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Tables: 3, Figs : 5

Alkaloids isolated from *Hortia superba* (Rutaceae) interact with spinach thylakoids inhibiting the electron transport chain

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ABSTRACT

Flindersine (**1**) and *N*-methyl-flindersine (**2**) were isolated from *Hortia superba.* Both compounds inhibited the synthesis of ATP and non-cyclic electron transport, i.e. they behaved as Hill reaction inhibitors. Both alkaloids inhibited electron flow through PSII. The effect of the alkaloids on the partial PSII reactions was measured as under: from water to oxidized DCPIP, water to sodium silicomolybdate and from reduced DPC to oxidized DCPIP. The results demonstrated that their inhibition site was at Q_{B-}, since the ectron flow from water to Pheophytin was not affected. Furthermore, alkaloid **2** had another site of inhibition located at PQH_2 oxidation site, the b_6f complex. The polarographic results were corroborated by Chl *a* fluorescence measurements; in thylakoids, the alkaloids changed the shape of the Kautsky curve. The OJIP test indicated that the behavior of alkaloids **1** and **2** was similar to DCMU. In addition, alkaloid **2** was less active than **1.** The main inhibition site of **2** was located at the PQH_2 site of oxidation within the b_6f complex.

Key words: Alkaloids, chlorophyll *a,* findersine, fluorescence, *Hortia superba,* *N*-methyl-flindersine, , PS II and b_6f inhibitors.

INTRODUCTION

The genus *Hortia* contains 10 species distributed from Panama to South America (especially in Amazonia) reaching central Brazil. The following six *Hortia* species are found in Brazil: *H. arborea* (3,23), *H. badinni* (9,11), *H. longifolia* (10), *H. brasiliana* (25), *H. colombiana* (30,31) and *H. regia* (19). Phytochemical studies have demonstrated that dihydrocinnamic acids, alkaloids, limonoids, coumarins, terpenes, amides and flavonoids are present in the *Hortia* genus (12,33). Alkaloids are major group of natural products with diverse structures. Although many classical alkaloids were isolated and characterized long ago, but still many new and important compounds are being extracted. Their useful biological properties, coupled with their interesting and challenging structures, make them targets for extensive synthetic studies. Biosynthetic pathways for alkaloids are the focus of intense study (20). Alkaloids are bio-active, e.g. quinolizidine

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alkaloids can be a chemical defense against pathogens (34), the alkaloids scopolamine and hyoscyamine are potentially involved in allelophatic activity (21). Plants with high alkaloid concentrations inhibit germination and radicle elongation of nearby seeds (21).

The alkaloid flindersine (**1**) was first isolated from *Flindersia australis* (Rutaceae) tree in 1914 (22), but was not completely purified. An alkaloid with very similar properties was isolated from this tree as it was apparently identical to the earlier base; the name 'flindersine' was retained (6). These compounds are very common in the plants of family Rutaceae (1,14). Flindersine have antifungal activity against *P. obscurans*, *C. gloeosporioides*, *C. fragariae*, *C. acutatum* and *F. oxysporum*, (7). However, in previous studies, it was not analyzed whether this alkaloid participates in plant-plant interactions or exhibits activity on photosynthesis. To search the natural compounds that affect photosynthetic activities, the alkaloid flindersine and its N-methyl derivative were studied. If these alkaloids affect photosynthesis, then this may be the mechanism of allelopathic compounds to act on other plants and it would be desirable to conduct further research to characterize their allelopathic behavior.

MATERIALS AND METHODS

Flindersine (**1**) and *N*-methyl-flindersine (**2**) (Fig. 1) were isolated from *Hortia superba* as per previously published procedure (5,6). Stock solutions of compounds **1** and **2** were prepared in dimethyl sulfoxide. The maximum concentration of solvent in the reaction medium was always less than 1%. In screening studies compounds **1** and **2** were tested as inhibitor against photophosphorylation activity.

