharmacological and ethnobotany methods is an interesting way. Plant resources have already shown their interest, because the origin of two major antimalarial drugs discovery: quinine from to quinine bark (*Cinchona* sp) and artemisinin from *Artemisia annua*. Both these natural products were the basis for many major antimalarial hemisynthesis drugs such as chloroquine, amodiaquine, mefloquine and primaquine from quinine and artesunate, artemether and the artheter from artemisinin [3].

“Tropical African forests were packed considerable plant genetic wealth” (Pauwels, 1993). However, the deforestation of many forest ecosystems for country needs, firewood, construction, artwork, etc. and domestication difficulties of most plant species lead to a reduction in biodiversity and consequently of plant protection potential of these ecosystems that are perceived as valuable losses [7].

Phyllanthus species, for example *Phyllanthus odontadenius*, are among the most important medicinal plants of *Phyllanthaceae* family

(Martynov, 2009). They are used in the Democratic Republic of Congo (DRC) and in several regions of the world for the treatment of various diseases [8,9,10,11].

Obtaining new varieties of plants with interest agronomic or pharmaceutical traits is feasible through the use of physical or chemical mutagens [12,13]. These mutagens could produce a random or targeted biochemical or physiological mechanism of the plant. Sometimes, they allow accumulation of adaptive genes in different conditions [14,15].

Many studies have shown the mutagenic capacity of sodium azide on many crops [16,17,18,19]. The effect of sodium azide (NaN_3) on improving properties of secondary metabolites synthesized by the plants has been little studied, particularly in regards to the *in vitro* antimalarial activities of extracts obtained from plants grown from seeds soaked in sodium azide solutions.

In the present study, we investigated the *in vitro* antimalarial activity of *P. odontadenius* extracts collected from the second generation plants (M2) of seeds soaked in sodium azide solutions, in order to: (i) validate the traditional use of *Phyllanthus* in the treatment of malaria; (ii) show the usefulness of chemical mutagenesis techniques in enhancing the *in vitro* antimalarial activity for subsequent phytochemical study of active plants leading to the identification of non-toxic active compounds.

The aims of the present study was to monitor the effects of sodium azide (SA) in second generation (M2) on the production of active secondary metabolites in *P. odontadenius* aerial parts in order to amplify those with *in vitro* high antimalarial activity.

2. MATERIALS AND METHODS

2.1 Biological Material

2.1.1 *Phyllanthus odontadenius*

The species of the genus *Phyllanthus* used plant material was harvested within the General Atomic Energy Commission/Regional Center for Nuclear Studies of Kinshasa (GAEC / RCNS-K) to the Kinshasa University at an altitude of 425 meters, 4 ° 24'38 '' latitude and 15 ° 20'45 '' longitude. The *Phyllanthus* species was first identified by a guide of the herbarium INERA to the Biology Department, Faculty of Science at University of Kinshasa and confirmed to the Laboratory of Systematic Botany and Plant Ecology in the same Department of Biology.

2.1.2 Parasite strain

The multi-resistant K1 strain of *P. falciparum* (Thailand, MR4-ATCC) was used. K1 is a multi-drug resistant strain and has an IC_{50} value with respect to chloroquine of 1156.50 (± 190.42) nM. The study was conducted by UMR-MD3, Aix-Marseille University/Marseille, France.

2.2 Methods

2.2.1 Treatments and culturing seeds

Seeds of *P. odontadenius* for the treatment of sodium azide (SA) (NaN_3) were placed in Eppendorf tubes and soaked in tap water for 1 hour. They were then soaked in different solutions of SA (Merck) obtained by dissolving SA in 1 M phosphate buffer [(Na_2HPO_4 (2 M) + NaH_2PO_4 (2 M)], pH 3 for 2h30 [20]. SA concentrations were 0, 2.5, 5.0, 7.5, 10, 12.5, 15, 17.5 and 20 mM–and were recorded T0, T1, T2, T3, T4, T5, T6, T7 and T8, respectively. After treatment in various solutions of SA, M1 seeds were then rinsed with sterile water for 1 h [17,18,21]. They were immersed in gibberellic acid solution (2 mg/l) for 4h and drained under the laminar flow hood (POLON kind KL - 21) overnight before culturing them in black polyethylene bags containing 300g of soil [22,23]. Ten Bags were then buried in ¾ in randomized complexes block (RCB) design with 3 replicates [24,17,25]. Plantlets were watered three times a week, the odd days, with the same amount of water. Plants from M1 seeds produce M2 seeds which are sown in two separate fields (M2.1 and M2.2) as described above. These are the M2 plants (M2.1 and M2.2) that were used as biological material study. Samples of *P. odontadenius* M2, aerial material, were harvested after four months.

