# Antiproliferative Effects of Several Compounds Isolated from *Amburana cearensis* A. C. Smith

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*Amburana cearensis* a common tree found in Northeastern Brazil is widely used in folk medicine. The present work evaluated the cytotoxicity of kaempferol, isokaempferide, amburoside A and protocatechuic acid isolated from the ethanol extract of the trunk bark of *A. cearensis*. The compounds were tested for their cytotoxicity on the sea urchin egg development, hemolysis assay and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide assay using tumor cell lines. Isokaempferide and kaempferol, but not amburoside A and protocatechuic acid, inhibited the sea urchin egg development as well as tumor cell lines, but in this assay isokaempferide was more potent than kaempferol. Protocatechuic acid was the only compound able to induce hemolysis of mouse erythrocytes, suggesting that the cytotoxicity of kaempferol and isokaempeferide was not related to membrane damage.

*Key words: Amburana cearensis*, Kaempferol, Isokaempferide

## Introduction

*Amburana cearensis* (syn. *Torresea cearensis* Fr. All.) A. C.Smith (Fabaceae) is a tree commonly found in Northeastern Brazil, where it is popularly known as "cumaru". The trunk bark and seeds of this tree are extensively used in Brazilian folk medicine to treat respiratory diseases (Correa, 1984). Previous studies have demonstrated that the hydroalcoholic extract of *A. cearensis* and coumarin, its major component, possessed antinociceptive, antiinflammatory and bronchodilator activities (Leal *et al*., 1997; 2000). Besides coumarin, several other compounds such as protocatechuic acid, isokaempferide, kaempferol, amburoside A and B, have been isolated from the trunk bark of *A. cearensis* (Canuto and Silveira, 1998; Bravo *et al*., 1999).

Protecatechuic acid is a phenolic compound widely distributed in plant species. Its occurrence was reported for many species, like *Hibiscus sabdariffa* and *Sebastiania brasiliensis* (Tseng *et al.*, 2000; Penna *et al*., 2001). Previous studies have demonstrated that this compound showed strong antioxidant and antitumor activity (Tseng *et al*., 1996; 2000), induced apoptosis in human leukemia cells (Tseng *et al*., 2000), possessed *in vitro* protective effect on *tert*-butyl hydroperoxide-induced rat hepatotoxicity (Liu *et al*., 2002), and exhibited anticarcinogenic activity in several animal models (Hirose *et al*., 1995).

Kaempferol is one of the most studied flavonoids and is widely distributed among plants (Middleton Jr *et al*., 2000). This flavonoid showed a strong antioxidant activity in several models, and a weak cytotoxicity in human cell lines (Cos *et al*., 2001). Isokaempferide, the 3-methoxyl derivative of kaempferol, is also found in different plant species (Ahmed *et al*., 1994; Banskota *et al.*, 2000a). Pharmacological studies with isokaempferide have demonstrated that this compound was cytotoxic to the murine colon 26-L5 carcinoma cells (Banskota *et al*., 2000a), possessed antimicrobial acitivity (Wang *et al*., 1989) and showed hepatoprotective properties both *in vitro* and *in vivo* (Banskota *et al*., 2000b).

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The amburosides A and B, phenol glycoside compounds, were first isolated from *A. cearensis*, and the *in vitro* evaluation of antimalarial, antiprotozoal, antifungal and antibacterial activities demonstrated just moderate antimalarial and antiprotozoal activities (Bravo *et al.*, 1999).

Despite the important studies performed with these compounds, much of their biological effects have not so far been elucidated. The aim of the present study was to evaluate the cytotoxicity of these four compounds on five different tumor cell lines and on the sea urchin egg development, as well as their lytic properties on mouse erythrocytes. It is worthwhile to mention that the study of alterations in sea urchin egg development is a suitable model for detecting cytotoxic, teratogenic and antineoplastic activities of new compounds, and it has also been extensively used as a model for developmental toxicology evaluation (Jacobs and Wilson, 1986; Costa-Lotufo *et al.*, 2002).

