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Furoquinoline alkaloids isolated from *Balfourodendron riedelianum* as photosynthetic inhibitors in spinach chloroplasts

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ABSTRACT

In the search for natural inhibitors of plant growth, we investigate the mechanism of action of the natural furoquinoline alkaloids isolated from *Balfourodendron riedelianum* (Rutaceae): evolitrine (**1**), kokusaginine (**2**), γ-fagarine (**3**), skimmianine (**4**) and maculosidine (**5**) on the photosynthesis light reactions. Their effect on the electron transport chain on thylakoids was analyzed. Alkaloids **1**, **2**, **4** and **5** inhibited ATP synthesis, basal, phosphorylating and uncoupled electron transport acting as Hill reaction inhibitors on spinach chloroplasts. Alkaloid **3** was not active. The inhibition and interaction site of alkaloids **1, 2, 4** and **5** on the non-cyclic electron transport chain was studied by polarography and fluorescence of the chlorophyll *a* (Chl *a*). The results indicate that the target for **1** was localized on the donor and acceptor side of PS II. In addition alkaloids **2** and **5** affect the PS I electron acceptors on leaf discs.

1. Introduction

There has been a growing recognition that many synthetic herbicides display a variety of adverse effects. In addition, it is becoming very difficult to discover new leads structures solely by synthetic means [1]. Therefore, modern strategies for discovering new agrochemical, i.e. insecticides, herbicides, etc. are focused on natural products, because they often have a reduced environmental impact, are biodegradable and may have new sites of interaction and specificity. In a program oriented towards the discovery of novel photosynthetic inhibitors, we identified diverse secondary metabolites such as chromenes, benzofurans, coumarins, phenylpropanoids, lactones, and terpenes from selected Mexican plants [2]. Furoquinoline alkaloids have attracted much interest due to their broad range of biological activities including various physiological and pharmacological activities [3,4]. In a previous work bioactive constituents from *Swinglea glutinosa* (Rutaceae) [4–7], and from *Hortia superba* (Rutaceae) were isolated and characterized. Quinolone alkaloids, flindersine, N-methyl-flindersine and acridone alkaloids inhibit the light reactions of photosynthesis [8]. The acridone alkaloids that contain 2,3,5,6-dehydro-4-oxopiperidine nucleus were the most active. As a contribution on the research of the Rutaceae family, in this work we report the isolation of five furoquinoline alkaloids: evolitrine (**1**), kokusaginine (**2**), γ-fagarine (**3**), skimmianine (**4**) and maculosidine (**5**) (Fig. 1) from a Brazilian tree *Balfourodendron riedelianum*. The plant is used in the Brazilian folk medicine to treat gastrointestinal ailments [9]. In the search for natural product that may have herbicide activity, we studied five furoquinoline alkaloids and found that four of them act as photosynthetic inhibitors. We investigated the mechanism of action of furoquinoline alkaloids **1**–**5** on the light reaction of photosynthesis and their possible targets on the electron transport chain on thylakoids and leaves discs. Among the techniques suitable for assessing the electron transport flow in the light reactions of photosynthesis on plants subjected to chemical stress, polarography (measuring oxygen evolution and using appropriate electron acceptors, donors and inhibitors) and fluorescence induction curves of chlorophyll *a* of photosystem II (PS II) were employed in the present work. Chlorophyll *a* fluorescence data were analyzed by the "JIP-test analysis" [10].

2. Materials and methods

2.1. Tested material

The natural furoquinoline alkaloids: evolitrine (**1**), kokusaginine (**2**), γ-fagarin (**3**), skimmianine (**4**) and maculosidine (**5**) (Fig. 1)

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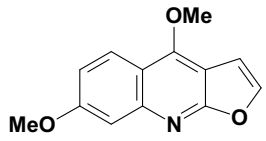
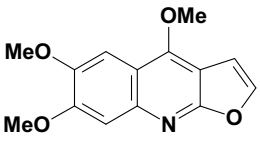


Evolitrine (**1**)

Log P = 3.20 ± 1.27

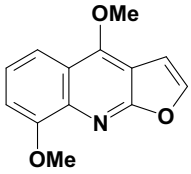
Molar Vol = 181.6 ± 3.0 cm^3



Kokusaginine (**2**)

Log P = 3.19 ± 1.47

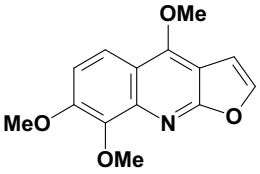
Molar Vol = 205.6 ± 3.0 cm^3



γ-Fagarine (**3**)

Log P = 2.70 ± 1.27

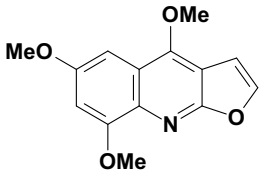
Molar Vol = 181.6 ± 3.0 cm^3



Skimmianine (**4**)

Log P = 2.86 ± 1.47

Molar Vol = 205.6 ± 3.0 cm^3



maculosidine (**5**)

Log P = 2.67 ± 1.47

Molar Vol = 205.6 ± 3.0 cm^3

Fig. 1. Structures of the isolated furoquinoline alkaloids (**1**–**5**).

were isolated from *Balfourodendron riedelianum* (Rutaceae) as previously published [11] and were assayed on the light reaction of photosynthesis in order to evaluate their activity. Stock solutions of **1**–**5** were prepared using DMSO (dimethyl sulfoxide) as solvent and its maximum concentration in the medium was less than 0.5%. Log *P* Parameters and Molar volume of the alkaloids were determined for each compound by the program Chem stech from www.ACD labs.com.

