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Article

# Caesalpinioflavone, a New Cytotoxic Biflavonoid Isolated from *Caesalpinia pluviosa* var. *peltophoroides*

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The present study aimed to investigate the presence of compounds with antitumor activity in the plant *Caesalpinia pluviosa* var. *peltophoroides*. From bioactivity guided studies it was possible to isolate a new biflavonoid, named caesalpinioflavone, whose chemical structure was determined by spectroscopic ($^{1}$H and $^{13}$C nucler magnetic ressonace, homonuclear correlation spectroscopy and heteronuclear multiple-bond correlation spectroscopy) and spectrometric (high resolution electrospray ionization mass) methods. According to *in vitro* assays, caesalpinioflavone was effective in reducing the cell viability of tumor cell lines A549, MCF7, Hst578T and HTC. This effect was consequent of cell cycle arrest in G1/S transition (A549 and MCF7) and cytotoxic activity (Hs578T and HTC). Taken together, these data indicate that caesalpinioflavone has a promising antitumor activity.

**Keywords:** *Caesalpinia pluviosa* var. *peltophoroides*, biflavonoid, antiproliferative activity, cancer

## Introduction

*Caesalpinia* L. is a genus of Fabaceae plants belonging to the Caesalpinioideae sub-family, which is found in tropical and subtropical zones, and includes about 500 species, most of which have not been investigated in relation to their chemical compositions and biological properties. *Caesalpinia pulcherrima* presents emmenagogue and abortifacient effects,[1] *Caesalpinia bonduc* (L.) Roxb. displays anthelmintic, anticancer and antimalarial properties,[2] and *Caesalpinia sappan* has been used as an anti-inflammatory agent.[2-4] In addition, it has been described that sappanchalcone, extracted from *C. sappan,* suppresses oral cancer cell growth and induces apoptosis in oral squamous cell carcinoma.[5] Several classes of natural compounds have been isolated from the *Caesalpinia* genus, including flavonoids, diterpenes, steroids, organic acids and sugars.[6] Additionally, hydrolysable tannins were isolated from *Caesalpinia pluviosa* (synonym *Poincianella pluviosa*).[7]

Considering that *Caesalpinia* represents a valuable source for identifying new chemical compounds with therapeutic potential, in this meaning, this study aimed the identification of cytotoxic compounds from the stem bark methanol extract of *Caesalpinia pluviosa* var. *peltophoroides* using a bioguided fractionation procedure.

## Experimental

### General

All solvents and reagents used were analytically pure. Silica gel (Merck, 230-400 mesh) and sephadex LH-20 (Sigma-Aldrich) were used for column chromatographic (CC) separations while silica gel plates (0.25 mm) 60 $PF_{254}$ (Merck) were used for analytical thin layer

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chromatography (TLC). Plates were revealed using iodine vapor, vanillin-$H_2SO_4$ (3%), 1% $FeCl_3$ in ethanol or using ultraviolet radiation ($\lambda$ = 254 and 356 nm). Ultraviolet (UV) measurements were performed using an UV-spectrophotometer model UVvis2550 (Shimadzu). Melting point was determined using a PFM II Aaker apparatus. Infrared (IR) spectrum was obtained on Shimadzu IR-Prestig-21 and manipulated with the software IR-Solution. $^1$H (500 MHz) and $^{13}$C (125 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 500 MHz apparatus DRX spectrometer (Bruker BioSpin, Germany) using $CD_3OD$ as solvent. High resolution electrospray ionization mass (HRESIMS) was obtained using a Bruker MicroTOF II spectrometer (Bruker Daltonics, Germany) in negative mode. Optical rotation measurement was recorded at 25 °C on a Perkin Elmer model 343 polarimeter. High pressure liquid chromatography (HPLC) chromatograms were obtained on a UFLC Shimadzu 20 A, with a diode array detector (DAD) and a VP-ODS (Shimadzu) C-18 column (150 × 4.6 mm, 5 mm particle size).

### Plant material

Stem bark of *Caesalpinia pluviosa* var. *peltophoroides* was collected on the campus of the federal university of Alfenas-UNIFAL/MG (Latitude: 21° 25' 45'' south and longitude: 45° 56' 50'' west). The botanical identification was carried out at the federal university of Alfenas by professor Dr. Marcelo Polo. A voucher specimen is deposited at the herbarium of federal university of Alfenas under number of UALF-1634.

