

Fluorescence of Chlorophyll *a* for Discovering Inhibitors of Photosynthesis in Plant Extracts

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Abstract

Aims: Twelve crude extracts were prepared from different parts of *Pluchea sagittalis*, *Cecropia palmata* and *Brachiaria brizantha*, and their effect on the light reaction of photosynthesis. **Study Design:** Use of fluorescence of ChL *a* (Chlorophyll *a*) to detect inhibitors of photosynthesis in plant extracts. **Place and Duration of Study:** Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Departamento de Ciências Exatas e da Terra, Universidade Federal de São Paulo (UNIFESP), between July 2015 and January 2016. **Methodology:** The samples had their effect on the light reaction of photosynthesis studied by Chlorophyll *a* (Chl *a*) fluorescence transient by the use of chloroplasts isolated from spinach leaves. As negative controls we used the same amounts of DMSO that have been employed to solubilize the samples, and as positive control we have used 10 μ M of the commercial herbicide DCMU (Diuron). **Results:** The extracts inhibited the photosynthesis, and their inhibition sites were located in the range of electron flow from OEC complex and between P₆₈₀ to Q_A of PS II, and inhibited the photosystem II (PS II) by inducing the appearance of the transient bands K and J. **Conclusion:** Our results suggest an indicative that the photochemical apparatus probably is failing at the donor and acceptor sides of PSII, causing damage to electron transport during the photosynthesis phenomenon.

Keywords

Fluorescence of ChL *a*, Dereplication, Photosynthesis, Natural Products

1. Introduction

The world population is projected to grow to 9.7 billion in 2050 [1]. In this context, food, fibre and fuel de-

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mands tend to increase, and the development of weeds management practices is the need on control of weeds and pests, responsible for reducing the yield of food crops. The weeds compete with food crops for nutrients, water and space, being able to drain significantly the quality of crops [2]. Although the pesticide industries developed a lot of synthetic herbicides to control weeds, exploring sundry of action mechanisms, the search is continuous for new herbicides more efficient, of wide spectrum, lower use rates and safe to crop and environment.

One of the strategies used to identify new chemical agents to control weeds is about the exploration of natural products either directly as herbicides [3] or as leads for the development of new commercial products. The most of compounds isolated from nature have not been evaluated for agrochemical purpose and for this reason this strategy is considered attractive.

The most natural products are water soluble while the majority of synthetic agrochemicals are not. Moreover, due to natural selection these compounds can present bioactivity in very low concentration. The great variety of chemical structures found in nature can afford chemical agents for weed control that are toxicologically and environmentally safe. The molecular sites of natural products can be quite different from known molecular targets and this fact is important for solving the resistance problem [4]. For instance, few natural products lead to commercial herbicides as mesotrione, sulcotrione, cinmethylin, bialaphos and glyphosate. These pesticides act on molecular sites that were not known before they were introduced [5]-[8].

Furthermore, an increasing number of other natural products have been reported in the literature as models for the development of chemical agents for weed control as coumarins, benzoquinones, flavonoids, terpenoids and lactones [9]. The natural product research has been explored to develop new models for inhibitors of photosynthesis and the advances in this field are based on approaches like advancements in automation (high-throughput screening) and isolation technologies resulting in a development at the natural products area both in the fields of human health and agriculture [10].

The crucial step in the discovery of new chemical entities is the fast identification of substances in the real biological screening libraries: natural products, for instance, plants. Therefore, exploring plant's potential is a limited deal to experimental general manner of purification from crude extracts by time-consuming fractionation process, which also requires extensive chromatography. Dereplication approach permits early identification of known compounds without manual structure elucidation [11].

Dereplication is the process of differentiating those natural product extracts that contain nuisance compounds, or known specialized metabolites, from those that contain novel compounds that are of interest [11] and are identified comparing preliminary spectral data to spectral databases of known compounds. The most common approaches are LC-UV, LC-MS, LC-MS/MS, and LC-NMR, or combinations thereof. The dereplication based on LC-MS and LC-MS/MS is important because it is sensitive and provides structural information which is searchable in databases [12].

