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Flavonoids from leaves of *Derris urucu*: assessment of potential effects on seed germination and development of weeds

EWERTON A.S. DA SILVA¹, LÍVIA T. LÔBO¹, GEILSON A. DA SILVA¹,
ANTONIO PEDRO DA S. SOUZA FILHO², MILTON N. DA SILVA¹, ALBERTO C. ARRUDA¹,
GISELLE M.S.P. GUILHON¹, LOURIVALDO S. SANTOS¹ and MARA S.P. ARRUDA¹

¹Programa de Pós-Graduação em Química, Instituto de Ciências Exatas e Naturais,
Universidade Federal do Pará, Cidade Universitária Professor Jose da Silveira Netto, 66075-970 Belém, PA, Brasil

²Laboratório de Agroindústria, Embrapa Amazônia Oriental, Trav. Dr. Enéas Pinheiro, s/n, 66095-100 Belém, PA, Brasil

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ABSTRACT

In some previous studies, we described the isolation of nine compounds from leaves of *Derris urucu*, a species found widely in the Amazon rainforest, identified as five stilbenes and four dihydroflavonols. In this work, three of these dihydroflavonols [urucuol A (**1**), urucuol B (**2**) and isotirumalin (**3**)] were evaluated to identify their potential as allelochemicals, and we are also reporting the isolation and structural determination of a new flavonoid [5,3'-dihydroxy-4'-methoxy-(7,6:5'',6'')-2'',2''-dimethylpyranoflavanone (**4**)]. We investigated the effects of the dihydroflavonols **1-3** on seed germination and radicle and hypocotyl growth of the weed *Mimosa pudica*, using solutions at 150 mg.L⁻¹. Urucuol B, alone, was the substance with the greatest potential to inhibit seed germination (26%), while isotirumalin showed greater ability to reduce the development of the hypocotyl (25%), but none of the three substances showed the potential to inhibit radicle. When combined in pairs, the substances showed synergism for the development of root and hypocotyl and effects on seed germination that could be attributed to antagonism. When tested separately, the trend has become more intense effects on seed germination, while for the substances tested in pairs, the intensity of the effect was greater on development of weed.

Key words: *Derris urucu*, flavonoids, allelopathy, development of weeds, seed germination.

INTRODUCTION

Over the past few decades, in order to meet a growing demand for food, the so-called modern agriculture has been increasingly dependent on agrochemicals, such as fertilizers, and specially, agricultural defensives. In tropical regions, such as the Amazon, those needs are more present mainly due to acid soil conditions and low natural fertility, and

because environmental conditions are extremely favorable to endemic development. The use of these products has produced discontent of social order, mainly because they pollute natural resources, and jeopardize the quality of food of human diet, and for being associated with health problems (Anaya 1999). Besides, varieties of weeds resistant to commercial herbicides are more frequent in agricultural systems (Moreira et al. 2010). Such information shows that novel strategies are required for controlling weeds.

Correspondence to: Mara Silvia Pinheiro Arruda
E-mail: mspa@ufpa.br

Chemical substances produced by the plants themselves have been one of the many viable alternatives to face such issues. Like herbicides many of these substances act in the plant metabolism, thus a potential replacement to synthetic products available in the market (Duke et al. 2002). Another favorable topic is the similarity of molecular sites of action between natural products and synthetic products (Duke and Abbas 1995). Additionally, natural molecules have specificities which present low risk to natural resources and to the interests of society (Cutler 1988).

The Amazon forest known for its wealth and biological diversity of species, may offer opportunities for discoveries of innovative and efficient chemical molecules which may be used in large scale in most agricultural activities, including management of both agricultural pests, of economic importance, and weed control. As a result, during the last few years several studies have been conducted with native plants from the Amazon which have helped to identify powerful allelochemicals for possible agricultural use (Arruda et al. 2005, Souza Filho et al. 2005, 2006a, 2009, Santos et al. 2007, Lôbo et al. 2008, Vilhena et al. 2009).