2.2. Purification and Isolation of the furoquinoline alkaloids (*1*–*5*)

The dichloromethane extract (2.1 g) obtained from the stem barks of *B. riedelianum* was subjected to a counter current chromatography, in the RLCC (Rotation Locular Counter Chromatography, RLCC-1000, Eyela ceramic bomb VSP-3050) apparatus using chloroform/methanol/isopropanol/water (45:60:2:40), as stationary phase in an automatic collector (Eyela fraction collector DC-120). The apparatus loop was 3.0 mL to give a group of 24 fractions in a chloroform solution stationary phase (0.3626 g), which was subjected to a column over Sephadex LH-20 (430 mm × 30 mm, 300 g) and the elution was carried out with methanol (100%) to give 7 fractions. From the fraction 3 (0.0902 g) the alkaloid **3** was obtained and from the fraction 6 (0.0137 g) **1** and **2** were isolated, and the compound **4** was isolated from the fraction 5 (0.0437 g). These metabolites were identified by spectroscopic and spectrometric techniques: ^1H and ^{13}C NMR, including 2D experiments (HSQC and HMBC), the data were compared with previous report [12–14]. A solution of methanol/H_2O (25%) of the methanol extract (58.6 g) obtained from the stem bark of *Balfourodendrum riedelianum* and then subjected to a liquid extraction to get n-hexane (5.5 g), dichloromethane (26.4 g) and ethyl acetate (2.8 g) fractions. The dichloromethane fraction was subjected to a chromatographic column (silica flash, 47.0 × 5.5 cm). The gradient elution was carried out with hexane to methanol, giving eleven fractions. The fraction 9 (1.45 g) gave rise to a new chromatographic column [silica flash + florisil (1:1), 70.0 × 2.5 cm] with gradient of elution (hexane to methanol) providing seven fractions (I–VII). The fraction IV (0.95 g) was subjected to another silica flash/Florisil (1:1) column (71.0 × 2.5 cm, eluted with gradient of hexane – ethyl acetate), giving five new fractions (A–E). The fraction E (0.06 g) was subjected to a preparative thin-layer chromatography (20 × 20 cm, 1 mm), eluted with dichloromethane (100%) to isolate maculosidine (**5**) (0.02 g). This compound was identified with spectroscopic and spectrometric techniques: ^1H and ^{13}C NMR, as previously reported [12–14].

2.3. Chloroplasts isolation and chlorophyll determination

Intact chloroplasts were isolated from spinach leaves (*Spinacea oleracea* L.) obtained from a local market as previously described [15,16]. Chloroplasts were isolated with a medium that contained: 400 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, and buffered with 3 mM K^+-tricine at pH 8.0, then they were re-suspended in the same medium and stored as a concentrated suspension in the dark for 1 h at 0 °C.

2.4. Measurement of ATP synthesis

To measure ATP synthesis, intact chloroplasts (20 μg/mL of chlorophyll) were broken by osmotic rupture in the following low-buffered solution: 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN, 50 μM methylviologen (MV) (as electron acceptor at F_X level), 1 mM K^+-tricine (pH 8.0), and 1 mM ADP, pH adjusted to 8.0 with KOH. The ATP synthesis was determined titrimetrically using a microelectrode Orion model 8103 Ross connected to a Corning potentiometer model 12, with expanded scale as previously reported [15,17]. The linear rate of alkalization in the light was calibrated by back-titration with saturated HCl, the ATP formed was calculated as μmol ATP mg Chl^{-1} h^{-1}.

2.5. Measurement of non-cyclic electron transport rate

The Light-induced non-cyclic electron transport activity from water to MV was determined with an oxygen monitors YSI (Yellow Springs Instrument) model 5300 using a Clark type electrode as previously published [15,18].

Chloroplasts (equivalent of 20 μg/mL of chlorophyll) were freshly lysed in 3 mL of the basal electron transport reaction medium: 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM KCN, 30 mM K^+-tricine at pH 8.0, and 50 μM MV and measured the basal electron flow by polarography. Phosphorylating non-cyclic electron transport from water to MV was measured as basal non-cyclic electron transport medium 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 as the basal non-cyclic electron transport, and 6 mM NH_4Cl was added as uncoupler to the medium. All reaction mixture was illuminated for 1 min with an actinic light of a projector lamp (GAF 2660) passed through a 5 cm filter of $CuSO_4$ at 2% w/v.

2.6. Uncoupled photosystem II (PS II) and Uncoupled photosystem I (PS I) electron flow determination

Uncoupled PS II from water to 2,5-dichloro-1,4-benzoquinone (DCBQ) [19] was monitored polarographically. DCBQ accepts electrons at the D1 protein, and thus we measured the electron transport from water to Q_B. The reaction medium for assaying PS II activity was the same used for basal electron transport, except that MV was omitted and 1 μM 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB), 100 μM DCBQ and 6 mM NH_4Cl were added. Partial reaction of PS II electron transport from water to sodium silicomolybdate (SiMo) was determined as in PS II with the same basal electron transport medium without MV, and 50 μM SiMo and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were added [20]. These electron flow activities were monitored with a yellow spring instrument (YSI) oxygen monitor, model 5300 using a Clark type electrode.