Moreover, combined with the powerful analytical strategies to obtain unknown chemical entities is extremely important the selection of a sensitive, fast and wide technique for elucidating the mechanisms of action of a new candidate to herbicide. According to Meyer & Genty (1999) [13] the fluorescence of the chlorophyll *a* (ChL *a*) allows easily to verify the photosynthetic electron transport activity in leaves, providing a detailed analysis of the photosynthesis inhibition, for example information about the PSII (photosystem II) state. This technique is important to detect the efficacy of the studied compounds as inhibitors of PSII.

It can suggest the extent to which PSII is using the energy absorbed by chlorophyll and the extent to which it is being damaged by excess light. The flow of electrons through PSII is indicative, under many conditions, of the overall rate of photosynthesis. It gives us the potential to estimate photosynthetic performance, under conditions in which other methods would fail, in a manner that is almost instantaneous. PSII is also accepted to be the most vulnerable part of the photosynthetic apparatus to light-induced damage. Damage to PSII will often be the first manifestation of stress in a leaf [14].

The mechanism of action of several natural products have been suggested by fluorescence of ChL *a*. Measurements confirmed the behavior of the diterpene trachyloban-19-oic acid isolated from *Iostephane heterophylla* as QA-QB (Quinone A-Quinone B) inhibitor [15]. The triterpenes epifriedelinol and canophyllol affect the *in vivo* photosynthesis efficiency of the chloroplasts in leaves of *Lolium perenne* and *Physalis ixocarpa* as a response to a process of detoxification [16]. Isolated from *Maytenus imbricata*, 3,4-Seco-friedelan-3-oic acid showed the formation of the K-band, acting as inhibitor at the donor side of PSII [17]. The flavonoids chrysin and 7-dimethylnarangenin purified from propolis also had their mechanisms of action confirmed by fluorescence studies. These results indicated that both inhibited the acceptor side of PS II, exactly like the commercial herbi-

cide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) [18]. On the other hands, pachypodol inhibited the water-splitting enzyme activity [19].

Several reports about the chemical profile of the plants studied in this work revealed, for example, the presence of monoterpenes, sesquiterpenes, triterpenes and flavonoids in *Pluchea* genus [20]. Twelve eudesmane sesquiterpenes were isolated from *Pluchea sagittalis* [21]. Flavonoids as 3',4',5,7-tetrahydroxy-3,6,8-trimethoxy flavone, 3',5,7-trihydroxy-3,4',6-trimethoxy flavone, and 3',4',5-trihydroxy-3,6,7-trimethoxy also were isolated and identified [22]. The analgesic and anti-inflammatory activity of *Pluchea sagittalis* leaves is attributed to the presence of terpenic substances: stigmaterol, β -amyrin, taraxasterol and pseudo-taraxasterol [23]. The most commonly described substances in *Cecropia* genus are terpenoids, steroids [24] [25] and phenolic compounds, such as proanthocyanidins and flavonoids [26] [27]. From the dichloromethane extract of *Cecropia palmata* leaves were isolated escoparone and ursolic acid [28], as well as pomolic acid, α -amyrin, β -amyrin and derivatives of stigmaterol [29]. Plants of *Brachiaria* genus have presented inhibition effects on the growth of some plants species [30]. Moreover, the extracts from shoots of *B. brizantha* inhibited the seed germination of *Stylosanthes* species [31] and seedling growth of cress, lettuce, *Phleum pratense* and *Lolium multiflorum* [32]. Allelopathic substances were isolated from *Brachiaria brizantha*, (6R,9S)-3-oxo- α -ionol, (6R,9R)-3-oxo- α -ionol, 4-ketopinoresinol [32], friedelin and friedelanol [33].

Based on these contexts, we can suggest that the photosynthetic system is an excellent target for the discovery of new compounds since the photosynthesis inhibition is fatal for the invasive plant and therefore is the principal focus of the actual study guided by the analytical methods for a rapid purification of the specialized metabolites.

