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In vitro antiplasmodial activity of callus culture extracts and fractions from fresh apical stems of *Phyllanthus niruri* L. (Euphorbiaceae): part 2

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Abstract

The ethanolic extracts from fresh apical stems of *Phyllanthus niruri* L. (Euphorbiaceae) cultured on Murashige and Skoog (MS) medium supplemented with IBA/BAP/*Coco nucifera* L. milk for 1, 2, 4 and 6 months were phytochemically and biologically investigated and compared with intact plant part and whole plant extracts. Results from the in vitro antiplasmodial testing indicated that the EtOH extract of a 1-month-old callus culture (IC_{50} = 16.3 ± 2.5 μg/ml) exhibited a higher activity than the ethanolic extracts of the fresh apical stem (IC_{50} = 18.2 ± 2.4 μg/ml) and callus cultures of 2-, 4- and 6-months-old (25 μg/ml < IC_{50} < 40 μg/ml). These activities were however lower than that displayed by the ethanolic extract of the whole plant (IC_{50} < 3 μg/ml). The EtOH extract of 1-month-old callus culture (the most active) was fractionated with solvents of different polarities. Its CH_2Cl_2 fraction rich in terpenic constituents (IC_{50} = 9.2 ± 3.4 μg/ml) exhibited a higher antiplasmodial activity than its isoamylic alcohol fraction obtained at pH 2–3 (IC_{50} = 25.6 ± 2.3 μg/ml) rich in flavonoids. The activity of these two fractions was lower than that displayed by the same fractions from the whole plant (2 μg/ml < IC_{50} < 3 μg/ml). Alkaloidic fractions from the whole plant and 1-month-old callus culture of fresh apical stem were considered as inactive (IC_{50} > 100 μg/ml).

Keywords: *Phyllanthus niruri*; Euphorbiaceae; Apical stem; Callus cultures; Terpenes; Flavonoids; In vitro antiplasmodial activity

1. Introduction

It is well known that plant species synthesize and accumulate various secondary metabolites belonging to different phytochemical groups. In intact plants, the formation of these metabolites is regulated in a coordinated fashion. Differentiation of plant cells or tissues during development is implied in this process. On the other hand, plant cell cultures are widely used for the comparison of biological activities of extracts, fractions or isolated compounds from the intact plant material to that of cultured plant material obtained in some experimental conditions (Santos et al., 1994; Sokmen et al., 1999). Callus cultures are also initiated for analytic and quantitative comparative studies of secondary metabolites synthesis between the intact plant material and callus extracts (Bahorun et al., 1994; El-Bahr et al., 1997; Rady and Nazif, 1997; Balz et al., 1999; Zhentian et al., 1999).

Phyllanthus niruri L. (Euphorbiaceae) is a medicinal plant widely used in different regions in the world for the treatment of various diseases. An aqueous infusion of the whole plant, which is a typical preparation, is employed as a stomachic, aperitive, antispasmodic, laxative, diuretic, carminative, against constipation, fever including malaria, hepatitis B, dysentery, gonorrhea, syphilis, tuberculosis, cough, diarrhea and vaginitis (Olive-Bever, 1986; Paranjape, 2001). The in vitro and in vivo antiplasmodial activity of the ethanolic and dichloromethane extracts as well as the toxicity of the lyophilized aqueous extract from *Phyllanthus niruri* was previously reported (Tona et al., 1999, 2000).

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The present phytochemical and biological investigation deals with the definition of some callus culture conditions of fresh apical stems of this medicinal plant, the comparison of the chemical composition and the in vitro antiplasmodial activity of callus extracts obtained after different times of cultivation to that of the intact fresh apical stem, whole plant extracts and fractions.

2. Materials and methods

2.1. Plant material

Whole plant and fresh apical stems of *Phyllanthus niruri* L. (Euphorbiaceae) were collected in Kinshasa, Democratic Republic of Congo in January 1999. The plant was botanically identified by M.N. Nlandu of the Institut National d'Etudes et de Recherches en Agronomie (INERA) at the University of Kinshasa where a voucher specimen has been deposited (INERA 994244). The whole plant was dried under 40 °C and reduced to powder whereas apical stems were used in fresh state for callus cultures.