Uncoupled PS I electron transport was determined using the basal electron transport medium, plus 10 μM DCMU (to inhibit PSII at Q_B level), 100 μM of dichlorophenol indophenol (DCPIP) were reduced with 300 μM ascorbate and uncoupled with 6 mM NH_4Cl [21]. The I_{50} value for each activity was extrapolated using the graph of percentage of activity versus concentration of compounds. I_{50} is the concentration producing 50% inhibition of the activity.

2.7. Chlorophyll a fluorescence of PS II measurements in thylakoids

Chlorophyll *a* fluorescence transients were measured with a Handy-PEA (Plant Efficient Analyzer, from Hansatech, King's Lynn, Norfolk, UK) as previously described [16]. The maximum fluorescence yield from the sample was generated by illumination for 2 s with continuous light (650 nm peak wavelength, intensity equivalent of 2830 μmol photons m^{-2} s^{-1} and gain of 0.7.) provided by an array of three light-emitting diodes. The reaction medium used was that employed in basal non-cyclic electron transport measurements with MV. To monitor Chl *a* fluorescence transients, aliquots of dark-adapted thylakoids for 5 min containing 60 μg of Chl were transferred to filter paper by gravity with a dot-blot apparatus (Bio-Rad, United States) in order to ensure a homogeneous and reproducible distribution of thylakoids. The filter paper was dipped immediately in 3 mL of the medium with different concentrations of the tested compounds, and the control contained medium plus the amount of DMSO employed for each compound at the different used concentrations.

Chloroplasts infiltrated with 10 μM DCMU and chloroplasts previously treated with 0.8 M Tris ((hydroxymetyl)-aminomethan) were used as positive control. Chloroplasts were incubated with slight agitation for 30 min at 4 °C with 0.8 M Tris at pH 8.0 [22].

The OJIP transients were analyzed according to the JIP test and the measured parameters were: fluorescence intensity level (F_o) when plastoquinone electron acceptor pool (Q_A) is fully oxidized and fluorescence level when Q_A is transiently fully reduced (F_M). F_1 is the fluorescence intensity level at 0.05 ms, F_2 at 0.1 ms, F_3 at 0.3 ms, F_4 or F_J at 2 ms, F_5 at 30 ms. Area is the area over the curve between F_o and F_M, and relates the pool size of PS II electron transport acceptors [23]. From these parameters other derived parameters were calculated (Table 1) with the program Biolyzer HP3 (from Laboratory University of Geneva; available at http://www.unige.ch/sciences/biologie/bioen). The fluorescence of chlorophyll *a* transients were normalized using the equation $V_t = (F_t - F_o)/F_M - F_o)$, and the difference between the treated and control normalized transients is called "relative variable fluorescence", if plotted versus time revealed an increase in intensity between 2 and 4 ms appearing a J-band near to 2 ms, is an indication of the Q_A^- concentration is increased [10]. V_{OJ} is the relative variable fluorescence between F_o and F_J and is calculated as $V_{OJ}(t) = FVt/(F_J - F_o) = (F_t - F_o)/(F_J - F_o)$ and plotted versus time. If a K-band appeared near to 0.3 ms indicate a damaged in the water splitting system on thylakoids [10].

2.8. Chlorophyll a fluorescence measurements in spinach leaf discs

Chlorophyll *a* fluorescence on discs from dark-adapted spinach leaf was measured with the same equipment and conditions used for thylakoids. Fifteen spinach leaf discs (7 mm diameter) were placed in each Petri dishes with 10 mL of modified Krebs solution which contained: 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$ and 25 mM $NaHCO_3$ (pH 7.4), then were incubated for 4 h at room temperature, after this time, different concentrations of compound was added to each Petri dish. The leaf discs were incubated for 4 h more in the presence of the compounds, then, they were dark adapted for 30 min and afterwards the fluorescence of the chlorophyll *a* was measured. The same amount of dimethyl sulfoxide (DMSO) used for the samples treated with the analyzed compounds was added to the control samples for eliminating the solvent effect.

2.9. Plant material for in vivo assays

The seeds of *Physalis ixocarpa* and *Lolium perenne*, a weed species, were sown in 12 cm diameter pots and were watered daily in the greenhouse at 25–30 °C. After 15 and 18 days of emergence for *P. ixocarpa* and *L. perenne*, respectively, the plants were selected for similar size and were sprayed manually with the compounds **1** and **2** at concentrations of 150 and 300 μM. A stock of 20 mM of each compound was prepared in DMSO and an aliquot of this solution was taken to obtain the desired concentration in an aqueous suspension containing 0.05% w/v of polyoxyethylene sorbitan monolaurate (Tween 20). The control group was sprayed with distilled water containing the same amount of DMSO and Tween-20.

2.10. Chlorophyll a fluorescence determination in intact leaves in vivo

This was performed at room temperature with a portable Handy PEA apparatus [11] in the leaves of dark adapted-plants for 15 min both on the control and on the treated plants after 24, 40 and 72 h of being sprayed with the compounds.

3. Results and discussion

3.1. Inhibition of ATP synthesis

To know the effect of the furoquinoline alkaloids **1**–**5** on photophosphorylation, they were assayed on freshly lysed spinach chloroplasts. ATP synthesis was inhibited by **1**, **2, 4** and **5** in a concentration-dependent manner (Fig. 2). The I_{50} values for this activity were 83.5 and 59.0 μM for **1** and **2**, respectively; **4** and **5** had the same value 50.5 μM and **3** was almost inactive and it was not further studied (Fig. 2).

