Flavonoids from *Annona dioica* Leaves and their Effects in Ehrlich Carcinoma Cells, DNA-topoisomerase I and II

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A investigation fitoquímica do extrato metanólico das folhas de *Annona dioica* (Annonaceae) resultou na identificação dos flavonóides kaempferol (1), 3-O-[3",6"-di-O-p-hidroxicinnamoyl]-β-galactopiranossil-kaempferol (2), 6"-O-p-hidroxicinnamoyl-β-galactopiranossil-kaempferol (3) e 3-O-β-galactopiranossil-kaempferol (4). As estruturas foram caracterizadas inequivocamente através de análises espectroscópicas utilizando-se RMN de 1H e 13C uni e bi-dimensional. Os efeitos citotóxicos dos flavonóides e da fração flavonoidica (FF) foram avaliados frente a células do carcinoma de Ehrlich, utilizando-se o ensaio do MTT (brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium). Os resultados indicaram que 1, 2, 3 e FF apresentam ação anti-proliferativa importante quando comparados com a quercetina. A ação inibitória dos flavonóides sobre as DNA-topoisomerases I e II foi avaliada utilizando-se o ensaio de relaxação sobre o plasmídeo pBR322. Os resultados indicaram ação não seletiva dos flavonóides sobre as DNA-topoisomerases I e II.

Chemical investigation of methanol extract leaves from *Annona dioica* (Annonaceae) resulted in the identification of flavonoids kaempferol (1), 3-O-[3",6"-di-O-p-hidroxicinnamoyl]-β-galactopyranosil-kaempferol (2), 6"-O-p-hidroxicinnamoyl-β-galactopyranosil-kaempferol (3) and 3-O-β-galactopyranosil-kaempferol (4). The structures were unequivocally characterized by 1H and 13C NMR spectroscopic analyses using 1D and 2D experiments. The cytotoxic effects of the flavonoids and flavonoid fraction (FF) were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-difeniltetrazolium bromide) assay against Ehrlich carcinoma cells. The results indicated that 1, 2, 3 and FF exhibit significant antiproliferative action when compared to quercetin. The inhibitory action on DNA-topoisomerase I and II of all the flavonoids was evaluated by relaxation assays on pBR322 plasmid DNA. The results indicated the inhibitory and non-selective effects of the flavonoids on DNA-topoisomerase I and II.

**Keywords:** *Annona dioica*, Annonaceae, cytotoxic activity, flavonoids, DNA-topoisomerase

### Introduction

The Annonaceae represents a large pan tropical family comprising approximately 120 genera and 2,000-2,200 species. The family is extremely important since it is the source of several edible fruits; seeds of some plants may be used for the production of edible oils; wood from some annonaceous plants has been employed in alcohol production, and the fragrant flowers of *Cananga odorata* are an important raw material for perfumery. Further, many members of this family are used in folk medicine for different purposes. Chemical studies, and to a lesser extent pharmacological investigations, on Annonaceae plants involved the search of alkaloids in the past, and of the acetogenins in the last decade. However, amino acids, carbohydrates, lipids, proteins, polyphenols, essential oils, terpenes and aromatic compounds are also typically found in these plants.

Flavonoids, a heterogeneous group of ubiquitous plant polyphenols, are a frequent component of the human diet. A large number of biological activities has been attributed...
to these compounds, including anticancer activity.\textsuperscript{4,6} The flavonoid compounds on the diet may act as chemopreventive agents against the development of cancer in the alimentary tract.\textsuperscript{7} In fact, a number of studies has demonstrated anticancer activity associated with flavonoids.\textsuperscript{8} These compounds are constituents of herbal treatments used by lay practitioners for a long time, but their effects as individual compound have only recently been recognized.\textsuperscript{9}

DNA supercoiling is a precisely regulated process that influences DNA replication, transcription and packaging. DNA topoisomerases are enzymes that modulate the topological state of DNA. The interest in these enzymes has increased in the last few decades; and they have been shown to be the target enzymes for many effective drugs in cancer treatment.\textsuperscript{10}

As part of our continuing search for potential anticancer drug candidates from South American medicinal plants, we decided to study \textit{Annona dioica}, Annonaceae, with its very appreciable fruits, a shrub which is widely dispersed in South America, extending from Bolivia to Paraguay. In Brazil, the fruits and leaves are used against rheumatism and the seeds against diarrhea,\textsuperscript{11} and in Paraguay the leaves are used to make tea or to gargle as an anti-catarrhal, while the edible fruits possess sedative properties and their seeds are used as insecticides or in the treatment of parasitic infections of the skin.\textsuperscript{12}