2.2. Callus culture conditions

Fresh apical stems of *Phyllanthus niruri* rinsed with distilled water and were sterilized in 70% ethanol for 1 min, and then in calcium hypochlorite 9% (w/v) solution for 30 min. They were finally washed five times with sterile distilled water and aseptically cut in segments of 0.5 cm long. These explants were cultured in sterile polystyrene flasks containing 40 ml of Murashige and Skoog medium (MS) (Murashige and Skoog, 1962), according to the nature of the plant growth factors and the time of cultivation. The medium was supplemented separately with 2,4-dichlorophenoxyacetic acid (2,4-D, 4 mg/l), a mixture of indolebutyric acid (IBA) and benzylaminopurine (BAP, 2 mg/l each), a mixture of kinetine (Ki, 4 mg/l) and albumen liquid of *Coco nucifera* L. (100 ml/l), and a mixture of IBA/BAP (2 mg/l each) and albumen liquid five of *Coco nucifera* L. milk (100 ml/l). Prior to autoclaving at 120 °C (1.5 Kg/cm^2) for 30 min, the pH of all media was adjusted to 5.8 with 0.1N NaOH. All flasks were plugged with sterile cotton and cultures incubated at 27 °C in light or in darkness, or by alternative cycles of light for 12 h and darkness for 6 h in a phytotron incubator. The initiated calli were routinely subcultured onto a fresh medium during 2–3 weeks, not only to produce large amounts of calli, but also to ensure a normal growth and to prevent deterioration of explants characterized by the appearance of a brown color.

2.3. Preparation of extracts and fractions

Fifty grams of fresh intact apical stems and powdered dried whole plant were separately macerated with 300 ml EtOH (3 × 24 h). The mixture was filtered and the filtrate evaporated under reduced pressure yielding corresponding dried extracts denoted as PNT (2.873 g) and WP (5.376 g), respectively. Four callus cultures were initiated starting from 16.2 g, 16.5 g, 15.4 g, and 15.1 g of fresh explants, and cultivated for 1, 2, 4 and 6 months, respectively. Five grams of extract from each callus culture were separately treated in the same manner as described above yielding corresponding dried extracts denoted as PNC1 (0.2887 g), PNC2 (0.3024 g), PNC4 (0.3081 g) and PNC6 (0.2379 g), respectively. On the one hand, 150 mg of PNC1 was dissolved in 50 ml distilled water and filtered. The filtrate was exhaustively extracted with CH_2Cl_2, which was evaporated under reduced pressure to give dried extract denoted as PCN1.1 (42.3 mg). The aqueous phase was acidified (HCl 1N, pH 2–3), exhaustively extracted with isoamylic alcohol and treated as described above to give dried extract denoted as PNC1.2 (32.6 mg). The remaining aqueous phase was also evaporated under reduced pressure yielding a dried extract denoted as PNC1.3 (46.5 mg). On the other hand, 50 mg of PNC1 was dissolved in 25 ml distilled water and filtered. The filtrate was made basic (10 ml NH_4OH 25%, pH 10) and the mixture was exhaustively extracted with $CHCl_3$, which was evaporated under reduced pressure to give a dried extract denoted as PNC1.4 (16.2 mg). 200 mg of the ethanolic extract of *Phyllanthus niruri* whole plant (WP) was also fractionated in the same manner as described for the EtOH extract PNC1 yielding dried extracts denoted as WP1 (CH_2Cl_2 fraction: 64.5 mg), WP2 (isoamylic alcohol fraction: 37.40 mg), WP3 (aqueous fraction: 32.4 mg) and WP4 ($CHCl_3$ fraction, pH 10: 23.4 mg), respectively.

2.4. Measurements

Fresh weights of the calli were recorded immediately after the cultivation time and dried weights were determined by drying to a constant weight at 105 °C in an oven for 24 h.