3.2. Effect of the natural furoquinoline alkaloids on chloroplast electron transport rate

The light-dependent photophosphorylation is coupled to electron transport rate and can be inhibited by different ways: by blocking the electron transport, by direct inhibition of the H^+-ATPase, or by uncoupling the ATP synthesis process from the electron transport [24]. To elucidate the mechanism of action of the furoquinoline alkaloids on photosynthesis, their effects on non- cyclic electron transport (basal, phosphorylating, and uncoupled) from water to MV were investigated.

Table 1
Derived parameters, their description and formulae using data extracted from the chlorophyll *a* fluorescence (OJIP) transient presented in this paper.

Fluorescence parameters derived from the extracted data	
$M_o = 4(F_{300\mu s} - F_o)/(F_M - F_o)$	Approximated initial slope (in ms^{-1}) of the fluorescence transient $V = f(t)$
$Sm = (Area)/(F_M - F_o)$	Normalized total complementary area above the O-J-I-P transient (reflecting multiple turnover Q_A reduction events)
Yields or flux ratios	
$\varphi_{Po} = TR_o/ABS = [1 - F_o/F_M]$	Maximum quantum yield of primary photochemistry at $t = 0$
$\varphi_{Eo} = ET_o/ABS = [1 - (F_J/F_M)]$	Quantum yield for electron transport at $t = 0$
$\varphi_{Ro} = RE_o/ABS = \varphi_{Po}\psi_{Eo}\delta_{Ro} = 1 - (F_I/F_M)$	Quantum yield for the reduction of end acceptors of PSI per photon absorbed
$\psi_{Eo} = ET_o/TR_o = (1 - V_J)$	Probability (at $t = 0$) that a trapped exciton moves an electron into the electron transport chain beyond Q_{A^-}
$\delta_{Ro} = RE_o/ET_o = (1 - V_I)/(1 - V_J)$	Efficiency with which an electron can move from the reduced intersystem electron acceptors to the PS I end electron acceptors of PS I
$RE_o/TR_o = \psi_{Eo} \times \delta_{Ro}$	Efficiency with which a trapped exciton move an electron into the electron transport chain from Q_{A^-} to the PS I end electron acceptors
Specific fluxes or activities per reaction center (RC)	
$ABS/RC = M_o(1/V_J)(1/\varphi_{Po})$	Absorption per RC
$TR_o/RC = M_o/V_J$	Trapped energy flux per RC (at $t = 0$)
$ET_o/RC = M_o(1/V_J)\ \psi_{Eo}$	Electron transport flux per RC (at $t = 0$)
$DI_o/RC = ABS/RC - TR_o/RC$	Dissipated energy flux per RC at $t = 0$
$RE_o/RC = (RE_o/ET_o)(ET_o/RC)$	Reduction of end acceptors at PSI electron acceptor side per RC at $t = 0$
Phenomenological fluxes or activities per excited cross section	
$RE_o/CS = (RE_o/ET_o)(ET_o/CS_o)$	Reduction of end acceptors at PSI electron acceptor side per CS at $t = 0$
De-excitation rate constants	
Kp	Photochemical de-excitation rate constant
Kn	Non-photochemical de-excitation rate constant
Sum K	The sum of photochemical and non-photochemical rate constants
Performance index	
$PI_{ABS} = \frac{RC}{ABS} \times \frac{TR_O}{ABS-TR_O} \times \frac{ET_O}{TR_O-ET_O}$	Performance index on absorption basis
$PI_{total} = PI_{ABS} \times \frac{\delta_{RO}}{1-\delta_{Ro}} = \frac{RE_O}{ET_O-RE_O}$	Total PI, measuring the performance up to the PSI end electron acceptors

Furoquinoline alkaloids **1**, **2**, **4** and **5** inhibited basal, phosphorylating, and uncoupled electron transport as their concentrations increased up to 200 μM. The I_{50} values for uncoupled electron transport rate were 21.0, 18.6, 23.4 and 22.8 μM, respectively (Table 2). Compounds **1**, **2**, **4** and **5** inhibited the electron flow (in basal, phosphorylating and uncoupled conditions) and ATP synthesis; therefore they act as Hill reaction inhibitors. Other natural products that act as Hill reaction inhibitors are the biflavonoid isolated from *Seleginella lepydophylla*, robustaflavone [25] or the furanoditerpene canophyllol isolated from *Celastrus vulcanicola*) [16].

3.3. Localization of furoquinoline alkaloids interaction sites on the light reaction of photosystem PS II and photosystem PS I and partial reactions

To localize the inhibition site of the alkaloids on the thylakoid electron transport chain, their effects on PS II, PS I and photosystems partial reactions were tested using artificial electron donors and acceptors, as well as appropriate inhibitors [21]. Table 3 shows that **1**, **2, 4** and **5** inhibited the uncoupled PS II electron flow from water to DCBQ; **1** inhibited PS II 100% at a concentration of 300 μM, however, alkaloids **2, 4** and **5** inhibited partially this activity (81%, 67% and 89%, respectively) at the same concentration (300 μM); therefore, **1** was the most active compound inhibiting the PS II electron transport. To further localize the inhibition site of the furoquinolines on the PS II electron transport chain, their effects were measured on the electron flow from water to SiMo (SiMo accepts electrons from pheophytin, so in this way the electron flow measurements are from the water splitting enzyme to pheophytin). The results indicate that **1, 2, 4** and **5** did not affect this partial reaction, since the rate of treated thylakoids were similar to the control (data not shown), therefore, all active furoquinoline alkaloids inhibited the electron transport chain beyond the pheophytin site [20]. The polarographic measurements suggest that **1, 2, 4** and **5** inhibited electron transport between Q_A and Q_B of PS II, at the D1 protein by displacing Q_B, similar to how DCMU herbicide acts. The mechanism action of **1, 2, 4** and **5** is similar to other natural products like trachyloban-19-oic acid [26], flindersine [4].