## Material and Methods

### *Plant material*

The plant material was collected at Quixeramobim, Ceará, Brazil. Voucher specimens (# 837 and # 847) were deposited at the Prisco Bezerra Herbarium (EAC), Department of Biology, Federal University of Ceará.

200 g aliquots of powdered trunk bark of *Amburana cearensis* (3.4 kg) were individually extracted with MeOH, (0.5 l/200 g) in a glass sohxlet apparatus. After cooling to room temperature, a precipitate was formed, yielding sucrose (535.6 mg). All EtOH solutions were pooled together and rotoevaporated to yield a dark brown solid residue (250.0 g), designated ACCE, that included the characteristic coumarin odour.

ACCE was dissolved in $H_2O$ (330 ml) and then extracted with 15 portions (150 ml) of EtOAc. The aqueous phase yielded a brown solid mass (168.4 g) that had sucrose as the main constituent. The organic phase, dried over anhydrous $Na_2SO_4$ and evaporated, yielded a dark brown residue (74.0 g) that was then redissolved in MeOH (200 ml) and extracted with 9 portions of 100 ml hexane to yield a greenish residue (14.1 g). The MeOH solution was evaporated to half that volume and mixed with EtOAc (150 ml). Small portions of $H_2O$ was poured into the solution until two layers were formed (220 ml). The hydromethanol phase was extracted once with EtOAc. Upon usual procedures the hydromethanol solution was evaporated to yield a dark brown residue (ACCEAMI, 13.8 g) while the pooled EtOAc solutions also yielded a dark brown residue (ACCEAMA, 47.3 g).

ACCEAMA was coarsely chromatographed over silica gel (110 g). 100 ml fractions were collected, after elution with hexane/$CHCl_3$ 1:1, $CHCl_3$, $CHCl_3$/EtOAc 1:1 (v/v), EtOAc and finally MeOH, to yield F1 (13.5 g), F2 (4.2 g), F3 (13.4 g), F4 (8.5 g), F5 (5.8 g) and F6 (5.2 g).

Rechromatography over silica gel of F2 yielded afrormosin (Agrawal, 1989), (38.0 mg), protocatechuic acid (Pouchert and Behnke, 1993), **1** (213.0 mg), kaempferol (Agrawal, 1989), **2** (19.7 mg) and isokaempferide (Agrawal, 1989), **3** (114.1 mg). Rechromatography over silica gel yielded coumarin (1.0 g) and a mixture of glucosylated β-sitosterol and stigmasterol.

Exclusion chromatography by gel permeation on Sephadex LH-20® of F4, using MeOH as eluent, yielded amburoside A (Bravo *et al.,* 1999), **4** (1.02 g) and 4′-methoxyfisetin (Agrawal, 1989), (11.7 mg).

All structures (Fig. 1) have been determined by spectroscopy means, including one and two dimensional NMR such as COSY, HMQC, HMBC etc., physical properties and comparison with data from literature.

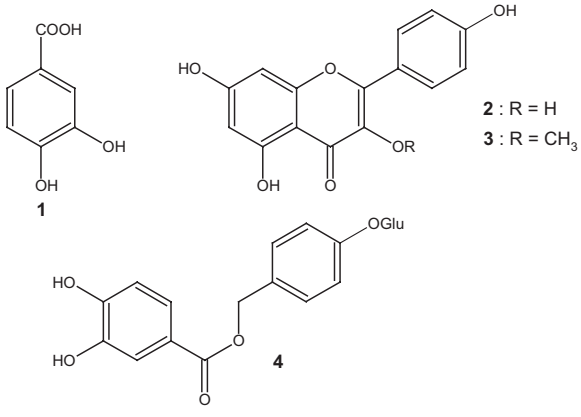


Fig. 1. Secondary metabolites isolated from the trunk bark of *Amburana cearensis.* **1**, protocatechuic acid; **2**, kaempferol; **3**, isokaempferide, and **4** amburoside A.