Among the many species and varieties of native plants occurring in the Amazon forest, with chemical molecules properties with potential use in agriculture are *Derris* (*Lonchocarpus*/*Deguelia*) genera. As a result, *Derris urucu*, known as timbó vermelho, is the species which has raised our interest for its pesticide properties (Hien et al. 2003), and has recently been studied by us, resulting in the isolation of substances belonging to the dihydroflavonol and stilbene classes (Lôbo et al. 2009, 2010). Following up on this study, a hypothesis was raised that the dihydroflavonols isolated from the leaves may present potential activity for use in weed management. Therefore, the urucuol A (**1**), urucuol B (**2**) and isotirumalin (**3**) substances (Fig. 1) were evaluated to characterize, isolated or in pairs, the potential allelopathic activity of these substances. We also describe the

isolation and structural determination of a new substance, the flavanone **4** (Fig. 1), also isolated from the leaves of this plant.

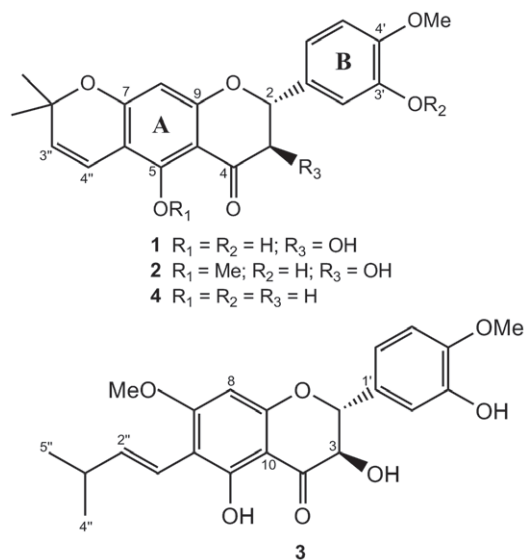


Fig. 1 - Chemical structure of flavonoids **1-4** isolated from *Derris urucu* leaves.

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURE

NMR spectra, including ¹H-¹H COSY, HMQC, HMBC experiments, were recorded on a Varian Mercury-300 spectrometer, operating at 300 MHz at ¹H and 75 MHz at ¹³C, using *d*-chloroform as solvent and internal standard. Mass spectral analyses were performed at high resolution analyses on microTOF II-ESI-TOF (Brucker, Daltonics Billerica MA, USA) only at the cationized ion region. HPLC was carried out in a preparative LC-8A Shimadzu system with SPD-10AV Shimadzu UV detector (Tokyo, Japan); using a Phenomenex Gemini C18 column (250 x10 mm, 5μ), an isocratic system of water/acetonitrile (38:62 for DU-3 fraction and 30:70 for DU-4 fraction) and a flow rate of 4.7 mL per min. Detection was performed at 280 and 320 nm. All solvents were filtered through a 0.45 μm membrane filter prior to analysis. Absorbance measurements were recorded on a Spectrum UV SP-220[®] spectrophotometer.

PLANT MATERIAL

The plants were collected at the Experimental Field of Embrapa Amazônia Oriental, located in Belém, Pará, Brazil when the plants were flowering. A fertile sample was obtained and stored at the the Botanical Laboratory, and deposited as a dried and pressed specimen under the IAN-179599 register. Approximately 2.0 kg *Derris urucu* (Killip & A.C.Sm.) green leaves were collected (Killip & A.C.Sm.) J. F. Macbr. (Leguminosae – Pap.) – being its synonym *Lonchocarpus urucu* (Killip & A.C.Sm.) and *Deguelia rufescens* var. *urucu* (Killip & A.C.Sm.) J.F. Macbr. (A.M.G. Azevedo Tozzi, unpublished data), - which were dried in a forced air greenhouse, at 40 °C, until constant weight was obtained. Next, trituration was performed with a knife mill.

EXTRACTION AND ISOLATION PROCEDURE

The dried and powdered leaves of *Derris urucu* (700 g) were extracted with ethanol at room temperature. Part of the crude extract obtained (30 g) was treated following the methodology described (Lôbo et al. 2009), giving rise, among others, to fractions DU-3 (5.17 g) and DU-4 (5.36 g). Fraction DU-3 (1.0 g) was purified on a HPLC system using a Phenomenex Gemini C18 column (250×10 mm, 5μ), an isocratic system of water: acetonitrile (38:62) and a flow rate of 4.7 mL min⁻¹, yielding five fractions. Fraction four showed chromatographic peak with retention time 19.24 and was identified as a flavanone **4** (11 mg). The fraction DU-4 (1.0 g) was purified on the same HPLC column using an isocratic system of water: acetonitrile (46:54) and a flow rate of 4.7 mL min⁻¹, yielding the compounds **1** (80 mg), **2** (28 mg) and **3** (20 mg), which showed chromatographic peaks with retention times 9.10, 7.84 and 16.27, respectively.