### Extraction and isolation

Fresh stem barks from *Caesalpinia pluviosa* var. *peltophoroides* were air-dried at 45 °C for 72 h, and the ground powder (3.0 kg) was extracted by maceration with ethanol (EtOH) at room temperature four times (4 × 7 L). The resulting solution was concentrated under reduced pressure to yield 100 g of crude EtOH extract, which was ressuspended in EtOH/$H_2O$ 3:1 and successively partitioned using n-hexane and ethyl acetate (EtOAc) to afford 62 g of n-hexane and 20 g of EtOAc phases. Cytotoxic assay on these both phases indicated that the bioactivity was concentrated on EtOAc phase. Part of this material (8 g) was subjected to CC on silica gel (135.5 g), using increasing amounts of EtOAc in n-hexane as eluent, affording twenty five fractions (250 mL each) which were pooled in five groups (A-E). Bioactive group C (2.4 g) was purified by CC over silica gel (80 g) using mixtures of n-hexane/EtOAc as eluent to give 105 fractions (50 mL each), which were pooled into 12 groups (C1-C12) being bioactivity concentered at group C6. This group (120 mg) was subjected to sephadex LH-20 (30 × 2 cm) column chromatography using methanol (MeOH) as eluent to afford 80 fractions (12 mL each) which were pooled in seven groups (C6/1-C6/7). Bioactive group C6/3 (40 mg) was composed by caesalpinioflavone as a dark yellow solid.

Caesalpinioflavone: $[\alpha]_D^{25}$ +135 (c 1.0, MeOH); mp 227 °C; $\lambda_{max}$/nm 264, 311; IR (KBr) $\nu_{max}$/cm$^{-1}$ 3415, 2952, 1653, 1615, 1512, 1445, 1273, 1242, 839; HRESIMS *m/z* 525.1185 $[M-H]^-$ (calcd. to $C_{30}H_{21}O_9$ 525.1186). $^1$H and $^{13}$C NMR spectra (see Table 1).

### Cytotoxic assays

Four tumor cell lines were used in this study: human lung carcinoma (A549), human breast carcinomas (MCF-7 and Hs578T) and rat hepatocellular carcinoma (HTC). The cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, CA, USA) supplemented with 10% fetal bovine serum (Vitrocell, Campinas, Brazil). Cells were grown in a 37 °C humidified incubator containing 5% $CO_2$ and seeded into 96 wells plates at 5 × $10^3$ (HTC and A549) or 1 × $10^4$ (MCF7 and Hs578T) cells *per* well. After attachment (24 h), the cells were treated for 48 h with caesalpinioflavone at different concentrations (5 - 160 μmol L$^{-1}$). The Promega non-radioactive cell proliferation assay was used to determinate the cell viability. This assay measures the amount of formazan produced from [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium, inner salt, MTS)] by the dehydrogenase enzymes of metabolically active cells. Thus, the quantity of formazan produced (as measured by the absorbance at 490 nm) is directly proportional to the number of living cells. Absorbance values of the treated cells were compared with the absorbance values of the untreated cells. The experiments were conducted in triplicate wells and repeated twice. The data are presented as the mean ± standard deviation (SD). Deoxyribonucleic acid (DNA) content was evaluated by flow cytometry (Attune, life technology) after 1 h of staining [2-phenylbenzimidazole-5-sulfonic acid (PBSA) containing propidium iodide (30 μg mL$^{-1}$) and RNAase (3 mg mL$^{-1}$)]. The data shown are representative of three independent experiments. The results presented in this study correspond to the average of three replicates (n = 3) ± standard deviation. The results were considered significantly different if they had values of $p < 0.05$, using an ANOVA followed by the Scott-Knott test in the SISVAR software.[8]