2.5. Phytochemical analysis of extracts and fractions

Different extracts and fractions from the partition of WP and PNC1 were subjected to phytochemical analysis using conventional protocol (Harborne, 1974). TLC was carried out on silica gel F_{254} plates (layer thickness 0.25 mm, Merck).

2.6. Antiplasmodial testing

The in vitro antiplasmodial screening of ethanolic extracts of *Phyllanthus niruri* whole plant, of its fresh apical stems and callus cultures, and their respective fractions was based on a modified Rieckman's method Rieckmann et al. (1968) as reported by Tona et al. (1999). Briefly, 2 mg of each dried extract and fraction was separately dissolved in 2 ml EtOH (stock solution). Each stock solution was diluted with liquid culture medium (RPMI 1640) supplemented with 23 mM $NaHCO_3$ and 25 mM HEPES [*N*-2(-hydroxylethyl)-piperazine-*N*-2-ethanesulfonic acid] (Sigma Chemical Co.). Each 96-well test microtitre plate contained human infected blood from male and female adult patients diagnosed with

acquired *Plasmodium falciparum* infection malaria at the Bondeko Clinic (Limete, Democratic Republic of Congo) (5% hematocrit and 1% parasitaemia), test sample (concentrations varying from 1.25 to 100 μg/ml) and culture medium. They were tested in triplicate. Control microtitre plates contained infected blood in culture medium mixed with EtOH without test sample (negative control) to ensure normal development of the parasite. All microtitre plates were gently stirred and then incubated for 48 h at 37 °C in an atmosphere of 4% CO_2, 5% O_2 and 92% N_2. Giemsa-stained thick blood films were prepared from each well. The number of schizonts with three or more nuclei per 200 asexual parasites were microscopically noted and compared with control plates for the determination of percentage inhibition of parasite growth. The IC_{50} value of each test sample was derived from linear curves of concentration versus percentage (%) inhibition.

2.7. Statistics

Data are expressed as mean ± standard deviation. Statistical analysis was performed using Student's *t*-test. *P* values lower than 0.05 were statistically considered as significant.

3. Results and discussion

A review on the chemistry, the pharmacology and the therapeutic potency of different *Phyllanthus* species has been reported (Calixto et al., 1998). *Phyllanthus niruri* has particularly shown some interesting biological activities related to its worldwide uses in traditional medicine. An aqueous extract of the whole plant has been reported to inhibit the endogenous DNA polymerase of hepatitis B virus in vitro and in vivo (Venkateswaran et al., 1987). Bioassay-guided fractionations of extracts from *Phyllanthus niruri* resulted in the isolation and characterization of some active constituents. For example, the inhibition of angiotensin-converting enzyme was attributed to geraniin (Ueno et al., 1988). This compound was also isolated from the leaves of *Phyllanthus sellowianus* and showed a potent analgesic effect (Miguel et al., 1996). It can be also considered as the component responsible for the same activity exhibited by the total *Phyllanthus niruri* extract (Santos et al., 1995), but not for callus culture extracts Santos et al. (1994) because it was not detected there (Ishimaru et al., 1992). Other *Phyllanthus niruri* constituents such as ellagic acid, brevifolin carboxylic acid and ethylbrevifolin have been shown to be inhibitors of aldose reductase (Shimizu et al., 1989).

Results from callus cultures elaborated in our experimental conditions showed that no development was observed in MS medium containing no growth factors while in different culture media supplemented with some growth factors, a development or/and a germination was observed at different levels (results not presented). Among these trial combinations of plant growth factors, MS medium containing IBA/BAP/*Coco nucifera* L. milk was the best callus intitiatior in light as well as in darkness. Fresh apical stem explants horizontally cultured showed a better development than that vertically cultured probably due to their direct contact with growth factors in the medium. The cellular multiplication started at the extremities before invading the complete explant surface. The proliferation was faster in light than in the dark. The physical appearance of all calli was friable and ranging from yellow to brown. For calli cultured in light, the presence of small green structures resembling plantlets that emerged individually suggesting the occurrence of photosynthesis was observed. This was not observed in calli cultured in the dark. Calli containing other growth factors in MS medium were neglected because of their bad development and their poor production. The growth of different cultures (1, 2, 4 and 6 months) expressed as the increase or decrease in fresh and dry weights was determined. Results presented in Table 1 indicate that the weights of fresh callus extracts significantly increased with the duration of cultivation ($P < 0.01$). Except for the dried ethanolic extract PNC6, this last observation is also valid for dried ethanolic extracts PNC1, PNC2 and PNC4. In spite of the variation in weight of dried callus extracts, no significant difference in loss on drying was observed ($P > 0.05$).