BIOASSAY EVALUATION OF ALLELOPATHIC ACTIVITY

The germination bioassay was developed in a controlled temperature chamber at 25 °C, for 12 hours of photoperiod. Germination was checked

every day for 10 days, with daily counting and the elimination of the germinated seeds. Seeds were considered germinated when they reached 2.0 cm or more in length of radicle. Each 9.0 cm diameter Petri dish covered with qualitative filter received 25 seeds.

The bioassays of the radicle and hypocotyl development were developed similar to the seed germination, except for the photoperiod which was now 24 hours. Each Petri dish covered with qualitative filter received three pre germinated seeds, with approximately two days of germination. After 10 days of growth, the radicle and hypocotyl length was measured.

OTHER EXPERIMENTAL PROCEDURES

The substances were tested separately and in pairs, under 150 mg.L⁻¹ concentrations. Each Petri dish received 3.0 mL of test solution. Specifically for those bioassays involving tests of substances in pairs, 50% of the volume was used for each substance. Following the eluent evaporation, the same volume of distilled water was added, thus, maintaining the original concentration. The solutions were added just once, at the beginning of the tests, and then, whenever necessary, only distilled water was added.

Malícia (*Mimosa pudica*) as a common weed in the Amazon, was chosen to evaluate the allelopathic potential of the substances investigated in this work. The seeds of that species were collected in cultivated pasture areas, in the Municipality of Terra Alta, State of Pará, and eventually cleaned and treated to break the dormancy, by immersing them into sulfuric acid for 20 minutes, as specified by Souza Filho et al. (1998).

EXPERIMENTAL DELINEATION AND STATISTICAL ANALYSIS

The experimental delineation was entirely randomized with four repetitions using distilled water as control treatment. The data were transformed to arc.

sine \sqrt{x} , to follow normal distribution. The values obtained were submitted to variance analysis, using F-test, and when treatment effects presented significant differences ($p < 0.05$), the means were compared using the Tukey test. We used the Statistical Analysis System (1990) to perform the data analysis.

RESULTS

PHYTOCHEMICAL INVESTIGATION

Fractionation of ethanol extract obtained from the leaves of *D. urucu*, allowed the isolation and structural identification of nine compounds (Lôbo et al. 2009, 2010). From the same extract, a new substance (**4**) was isolated as a white powder. The HRESIMS of **4** displayed a pseudomolecular ion $[M+Na]^+$ at m/z 391.1327, consistent with the molecular formula $C_{21}H_{20}O_6Na$, further corroborated by the NMR data of **4**. The 1H and ^{13}C NMR spectra of **4** (Table I) showed characteristic sets of signals at δ_H 5.30 (*dd*, $J=12.6$ and 2.7 Hz, H-2), 3.05 (*dd*, $J=17.4$ and 12.6 Hz, H-3 β) and 2.76 (*dd*, $J=17.4$ and 2.7 Hz, H-3 α) and at δ_C 78.8 (CH, C-2) and 43.1 (CH₂, C-3) of a flavanone skeleton (Jang et al. 2002). A low-field singlet at δ_H 12.29 indicated a C-5 OH group hydrogen-bonded to a carbonyl at C-4. Aromatic proton signals at δ_H 7.03 (*d*, $J=1.8$ Hz, H-2'), 6.92 (*dd*, $J=8.1$ and 1.8 Hz, H-6') and 6.87 (*d*, $J=8.1$ Hz, H-5') could be assigned as 1,3,4-trisubstituted aromatic ring B protons, as evident from the HMBC correlations from H-2' and H-6' to C-2 (Table I). The 3J correlations of H-2', H-6', and a singlet at δ_H 3.91 (3H) to C-4' (δ_C 145.8) indicated the attachment of a OCH₃ group at C-4'. The correlations of H-2' to both C-3' (δ_C 146.9) and C-4', and the presence of only one signal of OMe (δ_H 3.91) showed a OH group linked to C-3'. The 1H NMR signals at δ_H 5.49 (*d*, $J=9.9$ Hz, H-3''), 6.61 (*d*, $J=9.9$ Hz, H-4'') and 1.43 (*s*, 6H) showed 1J correlations with the ^{13}C NMR signals at δ_C 126.2, 115.2 and 28.4 (2Me), respectively, and were assigned to a dimethylchromene group. The key HMBC correlations between OH-5/C-5 (δ_C 158.3)