Previous phytochemical investigations of the \textit{Annona dioica} ethanolic extract of \textit{Annona dioica}, collected in Serra da Moeda (Minas Gerais state of Brazil), led to the isolation and characterization of six alkaloids.\textsuperscript{13} A taxonomic study of the Brazilian Annonaceae foliar material also indicated the presence of flavonoids.\textsuperscript{14}

In this paper we report for the first time the isolation and characterization of the four foliar flavonoids, kaempferol (1), 3-\textit{O}-\{3",6"-di-\textit{O}-p-hydroxycinnamoyl]-\textit{β}-galactopyranosyl-kaempferol (2), 6"-\textit{O}-\textit{p}-hydroxycinnamoyl-\textit{β}-galactopyranosyl-kaempferol (3) and 3-\textit{O}-\textit{β}-galactopyranosyl-kaempferol (4), from a methanolic extract of the leaves of \textit{Annona dioica} collected in Paraguay (see Figure 1). These flavonoids 1-4 were characterized by spectroscopic analyses involving IR, \textit{\textsuperscript{1}H} (1D and 2D \textit{\textsuperscript{1}H-\textsuperscript{1}H-COSY}), \textit{\textsuperscript{13}C} (\textit{\textsuperscript{1}H}- and APT), HMQC and HMBC NMR spectra and comparison to the previous literature data. In addition, these flavonoids (1-4) and the methanol fraction FF were evaluated for cytotoxic activities against Ehrlich carcinoma cells and DNA topoisomerase I and II-α inhibitory activities.

Figure 1. Flavonoids isolated from \textit{Annona dioica} leaves methanol extract.
Experimental

General

$^1$H-NMR and $^{13}$C-NMR spectra were recorded on a JEOL Eclipse (400 MHz and 100 MHz, respectively) spectrometer. Chemical shifts are given in $\delta$ units, relative to the tetramethylsilane (TMS) signal as an internal standard.

Plant material

Leaves of *A. dioica* were collected in Cerrado of Horqueta, Paraguay, in February of 1999. The voucher specimen (No. 8567) was deposited in the Departamento de Botanica of Universidad Nacional, Asunción, Paraguay.

Extraction and isolation

The leaves were extracted by maceration with hexane and methanol. The methanol extract (120.2 g) was partitioned between CH$_2$Cl$_2$ and MeOH:H$_2$O (2:1) to furnish hydroalcoholic and dichloromethane fractions. A portion of the methanolic fraction was separated for biological assays (FF) and the rest of the material, 50.7 g, was chromatographed on a silica gel column using a polarity gradient of CHCl$_3$/acetone/MeOH as eluents to yield 36 fractions. The 1-10 fractions (0.25 g) furnished a sitosterol and stigmasterol mixture, after elution with hexane:CH$_2$Cl$_2$ (8:2) as solvent system. The fraction 12 (0.075 g) was purified by preparative TLC using CH$_2$Cl$_2$:acetone (1:1) as solvent system, and furnished the flavonoid 2 (0.015 g, CAS number 606138-07-2, C$_{39}$H$_{32}$O$_{15}$, MW 740, mp 181-183 °C). The fractions 13-15 (0.14 g) were chromatographed on a Sephadex LH-20 column using MeOH as solvent affording the flavonoids 1 (0.018 g, CAS number 520-18-3, C$_{15}$H$_{10}$O$_6$, MW 286, mp 285-286 °C), 3 (0.024 g, CAS number 68170-52-2, C$_{30}$H$_{26}$O$_{13}$, MW 594, mp 269-271 °C) and 4 (0.004 g, CAS number 23627-87-4, C$_{21}$H$_{20}$O$_{11}$, MW 448, mp 230-232 °C).

Bioassays

Cell culture

Ehrlich carcinoma cells were maintained for 12-14 days in Swiss mice. The tumor cell cultures were started from mouse Ehrlich ascites with at least one passage *in vitro* prior to use.