Uncoupled PS I electron transport rate was measured by polarography from reduced DCPIP to MV by using increasing concentrations of the alkaloids tested, in order to find if they affect this photosystem. The results of these studies showed that the rate for control and treated samples were similar, therefore the furoquinoline alkaloids do not interact with PS I electron transport chain (data not shown).

3.4. Measurements of the Chl a fluorescence transient in thylakoids in the presence of the furoquinoline alkaloids

To corroborate the polarography results about the interaction site of the furoquinoline alkaloids at PS II, fluorescence of chlorophyll *a* were measured on freshly lysed spinach chloroplasts incubated for 5 min in the dark at room temperature. Control chloroplast contained the amount of DMSO used for each treatment, the treated chloroplasts contained various concentrations of the compounds (each concentration was measured three times); chloroplasts incubated with Tris and 10 μM DCMU were used as positive control (Fig. 3). Chlorophyll *a* induction curves of thylakoids control (Fig. 3) showed an OJIP sequence similar to that previously described for plants, green algae, and cyanobacteria [27] except that phase IP was in part suppressed due to the effect of MV [28]. The chloroplasts infiltrated with DCMU and the control samples showed almost identical F_M level (Table 4). However, the F_M level of treated chloroplasts decreased respect to the control, while the F_o was not been affected by almost all the compounds tested; only **1** increased lightly the F_o level. The fluorescence of chlorophyll *a* transients were normalized, and the difference between the treated and control normalized transients revealed an increase in intensity between 2 and 4 ms appearing a J-band (Fig. 4A). The fluorescence rise to the J-band provides information

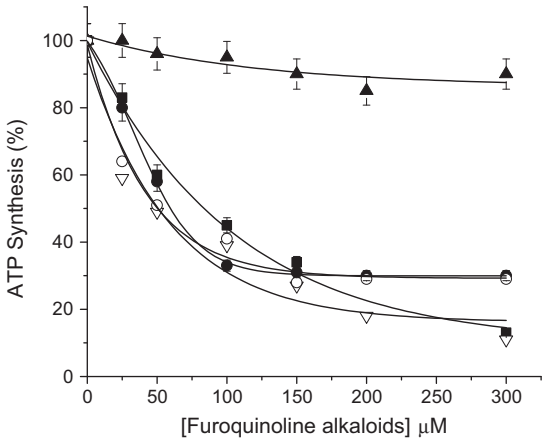


Fig. 2. Effect of **1** (■), **2** (●), **3** (▲), **4** (▽) and **5** (○) on ATP synthesis rate. Control rate value were 1113 for **1** and **2**, 1190 for **3**, 1110 for **4** and 1245 for **5** in μM ATP h^{-1} mg^{-1} Chl. Other conditions are as indicated in Materials and Methods. Each curve is the average of three replicates.

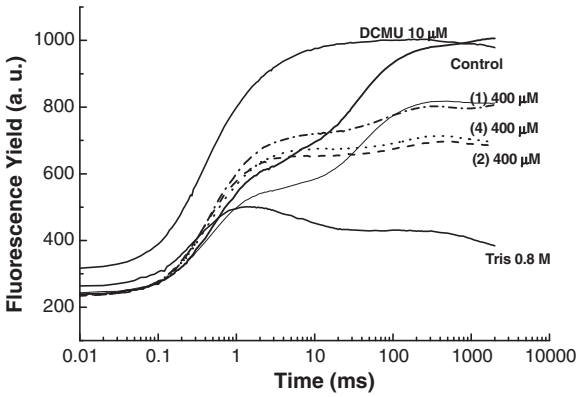


Fig. 3. Normalized fluorescence rise kinetics on F1 (0.05 ms) of freshly lysed broken chloroplasts infiltrated with 400 μM of **1**, **2** and **4**, 10 μM DCMU (dashed line) and Tris. Compound **5** was omitted because its curve was very similar to the curves for **2** and **4**. Control chloroplasts are shown for comparison. Chl *a* fluorescence induction curves were measured at room temperature. Details are described in Experimental Procedures. Data are results of three replicates.

Table 2
I_{50} values (μM) of **1**, **2**, **4**, and **5** obtained from basal, phosphorylating and uncoupled electron transport rate from water to MV in spinach chloroplasts. The data are the average of three replicates ± standard deviation.