## Results and Discussion

Caesalpinioflavone (Figure 1) was isolated as a yellow crystalline solid with an optical activity $[\alpha]_D^{25}$ of +135° (*c* 0.1, MeOH) and a melting point of 227 °C. The molecular formula was established as $C_{30}H_{22}O_9$ based on the negative mode HRESIMS (Figure S1), which exhibited the deprotonated molecule $[M-H]^-$ at *m/z* 525.1185 (calcd. 525.1186). The IR spectrum (Figure S2) showed strong absorptions at 3415 $cm^{-1}$ (OH), 2952 $cm^{-1}$ (CH=CH), and 1653/1615 $cm^{-1}$. The UV spectrum displayed absorptions with $\lambda_{max}$ at 264 and 311 nm, characteristic of flavonoids. The $^{13}C$ NMR spectrum (Table 1) showed carbonyl resonances at $\delta_C$ 203.5 (C-4") and 182.6 (C-4), oxygenated carbons at $\delta_C$ 166.3 (C-7), 165.9 (C-5), 165.8 (C-2), 163.3 (C-9"), 163.0 (C-7"), 161.1 (C4'), 159.3 (C-9), 156.9 (C-4"'), the p-substituted aromatic carbons at $\delta_C$ 131.5 (C-3' and C-5'), 131.4 (C-3"' and C-5"'), 116.3 (C-2' and C-6'), 116.1 (C-2"' and C-6"'), as well as saturated carbons at $\delta_C$ 49.2 (C-2") and $\delta_C$ 36.0 (C-3"). The presence of characteristic carbon signals of flavones and chalcones associated to HRESIMS, the occurrence of a biflavonoid derivative was defined.[9] $^1H$ NMR spectrum of caesalpinioflavone (Table 1) showed signals ranging from $\delta_H$ 7.00 to 6.50 (8H) which referred to the hydrogen atoms of two p-substituted aromatic rings. These data, associated to the presence of doublets at $\delta_H$ 6.24 (d, *J* 2.5 Hz, H-8) and $\delta_H$ 6.19 (d, *J* 2.5 Hz, H-6), indicated a subunit of apigenin.[10] Other signals were observed at $\delta_H$ 7.26 (d, *J* 8.7 Hz, H-5"), 6.19 (d, *J* 2.5 Hz, H-8") and 6.15 (dd, *J* 8.7 and 2.5 Hz, H-6"), indicating the presence of a 1,2,4-trisubstituted aromatic ring. Signals at $\delta_H$ 4.69 (dd, *J* 7.5 and 6.5 Hz, H-2"), 3.47 (dd, *J* 14.0 and 6.5 Hz, H-3a") and 3.09 (dd, *J* 14.0 and 7.5 Hz, H-3b") indicated the occurrence of a 4,2',4'-trihydroxychalcone unit. The homonuclear correlation spectroscopy (COSY) couplings between the signals at $\delta_H$ 6.19 (d, *J* 2.5 Hz, H-6) with $\delta_H$ 6.24 (d, *J* 2.5 Hz, H-8), $\delta_H$ 6.15 (dd, *J* 8.7 and 2.5 Hz, H-6") with $\delta_H$ 7.26 (d, *J* 8.7 Hz, H-5") and $\delta_H$ 6.19 (d, *J* 2.5 Hz, H-8"), and $\delta_H$ 4.69 (H-2") with $\delta_H$ 3.47 (H-3"a) and $\delta_H$ 3.09 (H-3"b) confirmed the proposed substructures. Finally, the heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum showed the correlation of the signal at $\delta_H$ 4.69 (H-2") with those at $\delta_C$ 182.6 (C-4), 36.0 (C-3"), 165.8 (C-2) and 203.5 (C-4"), and the correlations of the signals at $\delta_H$ 3.47/3.09 (H-3") with those at $\delta_C$ 36.0 (C-3") and 203.5 (C-4"), which confirmed the connection between C-2" and C-3 in the biflavonoid structure (Figure 2 and Figures S3-S7). All the observed correlations are shown in Table 1. The configuration at C-2" was not assigned.

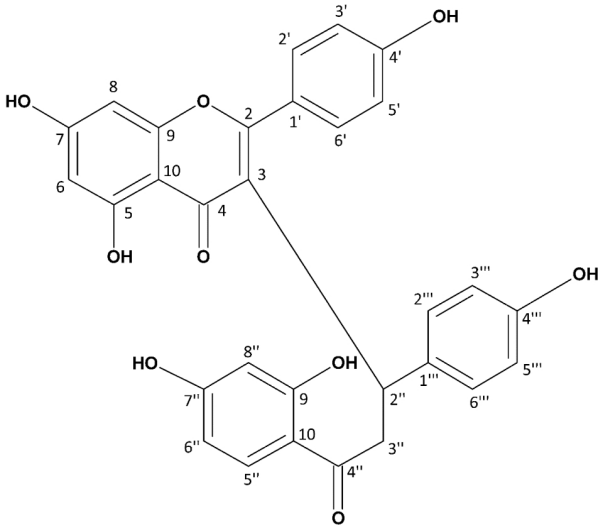


**Figure 1.** Chemical structure of caesalpinioflavone, a novel biflavonoid isolated from the stem bark of *Caesalpinia pluviosa var. peltophoroides*.