2.2.2 Seedling growth

Parameters such as collar diameter, shoot length, number of branches for the selected M2 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 for aerial parts after plants harvest was measured using DENVER APX-100 balance. The negative effects of SA (or negative values of

reduction parameters) on different parameters determined using adapted relationship [17] of length reduction of plants or emergence reduction of seeds.

> Negative effect (%) = (Average of parameter in the dose x 100) / Average of parameter in the control) – 100.

2.2.3 Phytochemical analysis

2.2.3.1 Preparation of crude extracts

The samples collected in the field consisting of the aerial parts of *P. odontadenius* from seeds soaked in SA were dried for 72 h at 45°C in oven (GCA Mechanical Convection Oven) then ground to THOMAS mill to obtain a powder of 0.5 mm in diameter. 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 45ºC 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 100ºC for 15 min, cooled, filtered and dried at 45ºC for 72 h [26,27].

2.2.3.2 Phytochemical screening

The chemical screening was carried out on all crude extracts. Alkaloids were detected with Draggendorff's and Mayer's reagents [28]. Saponins, tannins, flavonoids, anthocyanins, steroids and terpenoids were detected by different methods reported by [20]. Anthraquinones were detected using Börtranger's reagent.

The presence of the different chemical groups was confirmed by Thin Layer Chromatography (TLC) performed on silica gel plates GF254 (Merck, Germany) [29].

2.2.4 Parasite culture

The chloroquine-resistant *P. falciparum* K1 strain (IC_{50} = 1156.50 ± 190.42 nM) (Thailand) used was obtained from MR4-ATCC. The study was conducted by UMR-MD3, Aix-Marseille University, Marseille, France. *P. falciparum* strain K1 was cultured *in vitro* in RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES, 50 mg/l hypoxanthine, 25 mM $NaHCO_3$, 20 mg/l gentamycin and 10% human serum (complete medium), at 37°C and under an atmosphere containing 5% CO_2, 10% O_2 and 85% N_2 [30].

Parasites were cultured (0.5% parasitemia; 1.5% hematocrit) in the presence of increasing concentrations of extracts and chloroquine (used as reference drug) in 96-well plates (final volume: 200 µl/well). The plates (chemosensitivity plates) were incubated 72 h at 37°C under an atmosphere containing 5% CO_2, 10% O_2 and 85% N_2 and then frozen at -80°C. Samples of parasite culture in control wells (absence of extract or chloroquine) were taken at (T0) and 24 h (T0+24h) after the beginning of the incubation and frozen at -80°C to check the growth of parasites.

The revelation of parasite growth was realized using a homemade HRP2-based ELISA assay.

Briefly, ELISA plates (Nunc) were prepared by coating the anti-antibody HRP2 (MPFM- 55A, IgM, Immunology consultant Laboratories, Inc., USA) at 1 µg/ml in PBS (Gibco) overnight at +4°C. Wells were then saturated with 5% skimmed milk (Régilait) in PBS (Gibco) for 2 h at room temperature. After 3 washes with PBS-0.05% Tween 20 (v/v), the plates were dried, sealed with a plastic film and stored at -20°C. Chemosensitivity plates were thawed and 4 µl of each well were transferred to a well of ELISA plate and incubated for 1 h at room temperature. After 3 washes with PBS-Tween 0.05% (v/v), the second antibody conjugated with peroxidase (MPFG-55P, IgG, Immunology Consultants Laboratories, 100 µl/well) in PBS containing 1% Tween 20 and 5% skimmed milk was added. The plates were incubated 1 h at room temperature. After 3 washes with PBS-Tween 0.05% (v/v), tetramethylbenzidine (TMB Chromogen Solution Single, Zymed Lab., Inc., USA, 100) was added and the plates were incubated for 10 min in the dark. The reaction was stopped by adding 50 µl/well of sulfuric acid (1 M).

The absorbance was read at 450 nm with a Sunrise™ spectrophotometer (Tecan, Austria) using the Magellan™ data analysis software (Tecan, Austria). The 50% inhibitory concentrations (IC_{50}) were calculated by nonlinear regression analysis from the dose-response relationship as fitted by software-ICEstimator 1.2 (http://www.antimalarial-icestimator.net) [31].

2.3 Statistical Analysis

Data were subjected to Analysis of Variance (Anova) using MSTAT-C Software [24,32] and compared to the software Statistica with General

Linear and LSD test (Least Significant Difference) in order to identify difference between treatments. Means of different treatment were separated with LSD at 5% lever of probability.