and H-4''/C-5 required the placement of a chromene ring at C-6 and C-7. The configuration at C-2 was assigned as *S* based on a vicinal coupling constant of 12.6 Hz, in comparison to those of previously reported flavanones (Jang et al. 2002). Compound **4** was thus identified as 5,3'-dihydroxy-4'-methoxy-(7,6:5'',6'')-2'',2''-dimethylpyranoflavanone and was given the trivial name urucunone.

PHYTOTOXICITY OF DIHYDROFLAVONOLS ISOLATED

The potential phytotoxic promoted over seed germination (Fig. 2), of malicia as a receptor plant varied significantly ($p < 0.05$) between the substances tested (**1-3**). Substance **2** caused the most intense inhibiting factors, always significantly higher ($p < 0.05$), than those of **1** and **3**. However, the magnitudes of these effects were always lower than 30%. No significant difference was observed ($p > 0.05$) between the inhibitory potential of substances **1** and **3**. When analyzed in pairs, the intensity of inhibitions were always lower ($p < 0.05$) when compared to effects caused by each substance alone, what might indicate the existence of antagonism between both **1** and **2**, as well as between **1** and **3**, and **2** and **3**.

Figure 3 shows the effects over radicle development. The data show that there were no significant differences ($p > 0.05$) between the three substances, when tested separately, with effect intensity always below 20%. Comparatively to the inhibitory effects over seed germination, we observed that when the substances are administered separately, they presented more phytotoxic potential over seed germination, although this difference was not so significant. Under all conditions, when the substances were tested, inhibition was significantly ($p < 0.05$) higher than those promoted by the substances separately. In some combination, such as in **1+3**, the increase in relation to the two separated substances was 200%. For combination **1+2**, it was observed an increase in the phytotoxic capacity by 150%. These data sets show that there

TABLE I
¹H (300 MHz) and ¹³C (75 MHz) NMR spectral data for **4**, including heteronuclear 2D shift-correlated obtained by ¹H and ¹³C-COSY-ⁿJ_{CH} (n=1, HETCOR, n=2 and 3, HMBC) experiments, in CDCl₃ as solvent, chemical shifts (δ, ppm) and coupling constants (J, Hz, in parenthesis).^a

Position	¹ H- ¹³ C-COSY- ¹ J _{CH}		¹ H- ¹³ C-COSY- ⁿ J _{CH}	
	δ _H	δ _C	² J _{CH}	³ J _{CH}
2	5.30 (<i>dd</i> , 12.6 and 2.7)	78.8	H-3β/C-2	H-2'/C-2
3α	2.76 (<i>dd</i> , 17.4 and 2.7)	43.1		
3β	3.05 (<i>dd</i> , 17.4 and 12.6)			
4		195.4		
5		158.3	OH-5/C-5	
6		102.9		OH-5, H-8, H-3''/C-6
7		162.0	H-8/C-7	H-4''/C-7
8	5.95 (<i>s</i>)	96.2		
9		162.3	H-8/C-9	
10		103.1		H-8, OH-5/C-10
1'		131.5	H-6'/C-1'	H-5'/C-1'
2'	7.03 (<i>d</i> , 1.8)	112.6		
3'		146.9	H-2', OH-3'/C-3'	
4'		145.8	H-5/C-4'	H-2', H-6'/C-4'
5'	6.87 (<i>d</i> , 8.1)	110.5		
6'	6.92 (<i>dd</i> , 8.1 and 1.8)	118.1		H-2'/C-6'
2''		78.2	H-3'', 2Me-2''/C-2''	
3''	5.49 (<i>d</i> , 9.9)	126.2		2Me-2''/C-3''
4''	6.61 (<i>d</i> , 9.9)	115.2		
2Me-2''	1.43 (<i>s</i>)	28.4/28.3		
OMe-4'	3.91 (<i>s</i>)	56.0		
OH-5	12,29 (<i>s</i>)			
OH-3'	5,72 (<i>s</i>)			

^aNumber of hydrogens bound to carbon atoms deduced by comparative analysis of DEPT-¹³C NMR spectra.