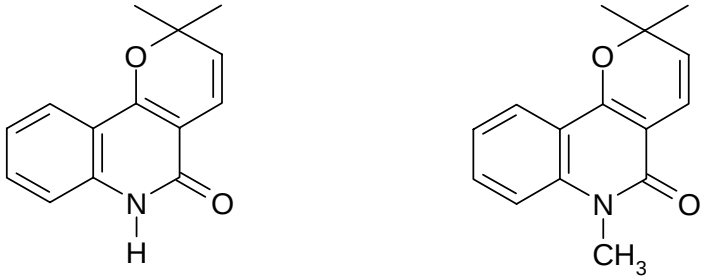

Figure 1. Chemical structures of the isolated alkaloids Flindersine (**1**) and *N*-methyl-flindersine (**2**).

Chloroplast isolation and chlorophyll determination: Intact chloroplasts were isolated from the spinach leaves (*Spinacea oleracea* L.), obtained from a local market. Chloroplasts were suspended in the following medium: 400 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, buffered with 30 mM K^+ -tricine (*N*-tris(hydroxymethyl)methylglicine) at pH 8.0 and stored as a concentrated suspension in the dark for 1 h at 0 ^oC. Intact chloroplasts were lysed to yield free thylakoids prior to each experiment by incubating them in the ATP

synthesis reaction medium or electron transport medium as indicated. Chlorophyll concentration was measured spectrophotometrically according to Strain *et al.* (27).

ATP synthesis: ATP synthesis was determined titrametrically using a microelectrode (Orion model 8103 Ross) connected to a Corning potentiometer model 12, with expanded scale as reported (13,24). The ATP synthesis reaction medium contained 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN, and 1 mM K^+- tricine (pH 8.0), 50 M methylviologen (MV) was used as electron acceptor. Chloroplasts were added to a final concentration of 20 g chlorophyll/mL.

Non-cyclic electron transport rate: Light-induced non-cyclic electron transport activity from water to MV was determined by using a Clark type electrode (24,26). Basal electron transport was determined by illuminating chloroplasts, 20 g chlorophyll/mL in 3.0 mL of the electron transport reaction medium: 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM KCN, 30 mM K^+-tricine, pH 8.0 plus 50 M MV (24,26). Phosphorylating non-cyclic electron transport was measured as basal non-cyclic electron transport except that 1 mM ADP and 3 mM KH_2PO_4 were added to the reaction medium. Uncoupled electron transport from water to MV was tested in the basal non-cyclic electron transport medium, and 6 mM NH_4Cl was added. The reaction mixture was illuminated for 1 min with actinic light from a projector lamp (GAF 2660) passed through a 5 cm filter of a 1 % $CuSO_4$ solution.

Uncoupled photosystem II (PSII) and photosystem I (PSI). Electron flow: Electron transport activity was monitored with an YSI (Yellow Springs Instrument) model 5300 oxygen monitor using a Clark type electrode. Uncoupled PSII from $H_2O \rightarrow$ DCPIP (2,6-dichlorophenol indophenol) was monitored polarographically; the reaction medium for PSII activity was the same as the basal electron transport medium except 1 M DBMIB (2,5-dibromo-3-methyl-6-isopropyl-1, 4-*p*-benzoquinone), 100 M DCPIP/300 M $K_3[Fe(CN)_6]$, and 6 mM NH_4Cl were added. Uncoupled partial electron transport of PS II from water to SiMo (sodium silicomolybdate) was determined as in PS II with a medium containing 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 15 mM tricine-KOH (pH 8.0), 200 M SiMo and 10 M DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (15). The uncoupled partial reaction of PSII from DPC to DCPIP was determined at 600 nm, the reduction of DCPIP was quantified using the molar extinction coefficient value of 21.8 10^{-3} cm^{-1} mol^{-1} (4). For this assay, Tris-treated thylakoids were used, that is, chloroplasts (300 g chlorophyll/ml) were incubated under slight agitation for 30 min at 4 ^{0}C in 0.8 M Tris (pH 8.0) (35). Then, the thylakoids were centrifuged at 5,000 x g (Sorvall super T21) for 2 min. The pellet was resuspended in 500 l of the isolation medium (400 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, 30 mM tricine-KOH (pH 8.0)) and was used in the assay after chlorophyll determination (32,16). The chloroplasts treatment with Tris inhibits the OEC complex, 100 M DPC was used as electron donor. DPC donates electrons to P_{680}. Similarly, the uncoupled PSI electron transport was determined measuring electron donation from reduced DCPIP to MV in basal non-cyclic electron transport medium. The following reagents were added: 10 M DCMU (3-(3,4-dichlorophenyl)-11-dimethylurea), 100 M DCPIP, 300 M ascorbate to reduce DCPIP and 6 mM NH_4Cl. The electron flow

from tetramethyl-*p*-benzoquinone ($TMQH_2$) to MV was determined polarographically (8,18). The I_{50} value for each activity was determined using the graph of percent activity versus concentration of compounds. I_{50} is the concentration producing 50% inhibition.