2. Experimental Part

2.1. Extracts Preparation, Tested Material, Chloroplasts Isolation and Chlorophyll Determination

Roots and leaves of *Pluchea sagittalis*, and leaves of *Cecropia palmata* and *Brachiaria brizantha* (Table 1) were harvested at Mina do Sossego/Canaã dos Carajás (Pará state, Brazil). The crude extracts were obtained from the plant material by automatic method using Dionex ASE 350 extractor in the following solvents in order of polarity, hexane (500 mL), ethyl acetate (500 mL) and methanol (500 mL). The solvents were eliminated under reduced pressure by using rotary evaporator R-300 (Büchi) to obtain twelve extracts (Table 1). Stock solutions for all extracts were prepared using a DMSO (dimethyl sulfoxide; the maximum concentration of solvent mixture in the media was less than 1%).

Intact chloroplasts were isolated from spinach leaves (*Spinacea oleracea* L.) obtained from a local market as previously described [34] [35]. Chloroplasts were suspended in the following medium: 400 mM sucrose, 5 mM

Table 1. Crude extracts obtained from *Pluchea sagittalis*, *Cecropia palmata* and *Brachiaria brizantha*.

Plant specie (Family)	Plant part	Extraction solvent	Identification	Obtained material (g)
<i>Pluchea sagittalis</i> (Lam.) Cabrera (ASTERACEAE)	Roots (Rt)	Hexane	P1Rt-Hex	1.3
		Ethyl acetate	P1Rt-EtA	1.5
		Methanol	P1Rt-Met	7.5
	Leaves (Lv)	Hexane	P1Lv-Hex	4.4
		Ethyl acetate	P1Lv-EtA	7.2
		Methanol	P1Lv-Met	16.5
<i>Cecropia palmata</i> Willd. (URTICACEAE)	Leaves (Lv)	Hexane	P2Lv-Hex	9.3
		Ethyl acetate	P2Lv-EtA	1.2
		Methanol	P2Lv-Met	12.2
<i>Brachiaria brizantha</i> (POACEAE)	Leaves (Lv)	Hexane	P3Lv-Hex	4.6
		Ethyl acetate	P3Lv-EtA	2.9
		Methanol	P3Lv-Met	70.2

MgCl₂, 10 mM KCl, and buffered with 0.03 M Na⁺-tricine at pH 8.0. They were stored as a concentrated suspension in the dark for 1 h at 4°C. Intact chloroplasts were efficiently lysed to yield free thylakoids prior to each experiment by incubating them in the following electron transport medium: 100 mM sorbitol, 10 mM KCl, 5 mM MgCl₂, 0.5 mM KCN, and 30 mM tricine ((N-tris[hydroxymethyl]methylglycine; N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine) buffer (pH 8 with the addition of KOH). Chlorophyll concentration was measured spectrophotometrically as reported [36] [37].

2.2. Chlorophyll *a* Fluorescence Determination

Chlorophyll *a* fluorescence was measured with a Hansatech Fluorescence Handy PEA (plant efficiency analyzer) in 10 min dark-adapted chloroplasts (20 µg·mL⁻¹) at room temperature in the presence of 100 µg·mL⁻¹ of each extract [34] [38], using red light intensity (broad band 650 nm) of 2800 µmol m⁻²·s⁻¹, provided by an array of three light emitting diodes. The pulse duration was 2 s. To monitor Chl *a* fluorescence transients, aliquots of dark adapted thylakoids were placed by gravity on filter paper with a dot-blot apparatus (Bio-Rad, United States) to ensure a homogeneous and reproducible distribution of thylakoids on the surface, then the filter paper were dipped immediately in 3 mL of electron transport medium with the tested sample.