3. RESULTS

3.1 Effects of SA on *P. odontadenius* Growth

The Fig. 1 shows the effects of SA on growth parameters of *P. odontadenius* in the second generation (M2).

The results reported in Fig. 1, show that increasing concentration of SA induce negative effects on all parameters (height, collar diameter and fresh biomass) over the control (0 mM) in the second generation (M2). Fresh biomass presented negative values [-0.85% at 5 mM to -22.66% at 15 mM] except with 2.5 mM SA which showed positive value (4.82%). The same results were observed for the height parameter that varied from -2.50% (25 mM) to -21.49% (15 mM) and collar diameter parameter with the lowest reduction observed at 10 mM SA (-11.42%) and the highest at 17.5 mM SA (-22.37%). However, these variations were not significantly different at 5% (P<0.05%).

3.2 Screening Phytochemical Extracts from Plants M2 (NaN_3)

The phytochemical screening extracts from the aerial parts of *P. odontadenius* plants M2 obtained after treatment with different concentrations of AS is shown in Table 1.

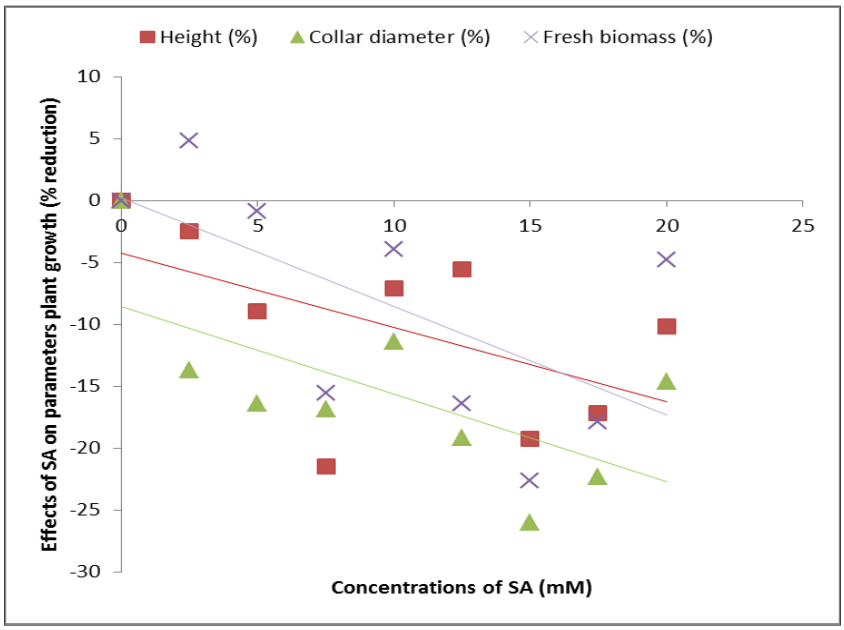

Fig. 1. Effects of SA on growth parameters: height, collar diameter and fresh biomass

Table 1. Screening phytochemical plant extracts of *P. odontadenius* plants M2

Chemical groups analysed	Concentrations (mM) of sodium azide (NaN_3)								
	0	2.5	5	7.5	10	12.5	15	17.5	20
Alkaloids	–	–	–	–	–	–	–	–	–
Saponins	–	+	–	–	–	+	–	–	+
Tannins	+	+	+	+	–	+	+	+	+
Anthocyanins	+	+	+	+	+	+	–	+	+
Free quinones	–	–	+	–	–	+/–	–	–	+/–
Flavonoids	+	+	+	+	+	+	+	+	+
Steroids and terpenoids	+	+	+	+	+	+	+	+	+
Anthraquinones	–	–	–	–	–	–	–	–	–
Polyphenols	+	+	+	+	+	+	+	+	+

Legend: + = Presence; – = Absence

The results reported in Table 1, show that alkaloids, saponins and anthraquinons were absent for all samples except 2.5, 12.5 and 20 mM for saponins. However, Tannins, anthocyanins, flavonoids and steroids combined terpenoids were present as well as polyphenols compounds except 10 mM for tannins and 12.5 mM for anthocyanins. Free quinones were almost absent with the exception of 5 mM dose's.

3.3 *In vitro* Antiplasmodial Activity

The effects of SA on the *in vitro* antiplasmodial activity of *P. odontadenius* plants M2 extracts are reported in Table 2.