Chlorophyll *a* fluorescence: Chlorophyll *a* fluorescence was measured with a Hansatech Fluorescence Handy PEA (plant efficiency analyzer) in 5 min dark-adapted chloroplasts (20 g /ml) at room temperature (24), using red light intensity (broad band 650 nm) of 3000 mol m^{-2} s^{-1}. The light was produced by an array of three light emitting diodes. The pulse duration was 2s. The reaction medium used was the one employed in basal non-cyclic electron transport measurements. To monitor Chl *a* fluorescence transients, aliquots of dark adapted thylakoids were sedimented by gravity on filter paper with a dot-blot apparatus (Bio-Rad, USA). This procedure ensured a homogeneous and reproducible distribution of thylakoids on the filter paper. The sample was dipped immediately in 3 ml of electron transport medium with 300 M of the test compound.

RESULTS AND DISCUSSION

ATP synthesis

In freshly lysed spinach chloroplasts, increasing concentrations of **1** and **2** inhibited the synthesis of ATP (Figure 2). The I_{50} values for **1** and **2** were 26.0 and 49.7 M, respectively.

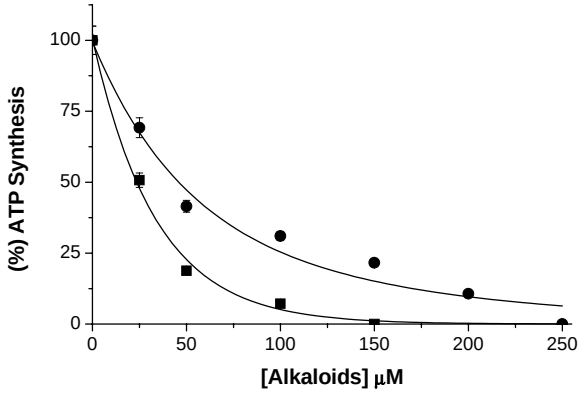

Figure 2. Effect of alkaloids **1** (■) and **2** (●), on ATP synthesis. Control rate value for **1** and **2** were 1610 and 1011 M $ATP \cdot h^{-1} \cdot mg^{-1}$ Chl, respectively. Other conditions as described in Material and methods.

The light-dependent synthesis of ATP by an illuminated thylakoid may be inhibited in many ways: a) by blocking electron transport, b) by uncoupling ATP synthesis from electron transport, or c) by blocking the phosphorylation reaction itself. Reagents that

block electron transport inhibit the synthesis of ATP because the generation of the transmembrane electrochemical gradient is not formed, i.e. the driving force for ATP synthesis is dependent upon electron flow. Chemicals that increase the proton permeability of thylakoid membranes uncouple phosphorylation from electron flow, i.e. uncoupling agents inhibit ATP synthesis by decreasing the proton gradient, even if electron transport occurs at higher rates. In contrast, direct inhibitors of photophosphorylation block phosphorylation at the level of the ATP synthase and besides slow down the electron transport as an indirect effect (17), in other words, the proton gradient established by electron transfer is not used by the synthase. Thus, it was decided to define the mechanism by which alkaloids **1** and **2** inhibit photophosphorylation.

To identify the mechanism of action of both compounds, we analyzed their effects on the rates of electron transport in different conditions (basal, phosphorylating, and uncoupled). Measurements were done in the absence or presence of ADP, Pi, or NH_4Cl and using MV as electron acceptor. Data (Fig. 3 and Table 1) show that flindersine (**1**) and *N*-methyl-flindersine (**2**) inhibited oxygen uptake by illuminated chloroplasts in all the conditions tested. Therefore, the results indicate that these alkaloids act as Hill reaction inhibitors. Furthermore, in the presence of **1** the highest inhibitions were observed on phosphorylating and uncoupled electron transport: 100 % at 10 and 25 M, respectively. Alkaloid **2** completely inhibited the rate of phosphorylating electron transport at 150 μM and inhibited 75 % the uncoupled electron transports rate at 300 μM. The concentration of **2** needed to inhibit the phosphorylating electron transport rate was fifteen times larger than the concentration of **1** needed to achieve the same effect (Table 1).