3. Results and Discussion

Chlorophyll *a* fluorescence of PSII measurements

To find the interaction sites of the extracts at PSII level, fluorescence of Chl *a* was 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 100 µg·mL⁻¹ of each crude sample (ten replicates); chloroplasts incubated with Tris (0.8 mol·L⁻¹) and 10 µM DCMU were used as positive controls. Chlorophyll *a* induction curves of thylakoids control showed an OJIP sequence similar to that previously described for plants, green algae, and cyanobacteria [39], except for the phase IP that was in part suppressed due to the effect of MV (methylviologen) [34].

The chloroplasts were infiltrated with DCMU, and the control samples showed almost identical F_M (fluorescence level when quinone A [Q_A] is transiently fully reduced) levels (Table 2). However, the F_M level of treated

Table 2. Effect of the extracts from *P. sagittalis*, *C. palmata* and *B. brizantha* at 100 µg/mL on fluorescence parameters of thylakoids previously incubated for 5 min in the dark with 10 µM of DCMU and 0.8 mol·L⁻¹ Tris, pH 8.0.

Samples	F ₀	F _m	F _v /F _m	Area
Control	1374 ± 69	3605 ± 180	0.622 ± 0.03	229,646 ± 1148
Tris 0.8 M	1245 ± 63	708 ± 35	0.412 ± 0.02	0
DCMU 10 µM	1595 ± 80	3265 ± 164	0.440 ± 0.02	13,685 ± 685
P1Rt-Hex	1371 ± 68	3632 ± 182	0.632 ± 0.03	275,320 ± 13,766
P1Rt-EtA	1572 ± 79	3438 ± 132	0.543 ± 0.02	225,978 ± 11,299
P1Rt-Met	1257 ± 63	3118 ± 156	0.597 ± 0.03	267,792 ± 13,390
P1Lv-Hex	1606 ± 80	3033 ± 152	0.470 ± 0.02	156,537 ± 7827
P1Lv-EtA	1195 ± 60	2771 ± 139	0.569 ± 0.03	165,586 ± 8280
P1Lv-Met	1523 ± 80	2253 ± 123	0.768 ± 0.04	122,153 ± 6108
P2Lv-Hex	1552 ± 78	2713 ± 136	0.797 ± 0.04	160,185 ± 8009
P2Lv-EtA	1468 ± 73	2404 ± 120	0.805 ± 0.04	158,655 ± 7933
P2Lv-Met	1421 ± 71	2034 ± 102	0.793 ± 0.04	113,150 ± 5658
P3Lv-Hex	1500 ± 75	2178 ± 109	0.770 ± 0.04	148,776 ± 7439
P3Lv-EtA	1475 ± 74	1889 ± 95	0.749 ± 0.04	97,085 ± 4854
P3Lv-Met	1505 ± 75	1189 ± 60	0.575 ± 0.03	52,909 ± 2645

chloroplasts decreased in comparison to the control, while the F_0 (fluorescence level when plastoquinone electron acceptor pool [Q_A] is fully oxidized) was not affected by most of the evaluated extracts.

The fluorescence of chlorophyll *a* transients (**Table 3**) 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 in the presence of the extracts from *P. sagittalis* (P1Rt-Hex, P1Rt-EtA, P1Rt-Met, P1Lv-Hex, P1Lv-EtA and P1Lv-Met). The *J*-band (**Figure 1**, panels A and B) provides information about single turnover events of the primary reactions of the photochemistry, mainly Q_A reduction, suggesting that the electron transport beyond Q_A is blocked at the Q_B level as DCMU does, indicating these samples contain substances that interact at this site and the unused energy is dissipated as heat [35].