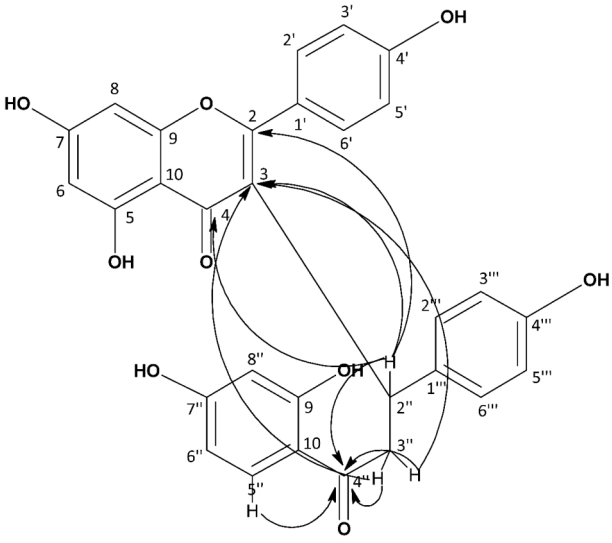


**Figure 2.** Relevant HMBC correlations (H → C) of caesalpinioflavone.

The cytotoxic potential of caesalpinioflavone was determined by MTS assay, and the results showed a drastic reduction of the cell viability in cells treated with caesalpinioflavone in concentrations upward of 40 μmol $L^{-1}$ (Figure 3). Among the cell lines studied, the hepatoma cell line (HTC) was the most responsive to treatment ($IC_{50}$ value = 48.00 ± 1.60 μmol $L^{-1}$). When HTC cells were treated under the same conditions with cisplatin, a powerful cytotoxic anticancer agent, the $IC_{50}$ value was found to be 38.53 ± 1.49 μmol $L^{-1}$. $IC_{50}$ values found for the other cell lines were 121 ± 9.7 μmol $L^{-1}$ (MCF7), 108 ± 7.6 μmol $L^{-1}$ (A549) and 97.65 ± 3.2 μmol $L^{-1}$ (Hs578T). These results indicate that caesalpinioflavone displays antiproliferative activity against HTC cells, which is in agreement with recent studies reporting that different flavonoids can

**Table 1.** NMR data for caesalpinioflavone in $CD_3OD$

| Position | $\delta_C$ | $\delta_H$ (m, *J* / Hz) | HMBC |
|---|---|---|---|
| **2** | 165.8 | - | H2” |
| **3** | 120.9 | - | H2”/H3” |
| **4** | 182.6 | - | H2” |
| **5** | 165.9 | - | H6 |
| **6** | 100.1 | 6.19 (d, 2.5) | H8 |
| **7** | 166.3 | - | H8 |
| **8** | 94.6 | 6.24 (d, 2.5) | H6 |
| **9** | 159.2 | - | H8 |
| **10** | 104.9 | - | H8 |
| **1’** | 124.6 | - | - |
| **2’; 6’** | 116.3 | 6.82 (d, 8.7) | - |
| **3’; 5’** | 131.5 | 7.02 (d, 8.7) | - |
| **4’** | 161.1 | - | H3’/H5’ |
| **2”** | 49.2 | 4.69 (dd, 7.5; 6.5) | H3” |
| **3a”** | 36.0 | 3.46 (dd, 14.0; 6.5) | H3b”/H2” |
| **3b”** | 36.0 | 3.09 (dd, 14.0; 7.5) | H3a”/H2” |
| **4”** | 203.5 | - | H2”/H3” |
| **5”** | 132.1 | 7.25 (d, 9.0) | H6” |
| **6”** | 108.5 | 6.15 (dd, 9.0; 2.5) | H5”/H8” |
| **7”** | 163.0 | - | H8” |
| **8”** | 103.9 | 6.19 (d, 2.5) | H6” |
| **9”** | 163.3 | - | H8” |
| **10”** | 113.7 | - | H5 |
| **1”’** | 132.1 | - | H2”/H2”’ |
| **2”’; 6”’** | 116.1 | 6.64 (d, 8.5) | - |
| **3”’; 5”’** | 131.4 | 6.95 (d, 8.5) | - |
| **4”’** | 156.8 | - | H3”’/H5”’ |

exert growth inhibition and/or cytotoxic activities on hepatocellular carcinoma cell lines.[9-12]