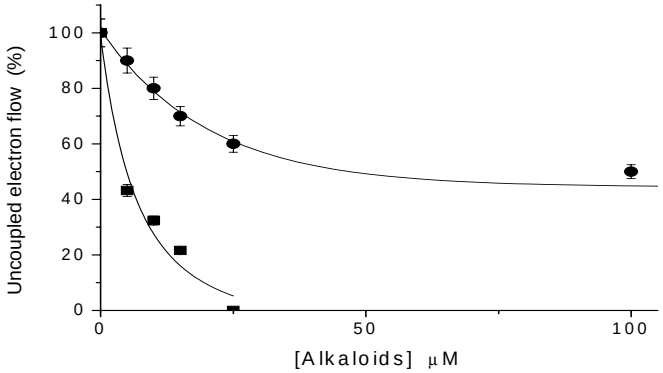

Figure 3. Effect of compounds **1** and **2** on uncoupled electron flow from water to MV in spinach chloroplasts. Control rate conditions for **1** (■) and **2** (●) were 1200 and 1400 equiv·e^-·h^{-1}·mg^{-1} Chl respectively.

Localization of flindersine (1) and *N*-methyl-flindersine (2) site(s) of interaction on PSI and PSII and partial reactions.

To determine the site(s) where **1** and **2** inhibited the thylakoid electron transport chain, their effects on the uncoupled PSII, PSI and partial reactions were determined using

appropriate artificial electron donors, acceptors and inhibitors (2). At 200 μM, **1**

Table 1. Effects of compounds **1** and **2** on basal and phosphorylating electron flow from water to MV in spinach chloroplasts

Compounds	Basal (%)	Phosphorylating (%)
[1] μM		
0	100.0	100.0
2.5	83.0	68.0
5.0	67.0	31.0
10.0	34.0	0
15.0	0	-
[2] μM		
0	100.0	100.0
10.0	43.0	72.0
25.0	29.0	59.0
50.0	14.0	35.0
100.0	0	18.0
150.0	-	0

Control rate conditions: 1 basal and phosphorylating rate was 320 and 650 equiv·e^{-}·h^{-1}·mg^{-1} Chl respectively. **2** basal and phosphorylating rates were 400 and 700 equiv·e^{-}·h^{-1}·mg^{-1} Chl respectively.

completely inhibited the uncoupled PSII electron transport rate from water to DCPIP (Table 2), while the electron flow from water to SiMo was not affected. Therefore, **1** inhibits below pheophytin, where SiMo accept electrons. Table 2 shows that the span of electron transport from P_{680} to Q_B was partially inhibited by **1** (38 % at 400 μM). The results suggest that **1** inhibits the photosystem electron transport chain between Q_A and Q_B, where DCPIP displaces PQ and accepts electrons. Therefore, the target of **1** was located at the acceptor side of PSII. Alkaloid **2** also inhibited the acceptor side of PSII, at 400 μM, **2** inhibited the electron transport rate from water to DCPIP by 100 % and at the same concentration it inhibited 44 % the PSII partial reactions from DPC to DCPIP (Table 2). By contrast, **2** did not exhibit any effect on the rate of electron flow from water to pheophytin, where SiMo accepts electrons. At 500 μM alkaloid **2** also completely inhibited the PSI electron transport flow from reduced $TMQH_2$ to MV. $TMQH_2$ donates electrons at the plastoquinone (PQH_2) oxidation site of the b_6f complex by displacing PQH_2. This reaction was totally sensitive to dibromothymoquinone inhibition, therefore, **2** inhibits the electron transport chain at the site where PQH_2 donates electrons. Thus alkaloid **2** has two sites of interaction and inhibition, one at the protein D_1 where Q_B interacts and the second site is at PQH_2 site of interaction located at the b_6f.complex. Furthermore, neither alkaloid **1** nor alkaloid **2** presented any effect on PSI electron transport from reduced (with ascorbate) DCPIP to MV. Therefore, polarographic measurements indicate that both alkaloids **1** and **2** showed inhibition at PSII, one at Q_B-D1 enzyme redox level and another at the PQH_2 site of oxidation of the b_6f complex. Maybe the hydrophobicity of **2** given for the methyl group at the nitrogen allows reaching the PQH_2 site of oxidation of b_6f complex.