Cytotoxicity assay

Aliquots of $5 \times 10^5$ tumor cells were seeded onto 96 well flat microplates in RPMI 1640 complete medium. The flavonoids 1-4 and quercetin (positive control) in 0.5% (v/v) DMSO:saline at final concentrations of 50, 25, 12.5 and 6.25 μmol L$^{-1}$, and FF at concentration of 30, 15, 7.5 and 3.75 ng mL$^{-1}$ were added to cells and incubated at 37 °C in the presence of 5% CO$_2$. The same volume was used as negative control. After 48h of cell culture, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the samples and the absorbance was read at 570 nm after 3h at 37 °C. The IC$_{50}$ values were reported as means ± SD of three independent experiments. Statistical significance was assessed by the Student’s $t$-test.

DNA-topoisomerase I assay

The topo I inhibition was determined by relaxation assay and was carried out as described in the TopoGEN screening kit. Briefly, 0.125 μg of the supercoiled pBR322 plasmid DNA (Invitrogen) was incubated with one unit of the enzyme in the presence or absence of flavonoids for 30 min at 37 °C, in reaction buffer. Reaction products were loaded onto a 1% agarose gel containing ethidium bromide. Electrophoresis was carried out in tris-acetate-EDTA pH 8.5 at 15V for 3.5h and then photographed with a digital camera by illumination.

DNA-topoisomerase II assay

The topo II-α inhibition was carried out as described in the TopoGEN screening kit. Briefly, the reaction mixture containing the flavonoid, 0.125 μg mL$^{-1}$ pBR322 (Invitrogen), assay buffer, 2U of topo II-α, and water was incubated at 37 °C for 30 min. The reaction products were submitted to electrophoresis using 1% agarose gel in 1× TAE buffer (50× stock: 242 g Tris base, 57.1 mL glacial acetic acid and 100 mL of 0.5 mol L$^{-1}$ EDTA) at 15V for 3.5h. Gels were stained with ethidium bromide (0.5 μg mL$^{-1}$) for 30-45 min, washed and photographed under UV light.

Results and Discussion

The flavonoids were isolated from methanol and hexane extracts of *Annona dioica* as indicated in Experimental section. The $^1$H NMR of flavonoids 1-4 showed typical signals of substitution for the standard kaempferol flavonoid. The A ring of flavonoid 1 showed two doublets (J 2.1 Hz, meta-coupling) at δ$_H$ 6.2 and 6.4 attributed to hydrogen atoms H-6 and H-8, respectively.

Analogously, the $^1$H NMR spectra of the flavonoids 2-4 showed the same substitution pattern for the A ring of 1, with signals for H-6 at δ$_H$ 6.3 (J 2.2 Hz, 2), 6.1 (J 1.9 Hz, 3) and 6.2 (J 1.9 Hz, 4).
Hz, 3) and 6.0 (J 2.2 Hz, 4), and for H-8 at δc 6.5 (J 2.2 Hz, 2), 6.3 (J 1.9 Hz, 3) and 6.2 (J 2.2 Hz, 4). Singlet signal at δc 12.37 was attributed to the hydroxyl group at carbon atom C-5 (HO-5, quelatogenic hydroxyl function). This 5,7-dihydroxy substitution pattern in the four flavonoids was confirmed by 13C chemical shifts of the methine carbon atoms CH-6 [δc 99.3 (1), 99.9 (2), 101.0 (3) and 99.7 (4)], CH-8 [δc 94.5 (1), 94.8 (2), 94.8 (3) and 94.5 (4)], and by the signals corresponding to quaternary carbons C-5, C-7, C-9 and C-10 (Table 1).

The presence of AA’BB’ system in the B ring (4'-hydroxylated) of the four flavonoids was revealed by 1H NMR spectra through doublet (J 8.8 to 8.9 Hz, ortho-coupling) signals corresponding to H-2’/H-6’ at δh 8.1 (1), 8.2 (2), 8.1 (3) and 8.1 (4), and H-3’/H-5’ at δh 6.9 (1), 7.0 (2), 6.8 (3) and 6.9 (4). The HMQC spectra showed cross-peaks indicating direct 2D-shift-correlations of these hydrogen signals with the corresponding 13C signals (Table 1) of the methine carbons CH-2’/CH6’ (δc 130.7 to 132.3) and CH-3’/CH5’ (δc 115.9 to 116.3).