Phytochemical analysis was carried out by TLC and indicated the presence of alkaloids, steroids, terpenes, coumarins, polyphenolic compounds such as phenolic acids and flavonoids in the ethanolic extracts of whole plant. Except for alkaloids which were detected only in traces, the remaining phytochemical groups cited above were also detected in the ethanolic extracts of the intact fresh apical stems and callus cultures of 1-, 2-, 4- and 6-months-old. Tannins were only detected in the whole plant extract. In general, the R_f values of the detected compounds on TLC were not the same (chromatograms not shown) suggesting that supplementation of culture media with some phytohormones is an important factor affecting the qualitative and to some extent

Table 1
Weights extracts (mg) of intact apical stem and callus cultures after 1, 2, 4 and 6 months in MS supplemented with IBA/BAP/*Coco nucifera* L. milk

	PNC1	PNC2	PNC4	PNC6
Fresh callus	2468.0 ± 3.1	2500.0 ± 2.3	2624.0 ± 1.7	2669.0 ± 3.4
Dried callus	142.5 ± 2.7	150.0 ± 1.8	162.0 ± 2.0	127.0 ± 2.7
Loss on drying (%)	94.0 ± 3.9*	94.0 ± 4.3*	94.0 ± 4.1*	95.0 ± 1.3*
Number of samples	7	9	6	11

PNC1, PNC2, PNC4 and PNC6: ethanolic extracts from callus culture OD 1-, 2-, 4- and 6-months-old, respectively. $P < 0.001$.
* $P > 0.05$.

Table 2
Phytochemical screening of whole plant, intact fresh apical stem and callus extracts of *Phyllanthus niruri*

Extracts	Alk. (alkaloids)	Anth. (anthocyanins)	Phen. (phenolic compounds, $FeCl_3$ positive)	Anthr. (anthraquinones)	Flav. (flavonoids)	Tan. (tannins)	Sap. (saponins)	Terp/Ster. (terpenes/steroids)
WP	+	–	+	–	+	+	–	+
PNT	±	–	+	–	+	–	–	+
PNC1	±	–	+	–	+	–	–	+
PNC2	±	–	+	–	+	–	–	+
PNC4	±	–	+	–	+	–	–	+
PNC6	±	–	+	–	+	–	–	+

WP, PNT, PNC1, PNC2, PNC4, and PNC6: ethanolic extracts of whole plant extract, intact fresh apical stem extract, callus extracts of 1-, 2-, 4- and 6-months-old, respectively, +: positive test; –: negative test in our experimental conditions.

the quantitative production of some secondary metabolites according to the time of cultivation. This finding corroborates with the observations deduced by other authors on the influence of some plant growth factors on the production of some metabolites in callus cultures of some medicinal plant parts (Kartnig et al., 1993; Bahorun et al., 1994; Saker and El Ashal, 1995; El-Bahr et al., 1997). Anthocyanins, anthraquinones and saponins were not detected in any extract and fraction (Table 2). Ishimaru et al. (1992) have reported the presence of phenolic compounds such as gallic acid, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin-3-*O*-gallate, (−)-epigallocatechin, (−)-epigallocatechin 3-*O*-gallate and (+)-gallocatechin in tissue culture of *Phyllanthus niruri* on MS medium containing 30 g/l sucrose. In our experimental conditions, it was observed that the phytochemical composition of callus cultured in the light and in the dark in MS medium supplemented with IBP/BAP/*Coco nucifera* milk was similar. They were thus combined for the biological testing according to their cultivation time.