Table 2. Effects of alkaloids **1** and **2** on uncoupled PSII electron transport from water to oxidized DCPIP, and the partial reactions of PSII, from water to oxidized SiMo and from DPC to DCPIP, and PSI electron transport from $TMQH_2$ to MV or reduced DCPIP to MV

Compound	Conc.	PSII H_2O to DCPIP		PSII H_2O to SiMo		PSII DPC to DCPIP		PSI DCPIPred to MV		PSI $TMQH_2$ to MV	
	(M)	a	b	a	b	a	c	a	b	a	b
	0	480	100	225	100	313	100	960	100	367	100
	25	-	-	-	-	231	74	-	-	-	-
	50	200	41	-	-	204	72	-	-	-	-
1	100	120	25	225	100	193	60	960	100	367	100
	150	80	17	225	100	-	-	960	100	367	100
	200	0	0	225	100	-	-	960	100	367	100
	0	480	100	225	100	500	100	960	100	367	100
	100	280	58	-	-	422	74	-	-	300	82
2	200	240	50	225	100	360	72	960	100	267	73
	300	150	33	225	100	279	56	960	100	167	45
	400	0	0							133	36

a Values in equiv e^- mg^{-1} Chl.; *b* Values in percent; *c* Values in M $DCPIP_{red}$ mg^{-1} Chl h^{-1}.

Chl *a* fluorescence

To corroborate the interaction site of alkaloids **1** and **2** at the PSII site (found by polarographic measurement), Chl *a* fluorescence was measured on freshly lysed chloroplasts, incubating them for 5 min in the dark at room temperature with different concentrations of alkaloids **1** and **2.** DCMU at 10 M, and 0.8 M Tris, were used as positive controls (Figure 4). Inhibition of PSII electron transport at the Q_B protein site by DCMU resulted in the rapid accumulation of Q_A^- during the first 2 ms of the induction curve (Figure 5). In contrast, control thylakoids required approximately 900 ms to completely close all PSII reaction centres. However, when the concentration of **2** increased the relative variable fluorescence yield also increased, as shown with the transient *J*[V(*J*)] value. A smaller proportion of *J*[V(*J*)] value was obtained with **2** at 200 M, this last result being indicative of losing the Q_A^- re-oxidation capacity similar to that observed with DCMU treated thylakoids (Figure 5) (28). To characterize the site of **1** and **2** interactions, different photosynthetic parameters associated to the PSII activity were evaluated: a) the normalized total complementary area above the O-J-I-P- transient (reflecting multiple-turnover Q_A reduction events) S_M = area / (F_M – F_0). B) the normalized total complementary area corresponding only to the O-J phase (reflecting single turnover Q_A reduction events), calculated as $S_S = V_J / M_0$, and c) the turnover N, which expresses how many times Q_A has been reduced in the time interval from 0 to tF_M defined as $N = S_M / S_S$ were calculated (29). S_S and N values decreased when the thylakoids are treated with DCMU. The thylakoids treated with Tris showed S_M and N values equal to zero, an indicator that they are not turnover of Q_A (Table 3). Thylakoids infiltrated with the alkaloids showed S_S and N lower values than control; here the normalized total area (S_S) was inversely proportional to the slope initial of the relative variable fluorescence (M_0). These results indicated that the number of times that Q_A was decreased is in the same way