Comparative analysis of the 1H (1D and 2D 1H-1H-COSY) and 13C ([1H] and APT) NMR spectra of 1 and 2-4 revealed the additional presence of signals which allowed the identification of the unsubstituted galactopyranosyl moiety (4), 3,6-di-O-p-hydroxycinnamoyl (2) and 3-O-p-hydroxycinnamoyl-3, represented by the doublet (J 8.0 to 7.5 Hz, axial-axial couplings) signals corresponding to anomic hydrogens H-1g at δh 5.4 (2), 5.1 (3), and 5.0 (4), which were confirmed by the HMQC heteronuclear correlations with the 13C signals at δc 105.5, 105.0 and 103.0, respectively (Table 1). This galactopyranosyl unit was located in the carbon C-3 (3-O-galactopyranosyl) based on the deshielding effect (e.g. Δδc = 158.7 (2) - 148.1 (1) = 10.7 ppm) observed in the chemical shifts (Table 1) of the carbon atom C-2 of 2 (δc 158.7), 3 (δc 159.1) and 4 (δc 157.9) when compared to C-2 of 1 (δc 148.1).

The 1H and 13C NMR spectra of flavonoid 2 and 3 (Table 1) showed additional signals attributed to one (3) and two p-hydroxycinnamoyl (2) units. The location of these p-hydroxycinnamoyl moieties at methylene group CH2-6g of 2 (δc 65.6) and 3 (δc 64.4) was established by conspicuous deshielding (Δδc = 3.5 (2) and 2.3 (3) ppm) of 2 (δc 65.6) and 3 (δc 64.4), observed when compared to the chemical shift of this group in 4 (δc 62.1). This was confirmed by the shielding (γ-effect) shown by the chemical shifts of the carbon atoms CH-5g [Δδc = 74.1 (2) - 78.3 (4) = -4.2 and 74.8 (3) - 78.3 (4) = -3.5 ppm]. The analogous γ-effects were observed in the carbon atoms CH-2g and CH-4g of 2, justifying the presence of an additional p-hydroxycinnamoyl at carbon CH3-3g (Table 1).

The presence of cinnamoyl units was recognized by 1H (1D and 2D 1H-1H-COSY) and 13C (Table 1) NMR spectra. The doublet (J 8.4 Hz) signals corresponding to H-2’/H-6’ at δh 7.6 and H-3’/H-5’ at δh 6.9, as well as H-2’/H-6’ at δh 7.4 and H-3’/H-5’ at δh 6.9. The trans (E) stereochemistries were deduced by coupling constants for H-7’/H-8’ at δh 7.7 and 6.3, respectively (J 15.8 Hz, 2), δh 7.5 and 6.2 for H-7’/H-8’ (J 16.1 Hz, 2), and for 3, signals corresponding to two doublets at δh 7.4 and 6.0 (J 15.8 Hz, 3) were observed. The complete 1H and 13C chemical shift assignments of these flavonoids were also based on the homonuclear 2D 1H-1H-COSY and heteronuclear 2D 1H-13C shift-correlated (HMQC and HMBC) experiments and compared to previous incomplete literature data.16-19

Table 1. 13C (100 MHz) NMR data for flavonoids 1-4 from A dioica in MeOH-d4 (1, 3 and 4) and acetone-d6 (2) as solvent. Chemical shifts in δc,*

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*Carbon signals corresponding to C, CH and CH2, deduced by comparative analysis of 1H and APT 13C NMR spectra. Heteronuclear 2D HMQC and HMBC spectra were also used for these assignments.
The cytotoxic activities of the flavonoids (1-4) and the methanolic fraction (FF) were assayed in vitro against murine Ehrlich carcinoma (1 × 10^6 cells). Cell viability was assayed in the presence or absence of 1-4, FF and quercetin, utilized as a positive control, using the Mossman assay. The flavonoids 1-3 and FF exhibited concentration-dependent growth inhibiting activities against cultured Ehrlich carcinoma cells (see Figure 2). The IC_{50} values were (7.8 ± 0.1), (10.9 ± 0.5) and (14.7 ± 1.4) mmol mL⁻¹ for 1, 2 and 3, respectively. The flavonoid 4 did not show significant cytotoxicity until the dose of 50 μmol L⁻¹, while the methanolic fraction FF showed the best activity with an IC_{50} value of 4.0 ± 0.1 μmol L⁻¹, suggesting a probable synergistic effect of flavonoids 1-3 (Table 2). The comparison of the IC_{50} values between the flavonols quercetin (IC_{50} = 44.0 ± 4.0) μmol L⁻¹ and kaempferol (1, IC_{50} = 7.8 ± 0.1 μmol L⁻¹) showed the importance of the hydroxylation pattern of the B ring. The absence of the p-hydroxycinnamoyl moiety linked to galactopyranosyl group located in the carbon C-3 of kaempferol led to the inactivity of 4.