¹H-¹H-COSY spectrum was also used in these assignments.

was a synergy between the three substances when administered in combination. The data differ from those observed over seed germination, where antagonistic effects might be involved.

For effects over the hypocotyl development (Figure 4), no significant differences were observed (*p*>0.05) between substances **1** and **2**. The inhibitions promoted by these two substances, when tested separately, were below 10%. When tested separately, substance **3** presented greater potential (*p*<0.05) to inhibit radicle development than **1** and **2**, promoting,

however, inhibition below 30%. When combined in pairs, the substances always promoted inhibitions of greater magnitude when compared to the effects achieved by the substances separately, indicating the existence of synergic effects among the three substances. This result replicates that observed on the radicle development (Figure 3) and is different from the effects promoted over seed germination (Figure 2), indicating possibility of antagonism. In general, the tendency observed was that the substances, when tested separately, promoted more

intense effects over seed germination, and when in pairs, they inhibited the radicle and the hypocotyl in a greater scale. Separately, substance **1** was more effective in inhibition of seed germination of malícia weed, while **3** showed that it was more effective on inhibition of hypocotyl development. No significant differences were observed ($p>0.05$) on inhibitions

promoted by allelochemicals **1** and **2**, on both the radicle and the hypocotyl development.

DISCUSSION

The rich Amazon plant biodiversity offers new and revealing opportunities to discover chemical molecules with potential use in weed management.

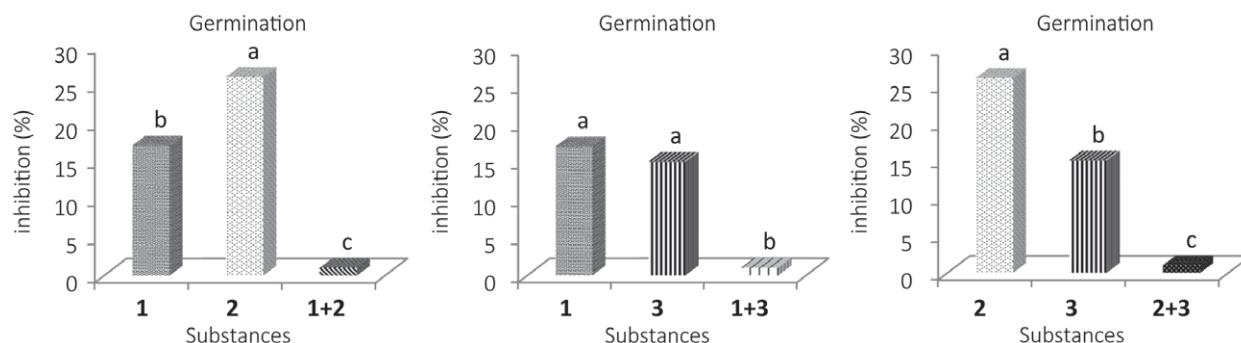


Fig. 2 - Effects of dihydroflavonols **1**, **2** and **3**, separately, and in pairs, over seed germination of malícia weed seedling. Data is shown in percentages of inhibition in relation to control treatment: distilled water. Letters show significant differences by the Tukey test (5%).