Concerning the antiplasmodial activiy of *Phyllanthus* species, aqueous extracts from the leaves and stem of *Phyllanthus reticulatus* Poir have been reported to be active against *Plasmodium falciparum* chloroquine-sensitive K67 (20 μg/ml < IC_{50} < 25 μg/ml) and chloroquine-resistant ENT36 strains (1 μg/ml < IC_{50} < 10 μg/ml) in vitro while the aqueous extract of the root was less active against both strains (IC_{50} > 100 μg/ml) (Omulokoli et al., 1997). The $CHCl_3$ extract from the partition of the aqueous extract of the root bark of *Phyllanthus decipiens* var. *antsihanakensis* Leandri has been reported to exhibit a good antiplasmodial activity in vitro against *Plasmodium falciparum* FCM 29/Cameroun chloroquine resistant strain (IC_{50} < 5 μg/ml) (Rasoanaivo et al., 1999). A preliminary study conducted by Dhar et al. (1968) demonstrated that an 50% ethanol extract of *Phyllanthus niruri* whole plant failed to reduce the parasitaemia in mice infected with *Plasmodium berghei berghei* while Tona et al. (1999, 2000) reported a good in vitro and in vivo antiplasmodial activity of the ethanolic and dichloromethane extracts of *Phyllanthus niruri* whole plant, the latter extract being more active than the first one. Usha et al. (2001) have also described a dose-dependent suppressive effect of the parasitaemia in mice infected with *Plasmodium berghei bergei* of an aqueous extract from the whole plant.

In the present investigation, the ethanolic extract of the callus cultures of fresh apical stems of *Phyllanthus niruri* in MS medium supplemented with IBA/BAP/*Coco nucifera* L. milk after 1-, 2-, 4- and 6-months-old (PNC1, PNC2, PNC4 and PNC6, respectively) were evaluated in vitro for their potential antiplasmodial activity. Results of the antiplasmodial testing indicated that all extracts exhibited a dose-dependent inhibitory effect on the development of trophozoits to schizonts when tested at a concentration from 11.5 to 18.75 μg/ml. At these concentrations, the ethanolic extract PNC1 produced 39 and 69% inhibition of *Plasmodium falciparum* growth, respectively, an activity which was higher ($P < 0.05$) than that of the ethanolic extracts PNT (37 and 61% inhibition), PNC2 (32 and 43% inhibition), PNC4 (32 and 34 % inhibition), and PNC6 (15 and 28% inhibition) tested at the same concentrations. To appreciate more, the antiplasmodial activity of these extracts, the IC_{50} values resulting in 50% inhibition of the parasite growth in vitro were derived by a linear curve concentration versus percentage (%) inhibition. The results showed that the intact fresh apical stem ethanolic extract (PNT, IC_{50} = 18.2 ± 2.4 μg/ml) exhibited a lower activity ($P < 0.05$) than the same extract from callus cultures of 1-month-old (PNC1, IC_{50} = 16.3 ± 2.5 μg/ml) (Table 3). This activity was however significantly higher ($P < 0.001$) than that of PNC2, PNC4 and PNC6 ethanolic extracts which showed a very low antiplasmodial effect with IC_{50} values of 27.5 ± 3.1 μg/ml, 34.7 ± 2.9 μg/ml and 36.2 ± 4.5 μg/ml, respectively. The results also showed that the activity significantly decreased with the duration of cultivation. This suggested that the production of active constituents in some experimental conditions decreases not only with a long time of the cultivation, but also seems to be influenced by the nature of growth factors and their concentration in the medium.