The images obtained by phase contrast microscopy (Figure 4) show the morphological features of the cell lines studied. The images clearly show lower cell densities in the treated cultures compared to the control cultures. To investigate whether the cytotoxic effects were a consequence of cell death induction or cell cycle arrest, we performed the cell cycle analysis by DNA quantification. Thus, the cultures were treated with caesalpinioflavone at 40 μmol $L^{-1}$ and 80 μmol $L^{-1}$, and the samples were analyzed by flow cytometry. The results showed that the effects of caesalpinioflavone on the cell cycle progression were concentration-dependent, and they varied depending on the cell type (Figure 5).

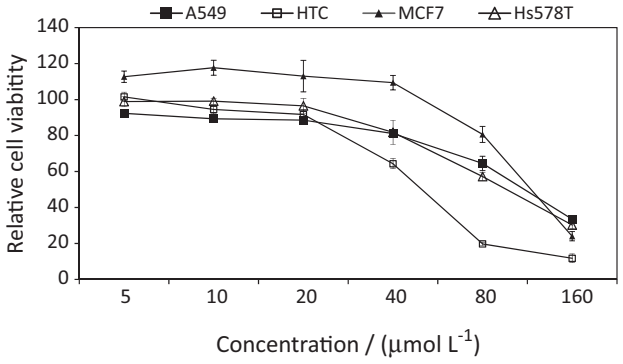


**Figure 3.** Relative cell viability of caesalpinioflavone against tumor cell lines A549, HCT, MCF7 and Hs578T, obtained by the MTS assay.

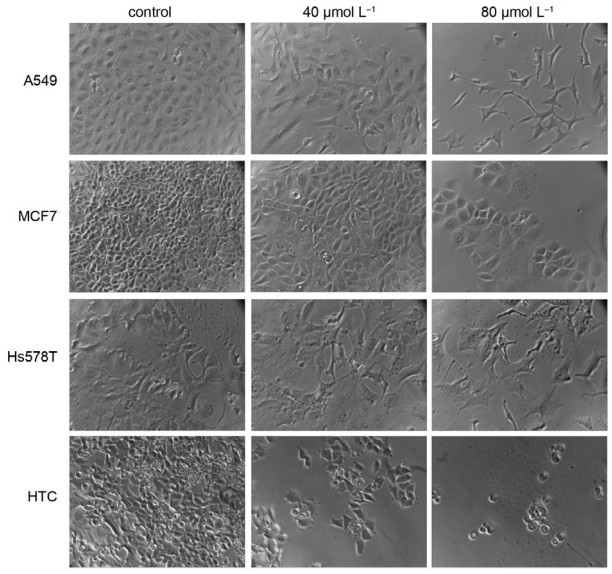


**Figure 4.** Images obtained by phase contrast microscopy showing the morphological features of the tumor cell lines A549, MCF7, Hs578T and HTC, after 48 h of treatment with caesalpinioflavone at 40 μmol $L^{-1}$ and 80 μmol $L^{-1}$.

In MCF7, we observed cell cycle arrest in the G1/S transition (at 40 μmol $L^{-1}$) and the G2/M arrest (at 80 μmol $L^{-1}$). In A549, both concentrations caused G1/S transition inhibition. It has been reported that phenolic compounds present pro-oxidant activity when used at high concentrations,[13-15] and may contribute for activating proteins associated to cell cycle control such as p53, p21 and GADD45.[16,17] In the present work, the cell lines with wild p53 displayed inhibition of the G1/S transition as a consequence of treatment, suggesting that the p53 pathway could be activated by caesalpinioflavone.

In Hs578T and HTC cells, an increased subG1 population was observed in addition to cell cycle arrest. When concentrations around the $IC_{50}$ were used (80 μmol $L^{-1}$ and 40 μmol $L^{-1}$ for Hs578T and HTC, respectively) cell cycle arrest in G2/M was observed. In these cell lines, at the same concentrations, the subG1 populations were 2.5-fold (Hs578T) and 5-fold (HTC) higher than in control cultures.