Table 2. *In vitro* antimalarial activities from extracts of *P. odontadenius* M2 (M2.1 and M2.2)

Concentration (mM)	IC_{50} (µg/ml) M2.1	IC_{50} (µg/ml) M2.2
0	3.82 ± 0.43^{bc}	11.98 ± 0.98^{a}
2.5	3.57 ± 0.52^{bc}	7.82 ± 0.40^{c}
5	10.11 ± 2.22^{a}	11.46 ± 1.00^{a}
7.5	3.26 ± 0.05^{bc}	4.87 ± 1.57^{d}
10	4.52 ± 0.12^{bc}	2.73 ± 1.67^{d}
12.5	8.83 ± 1.38^{a}	10.82 ± 3.37^{ab}
15	4.97 ± 0.05^{b}	12.66 ± 0.59^{a}
17.5	3.07 ± 0.48^{c}	8.77 ± 0.77^{bc}
20	9.06 ± 1.06^{a}	12.42 ± 2.30^{a}
LSD	1.5	2.67
CV (%)	14.54	13.55

For the same parameter, value with identical letters were not significantly different at 5% (p<0.05)
Legend: M2.1: Field 1 from seeds M2; M2.2: Field 2 from seeds M2

Result reported in Table 2 show that IC_{50} values (µg/ml) for M2.1 extract vary from 3.26 ± 0.05 µg/ml (7.5 mM) to 9.06 ± 1.06 µg/ml (20 mM). The IC_{50} values obtained for plants treated with SA at 2.5 mM (IC_{50}: 3.57 ± 0.52 µg/ml), 7.5 mM (3.26 ± 0.05 µg/ml), 10 mM (4.52 ± 0.12 µg/ml), 15 mM (4.97 ± 0.05 µg/ml) and 17.5 mM (3.07 ± 0.48 µg/ml) are not significantly different at the confidence level of 5% from that obtained for the control (0 mM SA; 3.82 ± 0.43 µg/ml).

For the M2.2 extract, the IC_{50} values vary from 2.73 ± 1.67 µg/ml (10 mM) to 12.42 ± 2.30 µg/ml (20 mM). The IC_{50} values obtained for plants treated with SA at 5 mM (IC_{50}: 11.46 ± 1.00 µg/ml), 12.5 mM (10.82 ± 3.37 µg/ml), 15 mM (12.66 ± 0.59 µg/ml) and 20 mM (12.42 ± 2.30 µg/ml) are not significantly different at the confidence level of 5% from that obtained for the control (0 mM SA; 11.98 ± 0.98 µg/ml).

For *in vitro* antimalarial activities M2.1, the control presented no statistically different at 5% between 2.5, 7.5, 10, 15 and 17.5 mM of SA. For M2.2, the *in vitro* antimalarial activity of the control is not significantly different at the 5% level ($p < 0.05$) of those obtained with SA concentrations of 5, 12.5, 15 and 20 mM, but significant difference was observed between the *in vitro* antimalarial activity of control and those obtained with SA concentrations of 2.5, 7.5, 10 and 17.5 mM.

4. DISCUSSION

Sodium azide (SA) is a mutagen and is known as one of the most powerful mutagens in crop plants. SA has been reported to affect plant physiology and decrease cyanide resistant respiration in tobacco callus [33]. Some authors reported that SA affects the rate of seeds germination, stem or root heights and delay seed germination [34,35,36,37]. For example, in their study, [21] showed that height of *Eruca sativa* (L.) varied from 5.01±1.06 cm (30 days) to 17.67±1.57 cm (60 days) for plants that were grown from seeds not treated with SA whereas height of plants grown from treated seeds with 5 mM of NaN_3 solution varied from 2.80±0.75 cm (30 days) to 10.36±1.53 cm (60 days).

Regarding our results, the three parameters (height, collar diameter and fresh biomass) were reduced generally with increasing doses of SA against the control; the height reduced from -2.5% (T1) to -21.49% (T3), Collar diameter reduced from -11.42% (T4) to - 26.03% (T6) and the fresh biomass reduced from -0.85% (T2) to -22.66% (T6) except for T1 which presented value exceeding those the control. However, the culturing parameters doesn't showed significant difference at 5% ($p<0.05$): the height varied from 20.38±2.00 cm for the control to 16.00±0.25 cm for T3 (7.5 mM); the collar diameter from 2.19±0.38 mm for the control to 1.62±0.26 mm for the T6 (15 mM) and finally, fresh biomass per plant varied from 3.53±0.43 g to 2.73±0.41 g for T6 (15 mM). The results of parameters (height, collar diameter and fresh biomass) obtained in this work decrease with increasing doses of SA.