The ethanolic extract of *Phyllanthus niruri* whole plant (WP, IC_{50} 2.5 ± 0.2 μg/ml) exhibited a higher antiplasmodial activity ($P < 0.001$) than the intact fresh apical stems (PNT) and callus culture of 1-month-old (PNC1) ethanolic extracts (Table 3). To locate active fractions containing at least one phytochemical group as major constituent, WP and PNC1 ethanolic extracts were fractionated as described above. The CH_2Cl_2 fraction (WP1, IC_{50} = 1.3 ± 0.3 μg/ml) from the *Phyllanthus niruri* whole plant ethanolic extract exhibited a

Table 3
Antiplasmodial activity of ethanolic extracts and fractions from *Phyllanthus niruri* whole plant and callus cultures of 1-month-old from fresh apical stems

Samples	IC_{50} (μg/ml)
Whole plant	
EtOH (WP)	2.5 ± 0.2*
CH_2Cl_2 (WP1)	1.3 ± 0.3
Isoamylic alcohol (WP2)	2.3 ± 0.5*
Residual aqueous fraction (WP3)	n.t.
$CHCl_3$ (WP4)	>100
Intact fresh apical stem	
EtOH (PNT)	18.2 ± 2.4
Fresh apical stem cultured of 1-month-old	
EtOH (PNC1)	16.3 ± 2.5
CH_2Cl_2 (PNC1.1)	9.2 ± 3.4
Isoamylic alcohol (PNC1.2)	25.6 ± 2.3
Residual aqueous fraction (PNC1.3)	n.t.
$CHCl_3$ (PNC1.4)	>100
Quinine dihydrochloride	0.25 ± 0.02

All isoamylic alcohol and chloroformic fractions were obtained at pH 2–3 and 10, respectively. n.t.: not tested. $P < 0.001$.
* $P > 0.05$.

higher antiplasmodial activity ($P < 0.001$) than the corresponding fraction (PNC1.1, IC_{50} = 9.2 ± 2.4 μg/ml) from the parent extract PNC1. The same observation is also valid ($P < 0.001$) for the isoamylic alcohol WP2 (IC_{50} = 2.3 ± 0.5 μg/ml) compared to the same fraction from PNC1 (PNC1.2, IC_{50} = 25.6 ± 2.3 μg/ml). The total alkaloid fractions from WP (WP4) and PNC1 (PNC1.4) did not show a significant antiplasmodial activity (IC_{50} > 100 μg/ml) (Table 3). From these results, the activity of the ethanolic extract from the whole plant and that of the callus cultures of 1-month-old from fresh apical stems was found to be located in the CH_2Cl_2, isoamylic alcohol and CH_2Cl_2 fractions, respectively. TLC analysis of these different fractions revealed the presence of terpenes and/or steroids in CH_2Cl_2 fractions, flavonoids as aglycones in isoamylic alcohol fractions and alkaloids in the $CHCl_3$ fractions obtained at pH 10 as their respective major constituents. Coumarins and/or phenolic acids were also detected in the parent extracts. Some constituents such as quercetin, lupeol, and ellagic acid were unambiguously identified by TLC and co-chromatography in the presence of reference products in extracts WP and PNC1 as well as in fractions according to the case, except lupeol which was not identified in the PNC1 extract and its CH_2Cl_2 fraction. These known compounds have already been reported to exhibit an antiplasmodial activity in vitro and/or in vivo (Alvez et al., 1997; Cimanga, 1997; Banzouzi et al., 2002) and could be considered at least partly responsible for the observed antiplasmodial activity in both plant samples. On the other hand, the influence of other phytochemical groups identified such as coumarins for the manifestation of this biological activity could not be excluded because some various constituents belonging to these groups have been reported to exhibit an antiplasmodial activity (Wright and Phillipson, 1990; Christensen and Kharazmi, 2001). The activity displayed by all plant samples in the present study was lower than that of quinine dihydrochloride used as a reference product (IC_{50} < 1 μg/ml).

On the basis of the results reported here, it was concluded that the phytohormone mixture IBA/BAP/albumen liquid *Coco nucifera* milk introduced onto the media for the callus cultures of fresh apical stems from *Phyllanthus niruri*, seems to be able to produce secondary metabolites with some antiplasmodial activity. Although, the activity of the EtOH extract and fractions from the whole plant was higher than that of the callus cultures of 1-month-old from fresh apical stems, the possibility to increase this activity by modifying the relative proportions of these plant growth regulators or by adding or removing one could not be excluded, and are being investigated.

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