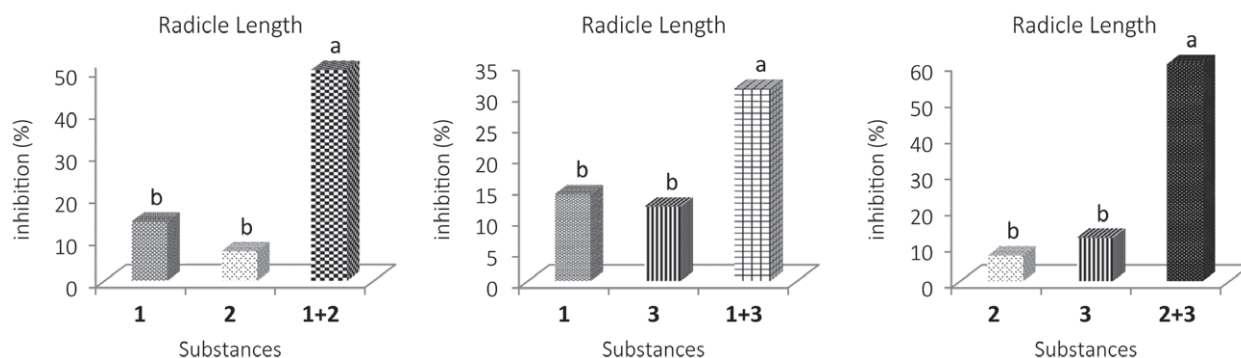


Fig. 3 - Effects of dihydroflavonols **1**, **2** and **3**, separately, and in pairs, over root development of malícia weed seedling. Data is shown in percentages of inhibition in relation to control treatment distilled water. Letters show significant differences by the Tukey test (5%).

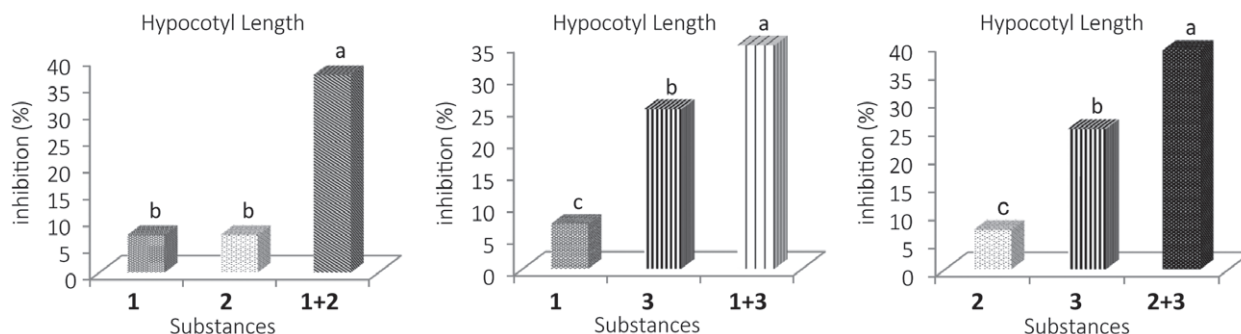


Fig. 4 - Effects of dihydroflavonols **1**, **2** e **3**, separately, and in pairs, over the hypocotyl development of malícia weed seedling. Data is shown in percentages of inhibition in relation to control treatment: distilled water. Letters show significant differences by the Tukey test (5%).

Timbó vermelho (*Derris urucu*) is a species found in South America and Asia, with large dispersion in the Amazon Region. Several chemical prospecting studies of this plant have been more restricted to its roots, being identified the presence of rotenoids rotenone, deguelin, tefrosin and rotenolone as major metabolites and minor compounds belonging to the flavanone, isoflavanone, chalcone, flavonol, pterocarpan, stilbene and saponin classes (Braz Filho et al. 1975, Parente and Mors 1980, Fang and Casida 1999, Pereira et al. 2000). Recent studies, including this one, involving the leaves of *D. urucu*, have shown the absence of rotenoids. Apparently, the occurrence of these substances is restricted to the roots and barks of this species.

Flavonoids form an important group with different biological activities. In terms of allelopathy, some information is found in the literature indicating this activity for different compounds of flavonoids (Anaya et al. 2003, Tseng et al. 2003, Perry et al. 2007, Simões et al. 2008). Recent studies (Cipollini et al. 2008, Simões et al. 2008) show the importance of flavonoids as allelopathic agents. There are no references concerning the allelopathic activity of isolated dihydroflavonoids in this study. Thus, the results found for other compounds of the group may serve as point of reference, facilitating assessment the potential of the three substances isolated and tested in this study. For instance, Lôbo et al. (2008) e Arruda et al. (2005) obtained, for lower concentration, effects much greater than those observed in this study, for the same weed specie indicating a wide variation in allelopathic activity of flavonoids. In addition allelopathic inhibition obtained for chemical substances from the leaves of timbó vermelho (*Derris urucu*) constitutes a factor that values the native flora of the Amazon rainforest and must be taken into consideration when speculating on the importance forest preservation in the Amazon as an alternative source of chemical molecules with potential use in weed management.