The conversion of supercoiled plasmid DNA to relaxed DNA by human topoisomerases I and II-α was examined in the presence of flavonoids (1-4) and FF. The activity of the flavonoids on DNA-topoisomerase was observed through relaxation assays of supercoiled pBR322 plasmid DNA. Quercetin, a well-known topoisomerase I enzyme inhibitor, was used as a positive control. The results were observed by the alteration of pBR322 electrophoretic mobility by the combined action of topoisomerase I and flavonoids. The analysis of the results was performed by ethidium bromide addition under UV light, and the results were photographed with a digital camera. As shown in Figure 3 all the tested flavonoids (with the exception of FF) indicated inhibitory relaxation effects, with 2 being the most effective. This observation was based on the direct measurement of fluorescence intensity of the respective gel areas by video-enhanced imaging (line 5).

### Table 2. IC_{50} (mmol mL⁻¹ and ng mL⁻¹) values of the flavonoids 1-4 and fraction FF against Ehrlich carcinoma cells

<table>
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<tr>
<th>Natural products</th>
<th>IC_{50} (± s.d.) mmol mL⁻¹</th>
<th>IC_{50} (± s.d.) ng mL⁻¹</th>
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<tr>
<td>Quercetin</td>
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<td>13.29 ± 1.3</td>
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<tr>
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<td>7.8 ± 0.1</td>
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<td>10.9 ± 0.5</td>
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<tr>
<td>3</td>
<td>14.7 ± 1.4</td>
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<td>na⁺</td>
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</tr>
<tr>
<td>FF</td>
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*sd: standard deviation; na: no activity until 50 mmol mL⁻¹.*
demonstrated inhibitory effects on human DNA-topoisomerase II-α similar to the pattern observed for etoposide, line 1, which was used as positive control.

The analysis of DNA topoisomerase inhibition and Ehrlich cells antiproliferative activity results, in which FF shows potent cytotoxic activity and only inhibits DNA-topoisomerase II, suggests the major importance of DNA topoisomerase II inhibition in determining the level of cytotoxic activity, and confirms the synergistic effect of the flavonoids 1-3 in this fraction.

Conclusions

The occurrence of flavonoids 1-4 in the genus *Annona* is described here for the first time, and as reported in this work, they act on human topoisomerases I and II-α inhibiting the DNA relaxation effect including the non-cytotoxic compound 4, reinforcing the potential importance of their presence in the human diet.

Supplementary Information

Supplementary data of flavonoid structures as 1 and 2D 1H and 13C NMR spectra and are available free of charge at http://jbcs.sbq.org.br, as PDF file.

Acknowledgments

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References

1. Leboeuf, M.; Cavé, A.; Bhaumik, P. K.; Mukherjee, B.; Mukherjee, R.; *Phytochemistry* 1982, 12, 2783.


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Figure S1. $^1$H NMR spectrum of flavonoid I (400 MHz, CD$_3$OD).

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Figure S2. $^{13}$C NMR spectrum of flavonoid 1 (100 MHz, CD$_3$OD).

Figure S3. $^1$H NMR spectrum of flavonoid 2 (400 MHz, acetone-D$_3$).
Figure S4. $^{13}$C NMR spectrum of flavonoid 2 (100 MHz, acetone-D$_6$).
Flavonoids from *Annona dioica* Leaves and their Effects in Ehrlich Carcinoma Cells


**Figure S5.** $^{13}$C NMR - APT of flavonoid 2 (100 MHz, acetone-D$_6$).

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Figure S5. $^{13}$C NMR - APT of flavonoid 2 (100 MHz, acetone-D$_6$).
Figure S6. NMR $^1$H–$^1$H–COSY spectrum of flavonoid 2.

Figure S7. $^1$H NMR spectrum of flavonoid 3 (400 MHz, CD$_3$OD).
Figure S8. $^{13}$C NMR spectrum of flavonoid 3 (100 MHz, CD$_3$OD).

Figure S9. NMR $^1$H spectrum of flavonoid 4 (400 MHz, CD$_3$OD).
Figure S10. $^1$H NMR spectrum expansion of flavonoid 4 de (400 MHz, CD$_3$OD).

Figure S11. $^{13}$C NMR spectrum of flavonoid 4 (100 MHz, CD$_3$OD).
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**Figure S12.** NMR $^1$H - $^{13}$C - HMBC (100 MHz, CD$_3$OD) of flavonoid 4.