Compounds	Basal	Phosphorylating	Uncoupled
1	25.0 ± 2	37.5 ± 2.3	21.0 ± 2
2	10.0 ± 4	10.0 ± 4	18.6 ± 1.35
4	29.5 ± 1.1	32.3 ± 1.33	23.4 ± 1.37
5	50.0 ± 3	42.6 ± 1.25	22.8 ± 1.66

about single turnover events of the primary reactions of the photochemistry, mainly Q_A reduction, indicating that the electron transport beyond Q_A is blocked at the Q_B level as DCMU does, suggesting that **1** interacts at this site and the unused energy is dissipated as heat [10]. In Fig. 4A, a small H-band was observed after 100 ms and closer to 1000 ms, this band is due to the electron acceptor MV effect [28]. In Fig. 4B, a K-band is observed in chloroplasts treated with Tris 0.8 M. Even though a small K-band is also observable in chloroplasts treated with DCMU, this band can be to the solvent effect of the ethanol where the DCMU was dissolved [28], however, the furoquinoline alkaloids were dissolved in DMSO as solvent and it have not any effect on the fluorescence induction curve.

Furthermore, the F_m value in the presence of DCMU plus MV is similar to the control sample (Table 4). In chloroplasts treated with furoquinolines alkaloids plus MV decreased Fm and the parameters derived from the JIP test (see Table 1) had similar behavior with DCMU treatment (Fig. 5), with the parameter δR_0 exception, [it reflects the efficiency with which an electron can move from the reduced electron acceptors coming of the intersystem to the PS I (reducing site)] [29]; this parameter was slightly increased by the furoquinoline treatments (with DCMU treatment it drastically diminished). Values like RE_o/CS, RE_o/RC and φ_{Ro} decreased with all treatments, being more drastic for DCMU. These results suggest that the furoquinoline alkaloids inhibited the electrons transport chain in two sites, one at donor and the other on the acceptor side in PS II (Fig. 5). The effect of furoquinoline alkaloids site at the donor side was detected only by the technique of fluorescence of chlorophyll *a* of PS II, and not by polarography technique [10].

3.5. Measurements of the Chl a fluorescence transient in leaf discs

To know if the alkaloids can reach their targets on the photosynthesis electron transport chain, fluorescence of chlorophyll *a* induction curves were measured in discs obtained from fresh spinach leaves. In Fig. 6A the OJIP sequences for control and discs treated with the most active furoquinoline **1** (in this case MV was omitted from the medium) showed a rise in the J peak. In discs treated with **1, 2**, and **5** at concentration of 400 μM, the differences between normalized curves and control showed a J-band (Fig. 6B). Furthermore **4** did not show any effect on the discs fluorescence (data not shown). J-band for **1** was bigger than for **2** and **5** (Fig. 6B). Also **1** and **2** showed a small K-band while compound **5** lacks this band (Fig. 6C). When the transients were normalized between F_M and F_5, a band in the IP region appeared (30 ms to about 300 ms) for compounds **2** and **5** (Fig. 6D). Corroborating with the derived parameters from the

Table 3
Effect of **1**, **2**, **4** and **5** on uncoupled PS II electron transport rate from water to DCBQ determined on freshly lysed spinach chloroplasts. The data are results of three replicates ± standard deviation.

Conc.	Furoquinoline alkaloids							
(μM)	**1**		**2**		**4**		**5**	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0	367 ± 18.4	100	400 ± 20.4	100	480 ± 24.0	100	411 ± 20.6	100
50	333 ± 16.7	91	324 ± 16.2	81	374 ± 18.7	78	165 ± 8.3	40
100	233 ± 11.7	64	240 ± 12.0	60	280 ± 14.0	58	94 ± 4.7	23
150	143 ± 7.1	39	200 ± 10.0	50	–	–	–	–
200	67 ± 3.4	18	124 ± 6.2	31	240 ± 12.0	50	82 ± 4.1	20
300	0	0	76 ± 3.8	19	160 ± 8.0	33	47 ± 2.4	11

a – Values are in μequiv e^- mg^{-1} Chl^{-1}.
b – Values are in percentage.

Table 4
Experimental average values ± standard deviation of chlorophyll *a* fluorescence obtained in control and treated chloroplasts with DCMU, Tris buffer and furoquinoline alkaloids. The values correspond to traces in Fig. 3.

	F_o	F_m	F1	F2	F3	F4	F5	Area
Control	228 ± 2.1	1000 ± 11.5	250 ± 4.5	270 ± 5.6	368 ± 10.8	602 ± 16.9	794 ± 16.5	40001 ± 2893
10 μM DCMU	275 ± 14.3	958 ± 22.7	321 ± 15.0	363 ± 17.6	535 ± 15.7	851 ± 17.0	950 ± 23.0	13400 ± 107
0.8 M Tris	254 ± 5.1	501 ± 11.4	280 ± 4.5	304 ± 5.6	399 ± 8.1	500 ± 10.0	440 ± 5.3	74 ± 1530
400 μM **1**	237 ± 3.6	824 ± 50.5	265 ± 4.5	291 ± 5.7	407 ± 6.8	665 ± 17.6	724 ± 37.8	25423 ± 4863
400 μM **2**	226 ± 13.0	728 ± 68.5	253 ± 13.8	278 ± 13.0	389 ± 15.9	634 ± 32.5	681 ± 62.8	6376 ± 7411
400 μM **4**	226 ± 5.86	745 ± 62.0	254 ± 9.3	280 ± 9.5	401 ± 18.5	650 ± 32.7	694 ± 51.8	6717 ± 3616
400 μM **5**	226 ± 4.51	723 ± 20.0	253 ± 4.4	279 ± 5.3	390 ± 8.5	645 ± 9.9	686 ± 13.6	6381 ± 998