On the other hand, the addition of $0.8 \text{ mol}\cdot\text{L}^{-1}$ Tris, pH 8.0, a well-known donor site inhibitor of PSII [36], resulted in the formation of a *K*-band followed by a dip; the *K* step arises when the electron flow to the acceptor side exceeds the electron flow from the donor side [37]. The *K*-band appears clearly and consists of a rapid rise to a maximum at $300 \mu\text{s}$ followed by a decrease to a level close to F_0 (**Figure 2(a)**). The other steps, *J* and *I*, are absent from the transient, as is shown by heat-treated samples [40], conditions in which the electron flow from P_{680} to Q_A results in the formation of the *K*-band. The fast initial fluorescence rise is due to the reduction of Q_A followed by the reduction of P_{680}^+ by Z without the direct participation of the OEC. The subsequent reduction in fluorescence yield apparently results from the opening of the reaction center by reoxidation of Q_A and/or accumulation of P_{680}^+ , both of which are effective fluorescence quenchers [37]. Furthermore, the analysis of the Chl *a* fluorescence transient in this work indicates that the water-splitting enzyme was blocked by the extracts P2Lv-Hex, P2Lv-EtA, P2Lv-Met, P3Lv-Hex, P3Lv-EtA and P3Lv-Met.

Table 3. Derived parameters, their description and formula using data extracted from the Chl *a* fluorescence (OJIP) transients.

Fluorescence parameters derived from the extracted data	
$dV/dt_0 = 4(F_{300\mu s} - F_0)/(F_M - F_0)$	Approximated initial slope (in ms^{-1}) of the fluorescence transient $V = f(t)$
$S_m = (\text{Area})/(F_M - F_0)$	Normalized total complementary area above the OJIP transient (reflecting multiple turnover Q_A reduction events)
Yields or flux ratios	
$\text{TR}_0/\text{ABS} = [1 - F_0/F_M]$	Maximum quantum yield of primary photochemistry at $t = 0$
$\varphi_{E_0} = \text{ET}_0/\text{ABS} = [1 - (F_J/F_M)]$	Quantum yield for electron transport at $t = 0$
$\psi_0 = \text{ET}_0/\text{TR}_0 = (1 - V_J)$	Probability (at $t = 0$) that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-
Specific fluxes or activities per reaction center (RC)	
$10\text{RC}/\text{ABS} = \text{Mo}/(1/V_J)(1/\varphi_{P_0})$	Absorption per RC
$\text{TR}_0/\text{RC} = \text{Mo}/V_J$	Trapped energy flux per RC (at $t = 0$)
$\text{ET}_0/\text{RC} = \text{Mo}/(1/V_J)\psi_0$	Electron transport flux per RC (at $t = 0$)
Phenomenological fluxes or activities per excited cross section (CS)	
$\text{ABS}/\text{CS}_0 \approx F_0$	Absorption flux per CS, approximated by F_0
$\text{TR}_0/\text{CS} = \varphi_{P_0}(\text{ABS}/\text{CS}_0)$	Trapped energy flux per CS (at $t = 0$)
$\text{ET}_0/\text{CS} = \varphi_{E_0}(\text{ABS}/\text{CS}_0)$	Electron transport flux per CS (at $t = 0$)
De-excitation rate constants	
K_p	Photochemical de-excitation rate constant
K_n	Non-photochemical de-excitation rate constant
Sum K	The sum of photochemical and non-photochemical rate constants
Performance index	
$\text{PI} = \text{RC}/(\text{ABSRC}) \times \varphi_{P_0}/(1 - \varphi_{P_0}) \times \psi_0/(1 - \psi_0)$	Performance index on absorption basis