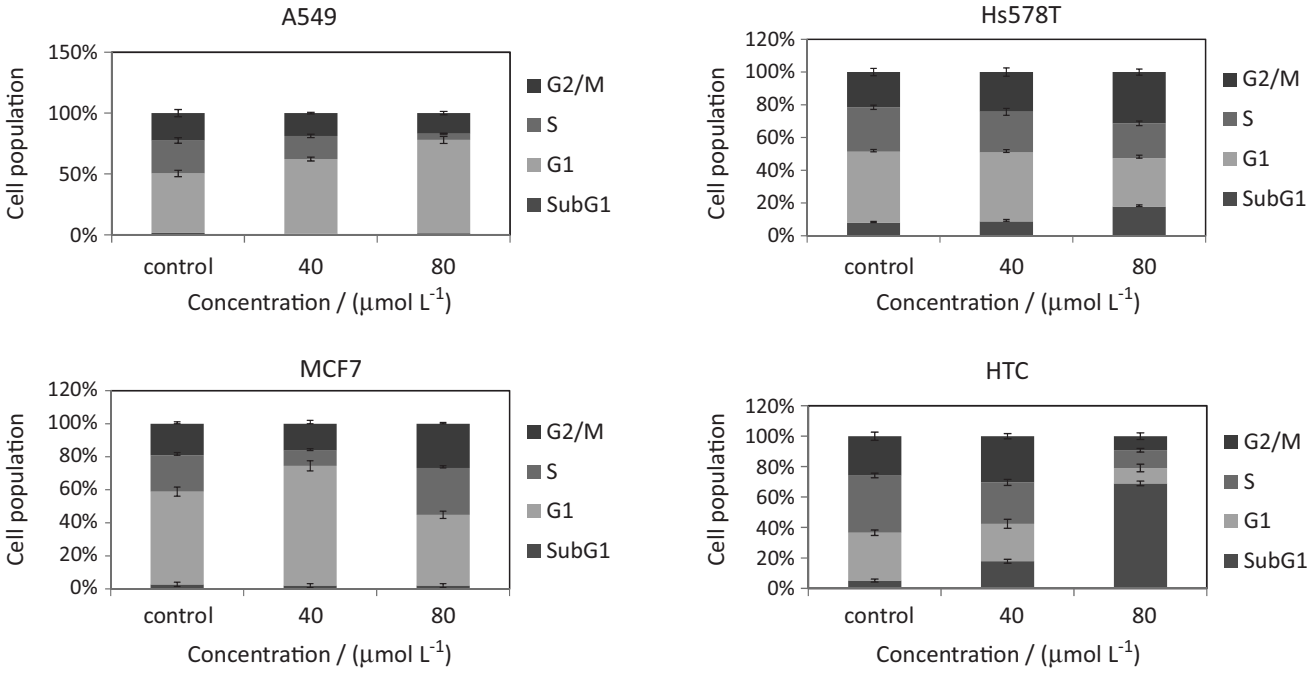


**Figure 5.** Cell cycle analysis of tumor cell lines A549, MCF7, Hs578T and HTC treated for 48 h with caesalpinioflavone at 40 µmol $L^{-1}$ and 80 µmol $L^{-1}$. According to ANOVA followed by the Scott-Knott test, $p < 0.05$.

You *et al.*[18] demonstrated that the increase in the subG1 population in human PC-3 (prostate cancer) cell cultures after treatment with ginkgetin (a biflavonoid) for 24 h was due to apoptosis induction. Phenolic compounds, such as caffeic acid phenethyl ester, induced apoptosis in glioma cells by the activation of the p53 pathway whereas cinnamic acid was effective in inducing apoptosis in melanoma cells by different pathways.[12,19,20] The results obtained in the present work showed that caesalpinioflavone inhibits cell proliferation of MCF7 and A549 cells, and has cytotoxic activity against Hs578T and HTC cell lines.

## Conclusions

A novel biflavonoid, named as caesalpinioflavone was isolated from the stem bark of *Caesalpinia pluviosa* var. *peltophoroides*. Caesalpinioflavone reduced cell viability of tumor cell lines A549, MCF7, Hst578T and HTC, as consequence of cell cycle arrest in G1/S transition (A549 and MCF7) and cytotoxic activity (Hs578T and HTC). Taken together, these data indicate that caesalpinioflavone has a promising antitumor activity.

## Supplementary Information

Supplementary information, including $^{1}$H NMR, $^{13}$C NMR, COSY, HSQC, and HMBC spectra, as well as mass and IR spectra, are available free of charge at http://jbcs.org.br as a PDF file.

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