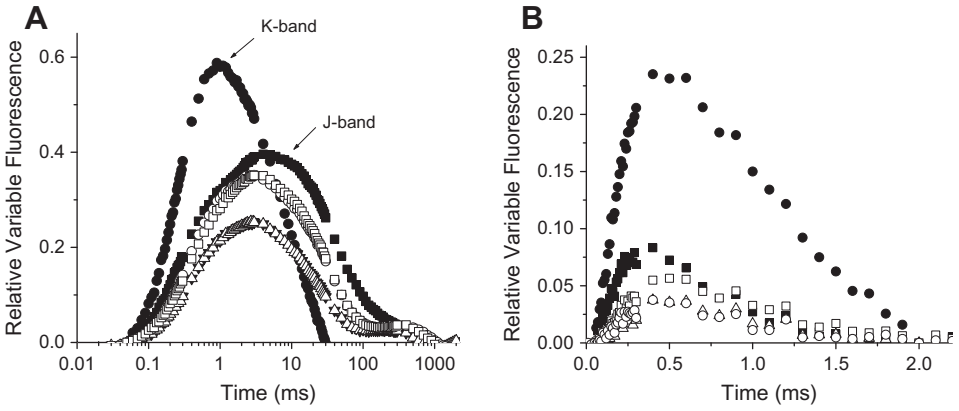


Fig. 4. (A) Appearance of J-band in the normalized relative variable fluorescence of chlorophyll *a* of thylakoids infiltrated with 10 μM DCMU (■), 400 μM of **1** (□), **4** (△) and **5** (○) and K-band obtained with thylakoid treated with Tris 0.8 M (●). (B) Appearance of K-band. Each curve is an average of three replicates.

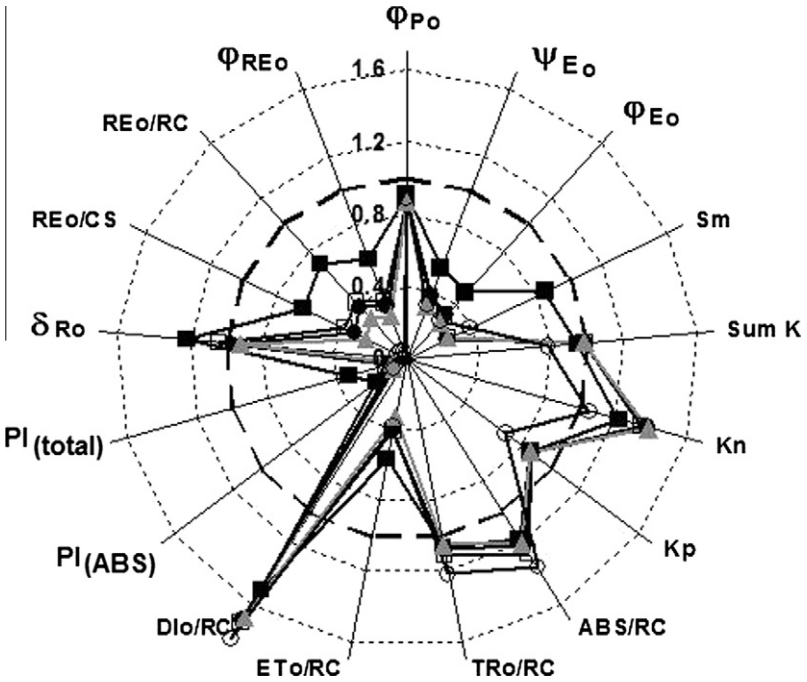


Fig. 5. Radar graph showing the effect of 400 μM of furoquinolines **1** (■), **2** (●), **4** (□) and **5** (▲) compared to 10 μM DCMU (○) on the derived parameters calculated from the OJIP transients presented by dark-adapted freshly lysed chloroplasts. Control is represented by a dashed line at the center of the radar.

transients obtained with **1** (Fig. 7), parameters as δ_{Ro} and φ_{Ro} increased up to 60%, indicating that the efficiency with which an electron can move from the reduced intersystem of electron acceptors to the PS I end acceptors increased. In addition, the quantum yield for the reduction of PSI end acceptors per photon absorbed also increased. These results suggest that end acceptors of PS I cannot be reduced because the electrons from PS II are not available. The parameters for **2** and **5** are similar to the control (Fig. 7) indicating that these compounds do not reach completely their target.