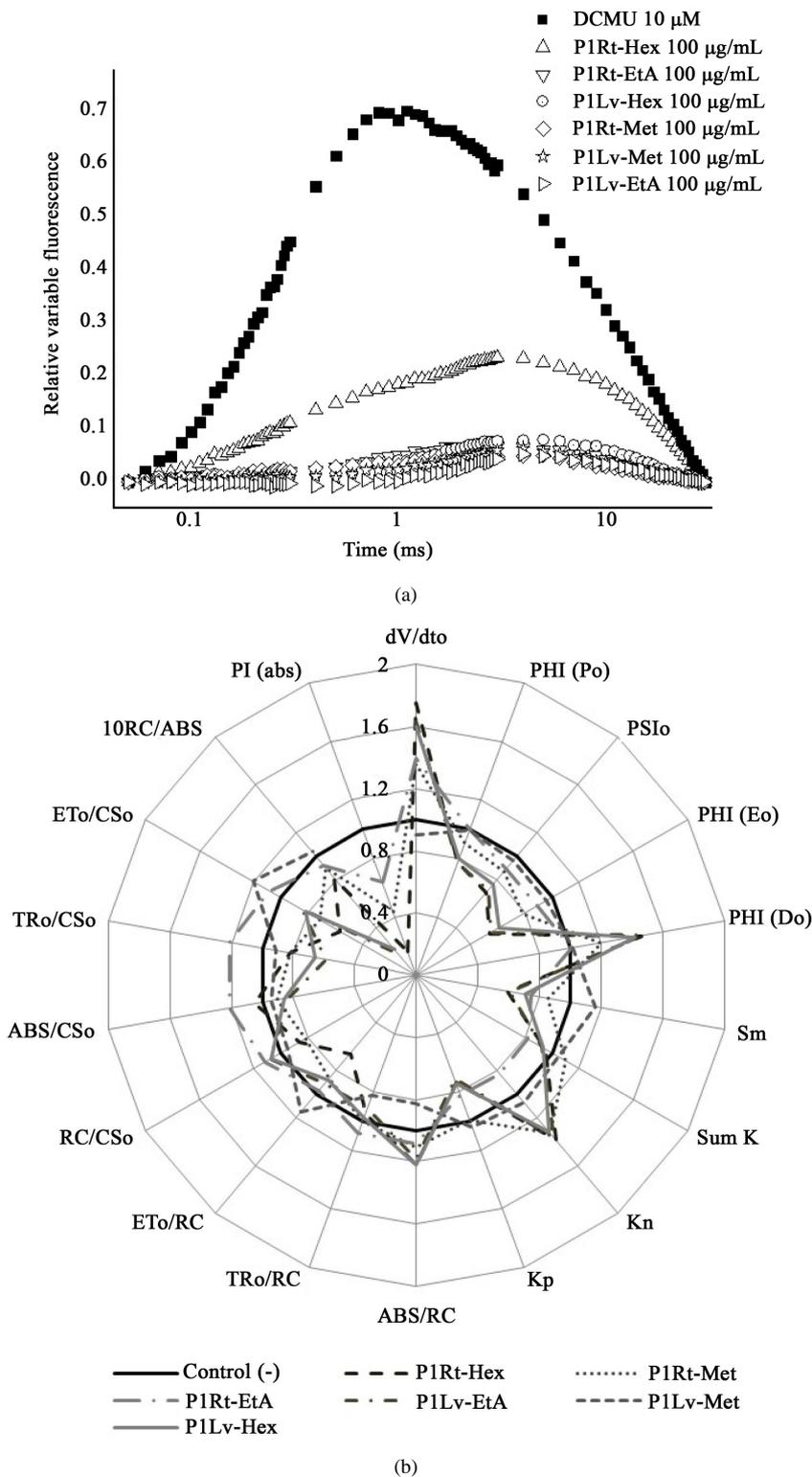


Figure 1. (a) Effect of extracts from *P. sagittalis* on different calculated parameters from OJIP curves measured in treated spinach chloroplasts. Appearance of the J-band at approximately 2000 μs in the presence of the samples at 100 $\mu\text{g}\cdot\text{mL}^{-1}$. Data are an average of ten replicates; (b) Radar plot showing the JIP-test analysis for extracts from roots and leaves of *Pluchea sagittalis*. (Notes: J-band appears when the compound inhibits the electron transport at the acceptor side of the PSII)