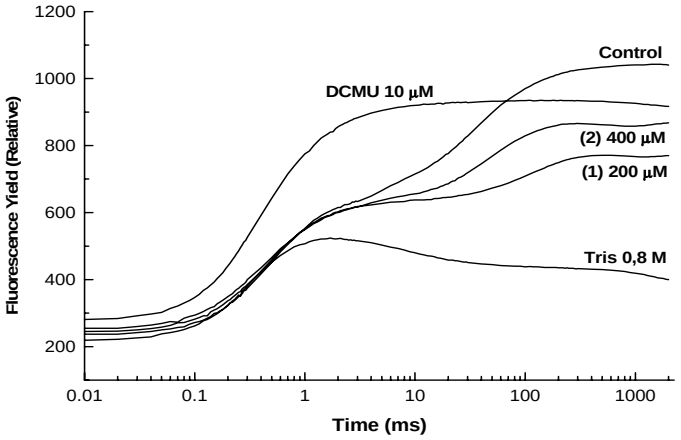

Figure 4. Fluorescence rise kinetics of freshly lysed broken chloroplasts infiltrated with **1** and **2** at 200 and 400 M, respectively, DCMU and Tris-treated thylakoid. Control chloroplasts are shown for comparison. Chl *a* fluorescence induction curves were measured at room temperature. Details are in Materials and methods. Data are of three replicates.

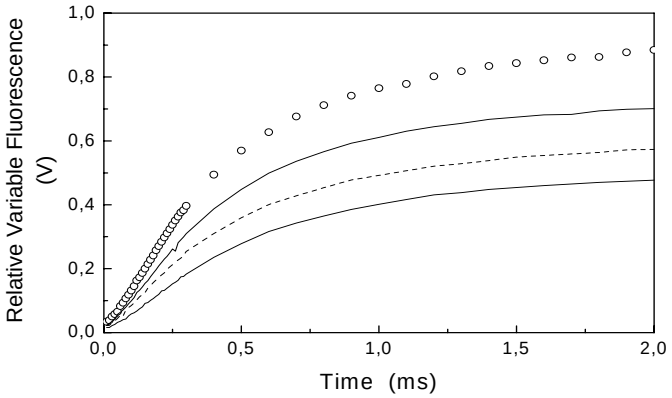

Figure 5. Normalized relative variable fluorescence rise of the photochemical phase. The curve with the lowest fluorescence yield represents control thylakoids. Open symbols represent control with acceptor side impairment after infiltration with DCMU. The intermediate curves represent thylakoids exposed to 200 M (**1**), intermediate dashed line curve represent thylakoids exposed to 400 M (**2**)

Table 3. Effects of alkaloids **1** and **2** on Fluorescence parameters on thylakoids previously incubated for 5 min in the dark and with the positive control 0.8 M Tris, pH 8.0

Compound	F_0	$F_{50\ s}$	$F_{300\ s}$	F_{2ms}	F_M	F_V/F_M	area	M_0	S_M	S_S	N
Control	224	250	374	615	1043	0.785	28600	0.63	36	0.74	49
10 M DCMU	258	302	526	856	934	0.724	800	1.42	1.3	0.6	2
0.8 M Tris	233	258	385	522	524	0.555	0	1.97	0	0.52	0
1 200 (M)	206	238	381	602	771	0.733	14800	1.0	28	0.64	44
2 400 (μM)	241	269	400	600	867	0.722	21200	0.88	35	0.63	55

that was observed with DCMU. However, the calculated N value for alkaloid **2** was 55, which is larger than the control N= 49 and for **1** where N= 44. Therefore, the last result with alkaloid **2** indicates that Q_A has been reduced more times than control and thus is less similar to DCMU. These last results corroborate the pollarographic results indicating that **2** interacts and inhibits at the D1 protein where Q_B are displaced with **2.**

CONCLUSIONS

These results demonstrated that the alkaloids Flindersine (**1**) and *N*-methyl-flindersine (**2**) were isolated from *Hortia superba* plant. The alkaloid **1** was very active (I_{50} = 25 M) and **2** was less active on inhibiting uncoupled PSII electron transport rate of chloroplasts. Therefore, in the exploration of a potential allelopathic plant, it is important to examine the allelochemical content in plants as we did in *Hortia superba* plant. Allelochemicals present in plants are released into the environment through living roots and affect the growth of other plants in their vicinity. Therefore, it is necessary to investigate in *Hortia superba* plant: (i) the content of alkaloids in the roots; (ii) the alkaloids contents in roots exudates and (iii) the alkaloids contents released through leaves into the environment. These studies would assist in the understanding the chemical basis of *Hortia superba* plant allelopathy.

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