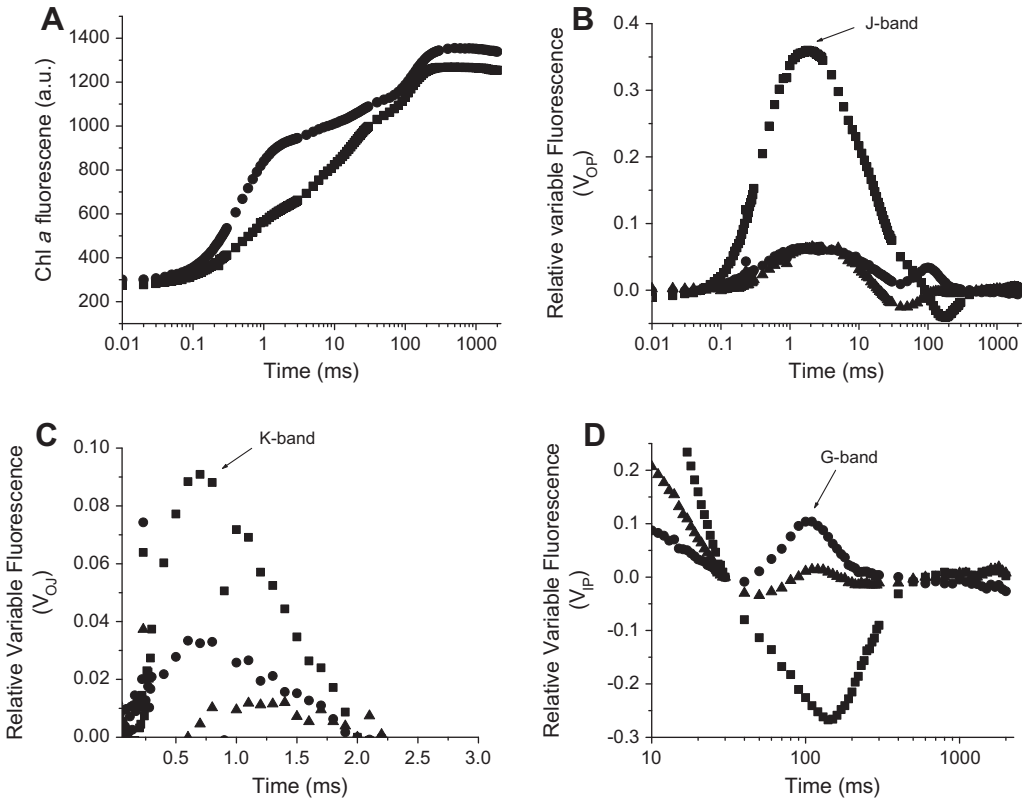


Fig. 6. (A) Chlorophyll a fluorescence (OJIP) transients of dark-adapted spinach leaf discs treated with 400 μM of furoquinoline **1** (●) and control (■). (B), (D) and (C) show the normalized relative variable fluorescence of chlorophyll *a* obtained with leaf discs infiltrated with 400 μM of **1** (■), **2** (●) and **5** (▲). Panel B, appearance of J-band; Panel C, appearance of K-band and panel D, appearance of a positive band in the IP region. Each curve is the average of ten replicates.

3.6. In vivo assays

In order to confirm if **1** can penetrate the intact whole leaf and affect the plants in vivo, this compound was sprayed on the leaves of *L. perenne* and *P. ixocarpa* plants after 15 days of growth in the green house. Studies of chlorophyll *a* Fluorescence were performed on the plants after 24, 48 and 72 h of being sprayed with **1** at 150 and 300 μM. The results showed non-significant inhibiting growth effect. These results suggest that **1** does not overcome the natural barriers provided by the plants, since the chlorophyll *a* and dry biomass of these plants were not affected by **1** (data not shown).

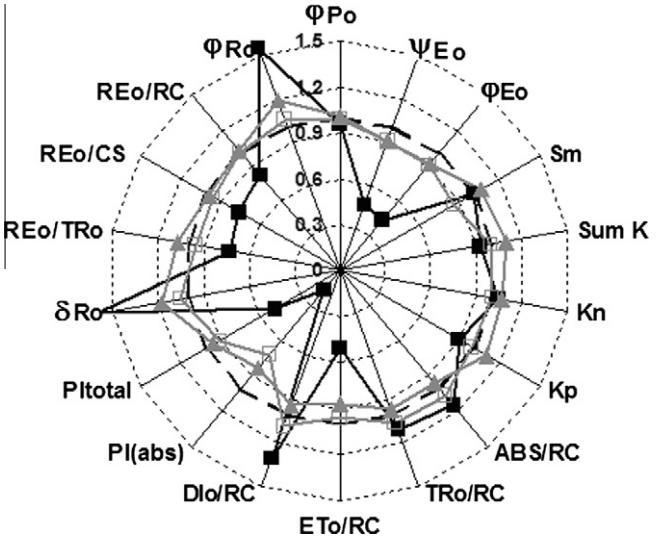


Fig. 7. Radar Graph showing the effects of 400 μM of furoquinolines **1** (■), **2** (□) and **5** (▲) on the derived parameters calculated from OJIP transients presented by dark adapted spinach leaf discs. Control is represented by a dashed line at the center of the radar.

4. Conclusions

The results indicated that furoquinoline alkaloids **1**, **2**, **4** and **5** inhibited ATP synthesis and electron transport in spinach chloroplasts while **3** was inactive. The inhibition site of alkaloids **1**, **2**, **4** and **5** was located at the acceptor side of PS II by polarographic technique. When these results were corroborated by chlorophyll *a* fluorescence of PS II data; we found that the donor side of PSII is inhibited by these furoquinoline alkaloids. Chlorophyll *a* fluorescence measurements on leaf discs treated with furoquinoline alkaloids showed changes in the induction curve of fluorescence only with **1**, transforming the OJIP trace at OJ step, showing a J band which indicates that only **1** can reach its inhibition target**.** However when **1** was sprayed on intact whole leaves, this compound could not penetrate the leaves plant natural barriers. Furthermore, **1** with two methoxy groups has the lowest molar volume unlike compounds **2**, **4** and **5** with 3 methoxy groups and therefore larger molar volumes which make more difficult their translocation in the leaves. Also alkaloid **3** has a lower molar volume, but it has a Log*P* value of 2.70, similar to compounds **4** and **5**. However, **1** has a higher Log*P* value of 3.20 suggesting that this compound fulfills the specific structure requirements for inhibiting PSII electron transport chain in leaf discs that the other furoquinoline alkaloids studied lack these characteristics.

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