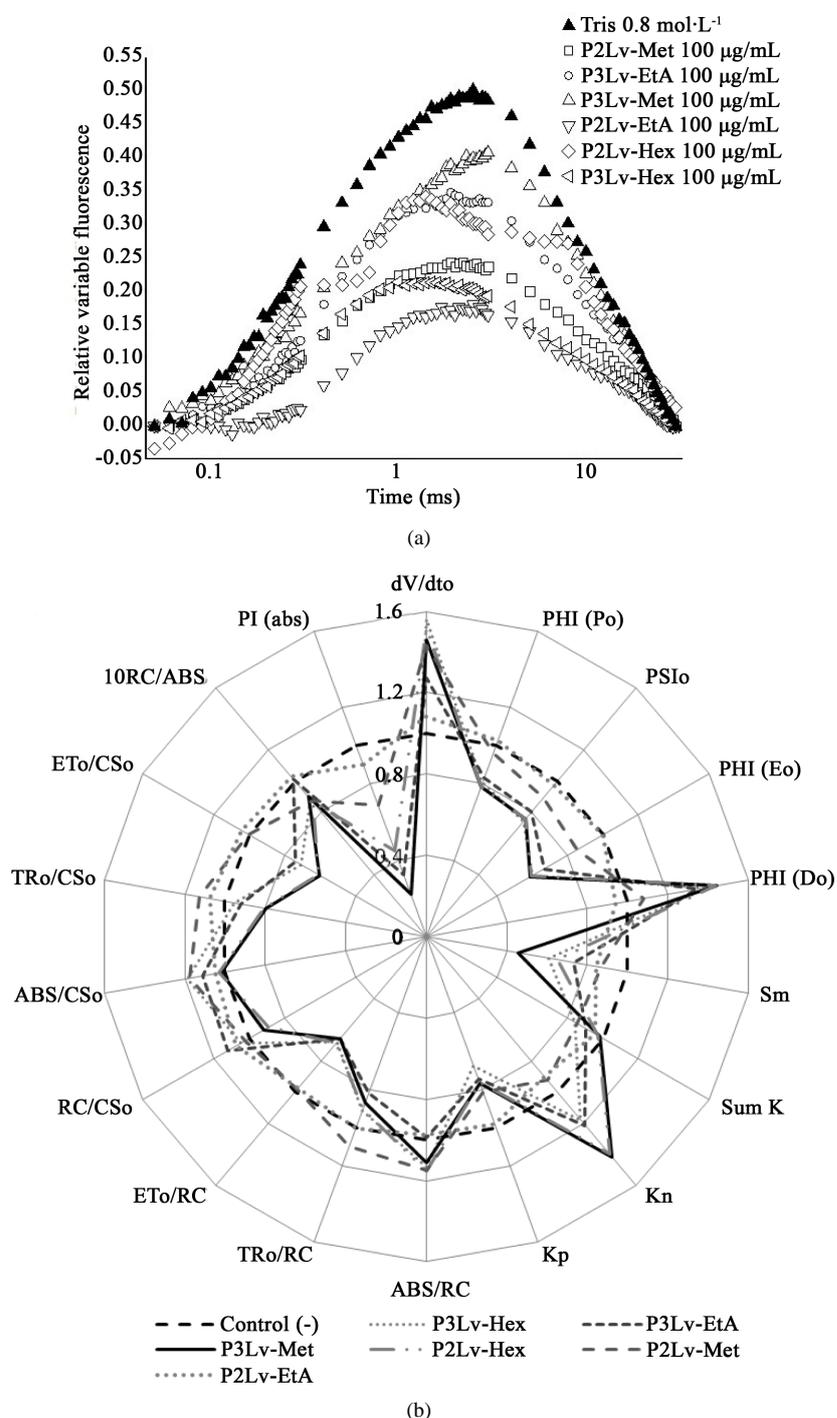


Figure 2. (a) Effect of extracts from *C. palmata* and *B. brizantha* on different calculated parameters from OJIP curves measured in treated spinach chloroplasts. Appearance of the K-band at approximately 300 µs in the presence of the samples at 100 µg·mL⁻¹. Data are an average of ten replicates; (b) Radar plot showing the JIP-test analysis for extracts from leaves of *Cecropia palmata* and *Brachiaria brizantha*. (Notes: K-band appears when the compound inhibits the electron transport at the donor side of the PSII; PI (abs), performance index; (ABS/RC), absorption per reaction center; PHI(Do), quantum yield for energy dissipation; dV/dto, water splitting enzyme function; ET0/CS0, electron transport per cross section; PHI(Po), maximum quantum yield of primary photochemistry; PSI0, trapped exciton moves an electron into the electron transport chain beyond Q_A⁻; RC/CS0, density of the reaction centers; TR0/CS0 and ET0/CS0, trapping and the electron transport per cross section)