[16] studied *in vitro* mutagenic effects of SA on development of four cultivars pea (*Pisum sativum* L.), they obtained a reduction of rate of germination, shoot length and root length when seeds soaked in 1, 2, 3, 4 and 5 mM SA solutions. The plant length, collar diameter and fresh biomass could be to subject at the same

expression of genes. A molecular disturbance which affects one or the group of genes will bring out the same effects on these three parameters. These genes could be those controlling hormones synthesis of plant such as auxins and cytokinins [38,7,34].

Decline in biomass could be awarded to the disruption of chlorophyll synthesis level, reliable molecular index for genetic effects assessment and the gas exchanges in the plant level [39,36].

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 [40,41,42]. [10] reported that *Phyllanthus niruri* plant contained alkaloids, polyphenol compounds, flavonoids, tannins and terpenoids/steroids, but do not contain anthraquinones and saponins. [42] also reported that *Phyllanthus emblica* L. contain alkaloids, saponins, flavonoids, phenolic compounds, triterpenoids and tannins and quinones were absent.

The phytochemical screening plant extracts of *P. odontadenius* from seeds soaked in SA reveals that the alkaloids and related quinones are absent when they met in the M1 [20]. Flavonoids and terpenoids and steroids are present in all samples as did M1 as was so clearly demonstrated [20]. Absence or presence of secondary metabolites could be explained by possible disturbance or stimulation of their genes involved in different biosynthesis ways.

Others results obtained by [31] showed that alkaloids were absent in M2 plants from irradiated seeds except 25, 50 and 75 Gy (grays), saponins were absent to control and to treated plants; tannins, flavonoids and terpenoids combined steroids were present except to 75 Gy for the last. For the *in vitro* antimalarial activities on *P. falciparum* K1 strain, all doses gave promising antimalarial activities ($5<IC_{50}<15$ µg/ml) with a single dose of irradiation (225 Gy) showed high antimalarial activity (IC_{50} = 3.91 µg/ml).

It also appears that the best *in vitro* antimalarial activity on multidrug-resistant strain K1 is observed for M2 extracts at 7.5 and 10 mM of SA with respective IC_{50} value of: 3.26±0.05 µg/ml and 4.52±0.12 µg/ml (M2.1) and 4.87±1.57 µg/ml and 2.73±1.67 µg/ml (M2.2). Therefore these extracts have high IC_{50} value that is to say than less 5 µg/ml. This confirms the linear regression equation (y = 85.27 + -3,210x) obtained in the chemosensitivity of *P. odontadenius* with SA which was used to determine the LD_{30} and LD_{50} that are located respectively between 4.78 mM and 10.99 mM [20]. The WHO classification according Jonville *et al.* (2008) and adopted by [43] shows that the different items have *in vitro* antiplasmodial activities (IC_{50}) which have proved to be high (CI_{50}≤5 µg/ml), is promising (5 µg/ml ≤CI_{50}≤15 µg/ml).

In this work, the *in vitro* antimalarial activities of M2.1 plants showed not statistically different between control and some treated plants such as 2.5, 7.5, 10, 15 and 17.5 mM concentration of SA in comparison with IC_{50} from M2.2 which showed significant difference with 7.5 and 10 mM which are doses included in LD_{30} and LD_{50}. This difference will be explained by the comportment for the individual plant towards mutagenesis agent and by intrinsic factors which caused the effects of mutagens for example those from to gene silencing [44]. However, regarding the IC_{50} of 7.5 and 10 mM for M2.1 and M2.2, the *in vitro* antimalarial activities values were ranged to high activity (IC_{50}<5 µg/ml) [43].

5. CONCLUSION

It turned out that SA have in general negative effects on growth parameters (height, collar diameter or fresh biomass) and enhances the *in vitro* antimalarial activities of *P. odontadeius* on *P. falciparum* multidrug-resistant strain K1 which are either high or promising according to WHO standards. The best *in vitro* antiplasmodial activities are encountered at SA doses between 7.5 mM (3.26±0.05 µg/ml and 4.87±1.57 µg/ml) and 10 mM (4.52±0,12 µg/ml and 2.73±1.67 µg/ml); confirming the values of LD_{30} (4.78 mM) and LD_{50} (10.99 mM) previously found in the chemosensitivity of NaN_3 on *P. odontadenius*.

CONSENT

It is not applicable. We don't use patient or laboratory animals during our work.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

The authors express their thanks to Professor Daniel PARZY of UMR-MD3, Aix-Marseille

University, France) for the acceptance of collaboration between her laboratory and Division of life Sciences through CGEA/CREN-K. M. Gabriel Nsonsa Ngudi-Mayala acknowledged for the technical assistance and others members of Division of life Sciences for their contribution.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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