Attempts to relate the allelopathic activity to the structure of a molecule are rare in the literature, as seen in a study by Reynolds (1987) is a good example. Small differences in the chemical structure of certain substances may be favorable to increase in allelopathic activity (Souza Filho et al. 2006b). According to Bitencourt et al. (2007), the presence or absence of certain functional groups in a molecule may represent the increase or decrease of allelopathic activity. Of the three dihydroflavonoids tested in this study (**1-3**), Urucuol A (**1**) and Urucuol B (**2**) present chemical structures extremely similar, with differences only in the group connected to C-5, OMe in **2** and OH in **1**. This difference produced effects more significant to Urucuol B (**2**), especially in relation to inhibition of seed germination. The Isotirumalin structure (**3**) differs from the other two substances due to the absence of ring 2,2-dimethylchromene linked to ring A, and its effects were more intense over the hypocotyl development. Apparently, the Urucuol B structure was more compatible to reduce weed germination while the Isotirumalin structure was more effective to reduce hypocotyl development, however, it is difficult to determine if only one functional group is operative and determinant of the molecule allelopathic activity, and it has not been clear yet, which specific factors act in each case.

In plant communities, the effects which may be attributed to the allelopathy phenomenon result not only from the action of a single allelochemical, but from various components acting simultaneously in the target plant. As a result, one may assume that allelopathic activity of a mixture of allelochemicals will be determined not only for its concentration, but also, by the positive or negative interaction which may exist among the substances. There is information in the literature suggesting the existence of synergism as a result from the combination of different allelochemicals (Kubo et al. 1992, Vokou et al. 2003). In most studies where this hypothesis has been tested, the combination among the allelochemicals

involve fixed concentration and the interferences are based in the increase of inhibitory activity in relation to effects promoted by each substances alone (leading us to conclude the existence of synergism) and the reduction in inhibitory activity (suggesting the occurrence of antagonism). The results observed for evaluation of the three dihydroflavonoids indicate the existence of synergic effects, as well as possible antagonist effects. In the seed germination bioassay, the predominant tendency was suggestive of antagonism, while synergic effects were observed in radicle and hypocotyl development. Apparently, the manifestation of one or the other of these two attributes depends not only in the capacity that the substances are thought to be able to augment each other's activity, but also the factor of the plant under analysis. It is known, for example, that in general, seed germination is less sensitive to allelochemical effects than the growth of seedlings (Reigosa and Malvido 2007), which, to some extent, may explain the variations observed in this study.

The set of results obtained once more indicate the Amazon forest as a source of chemical molecules with properties to be used in weed management, thus contributing to the aggregation of economic value of the two species and consequently for its preservation.

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RESUMO

Em estudos anteriores descrevemos o isolamento de nove substâncias, a partir das folhas de *Derris urucu*, uma espécie encontrada amplamente na floresta Amazônica, as quais foram identificadas como cinco

estilbenos e quatro diidroflavonóis. Neste trabalho, três desses diidroflavonóis [urucuol A (1), urucuol B (2) e isotirumalina (3)] foram avaliados para identificar seus potenciais como aleloquímicos. Estamos relatando também, o isolamento e a determinação estrutural de um novo flavonóide [5,3'-diidroxí-4'-metoxi-(7,6:5'',6'')-2'',2''-dimetilpiranoflavanona (4)]. Investigamos os efeitos dos diidroflavonóis 1-3 sobre a germinação de sementes e desenvolvimento da radícula e do hipocótilo da planta daninha *Mimosa pudica*, usando soluções a 150 mg.L⁻¹. Urucuol B, isoladamente, foi quem apresentou o maior potencial para inibir a germinação de sementes (26%), por sua vez, isotirumalina exibiu maior capacidade para reduzir o desenvolvimento do hipocótilo (25%), porém nenhuma das três substâncias mostrou potencial para inibir o desenvolvimento da radícula. Quando combinadas aos pares, as substâncias mostraram sinergismo ao desenvolvimento da radícula e do hipocótilo e efeitos, na germinação de sementes, que poderiam ser atribuídos a antagonismo. Quando testadas separadamente, as substâncias apresentaram maior tendência para inibir a germinação de sementes, enquanto que, quando testadas aos pares, observou-se aumento no efeito de inibição do desenvolvimento da planta daninha.

Palavras-chave: *Derris urucu*, flavonóides, alelopatia, desenvolvimento da planta, germinação de semente.

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