These observations have been confirmed by the analysis of **Figure 2(b)**. Chlorophyll *a* fluorescence is an intrinsic evidence of the photosynthetic system and the intensity of the fluorescence is a direct measure of the PS II activity. From the kinetics data of the fluorescence curve measured after treatment on chloroplasts and compared with the control, several parameters were calculated and plotted as a radar graph, a circular graphic with a series of spokes or rays projecting from a central point, with each ray label representing a different variable (**Figure 2(b)**). Data values were normalized to a common specific range or percentile values. The radar shows the parameters [35] affected by the evaluated extracts. When the extracts from *B. brizantha* (P3Lv-Hex, P3Lv-EtA and P3Lv-Met) have been evaluated at 100 $\mu\text{g}\cdot\text{mL}^{-1}$, PI (abs), performance index, decreased from 10 to 80%. The same parameter was less affected in the presence of the samples from *C. palmata* (P2Lv-Hex, P2Lv-EtA and P2Lv-Met) in a range between 10% to 50%.

On the other hand, under the presence of *B. brizantha* extracts we observed an increase of some parameters: 10% - 20% of absorption per reaction center (ABS/RC); up to 50% of the quantum yield for energy dissipation PHI (Do), and the indicator of the water splitting enzyme function (dV/dto) were 40% - 60% at 100 $\mu\text{g}\cdot\text{mL}^{-1}$. Furthermore, the ethyl acetate and methanol extracts from this plant have caused a decrease of 25% on the electron transport per cross section (ETo/CSO) and the maximum quantum yield of primary photochemistry [PHI(Po)] decreased around 20% (**Figure 2(b)**).

The probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (PSIO), the density of the reaction centers (RC/CSO), the trapping and the electron transport per cross section (TRo/CSO and ETo/CSO) decreased both of them around 10% and 50%. These observations suggested that some PSII RCs were transformed to “heat sinks” or “silent reaction centers”; these centers can neither reduce Q_A and their excitation energy is dissipated as heat [35], this is the reason why PHI(Do) values increased in some cases up to 150%, in the presence of the *B. brizantha* extracts.

In a similar behavior of some samples assayed here, many natural products also have presented a transient *J*-band (**Figure 1(a)**), like the alkaloids 1,3,5-trihydroxy-10-methyl-2,8-bis(3-methylbut-2-en-1-yl)-9(10H)-acridinone, (2R)-2-tert-butyl-3,10-dihydro-4,9-dihydroxy-11-methoxy-10-methylfuro-[3,2-b]acridin-5(2H)-one, (3R)-2,3,4,7-tetrahydro-3,5,8-trihydroxy-6-methoxy-2,2,7-trimethyl-12H-pyrano[2,3-a]acridin-12-one [41] and evolitrine [35]. On the other hand, specialized metabolites like labdane-8a,15-diol [42] and citrusine-I [41] indicated clearly the appearance of a *K*-band (**Figure 2(a)**).

4. Conclusion

Our data showed that the evaluated extracts have a great *in vitro* potential of inhibition on both sides of the photosystem II light reactions, once most of them have presented effect on photosynthesis under moderate concentrations (100 $\mu\text{g}\cdot\text{mL}^{-1}$). Based on these results we can show to the scientific community how powerful and sensitive the fluorescence of ChL *a* technique can be. Therefore, the main goal of our study is the use of dereplication of natural products approaches to isolate natural products from the active extracts to obtain models for herbicides, and then it is suggested that specialized metabolites from plants or microorganisms still can be employed on programs to discover good candidates of new lead molecules in the near future.

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