*Cytotoxicity on tumoral cell lines*

The cytotoxic activity was tested against five tumor cell lines: B-16 (murine skin cancer), HCT-8 (human colon cancer), MCF-7 (human breast cancer) CEM and HL-60 (leukemia cancer) cell lines (obtained from Children's Mercy Hospital, Kansas City, MO, USA). Cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C with 5% $CO_2$. For experiments, cells were plated in 96-well plates ($10^5$ cells/well for adherent cells or $0.5 \times 10^5$ cells/well for suspended cells in 100 μl of medium). After 24 h, the compounds (0.39 to 25 μg/ml) dissolved in DMSO (1%) was added to each well and incubated for 3 days (72 h). Control groups received the same amount of DMSO. The general viability of cultured cells was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) to a blue formazan product (Mosmann, 1983). After three hours of incubation, the formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). The drug effect was quantified as the percentage of control absorbance of the reduced dye at 550 nm.

*Antiproliferative assays on sea urchin eggs*

The test was performed according to the method described by Costa-Lotufo *et al.* (2002). Adult sea urchins (*Lytechinus variegatus*) were collected at Pecém beach on the northeastern coast of Brazil. The gamete elimination was induced by injecting 3.0 ml of 0.5 M KCl into the urchins coelomic cavity via the periostomial membrane. For fertilization, 1 ml of a sperm suspension (0.05 ml of collected sperm in 2.45 ml of filtered sea water) was added to every 50 ml of egg solution. Each well received 1 ml of fertilized egg suspension. The compounds were added immediately after fecundation (within 2 min) to get concentrations ranging from 10 to 100 μg/ml in a final volume of 2 ml. The plates were then shaken on a constant temperature water bath at 26 ± 2 °C. At appropriate intervals, aliquots of 200 μl were fixed in the same volume of 10% formaldehyde to obtain first and third cleavages, and blastulae. One hundred eggs or embryos were counted for each concentration of test substance to obtain the percentage of normal cells.

*Lytic activity on mouse erythrocytes*

The test was performed in 96-well plates following the method described by Costa-Lotufo *et al.* (2002). Each well received 100 μl of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well was the negative control that contained only the vehicle (distilled water or DMSO 10%). The compounds were tested at concentrations ranging from 1 to 250 μg/ml. The triton X-100 (0.1%) was used to obtain 100% hemolysis (positive control). Each well received 100 μl of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectroscopically at 540 nM.

*Statistical analysis*

Data are presented as mean ± S.E.M. The $IC_{50}$ or $EC_{50}$ values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). The differences between experimental groups were compared by Student's t test and the significance level was $p < 0.05$.

## Results and Discussion

The present study evaluated the cytotoxic activity of isokaempferide, kaempferol, amburoside A and protocatechuic acid, compounds isolated from the trunk bark of *Amburana cearensis*. The antimitotic activity was determined as the ability to inhibit sea urchin eggs development and five tumoral cells lines growth.

Isokaempferide and kaempferol induced a dose-dependent inhibition on egg development, while amburoside and protocatechuic acid did not modify normal egg cleavage, even at the higher tested concentration (100 μg/ml). The $IC_{50}$ values are presented in Table I.

The sea urchin egg development possess some peculiarities, making possible to suggest how the test substances acted. Isokaempeferide and

Table I. Antimitotic activity of compounds isolated from *Amburana cearensis* on sea urchin (*Lytechinus variegatus*) eggs development. Data are presented as $IC_{50}$ values and 95% confidence interval for first and third cleavages and blastulae obtained by non-linear regression.

| Substances | 1st cleavage μg/ml [μM] | 3rd cleavage μg/ml [μM] | Blastulae μg/ml [μM] | *n* |
|---|---|---|---|---|
| Isokaempferide | 22.6* (75.3) | 6.9* (23.0) | 16.6* (55.3) | 7 |
| | 17.2–29.6 | 5.3–8.8 | 14.6–19.0 | |
| Protecatechuic acid | > 100.0 (648.0) | > 100.0 (648.0) | > 100.0 (648.0) | 4 |
| Amburoside | > 100.0 (236.0) | > 100.0 (236.0) | > 100.0 (236.0) | 4 |
| Kaempferol | 36.2 (126.6) | 2.9 (10.1) | 1.4 (4.9) | 4 |
| | 23.1–56.7 | 1.8–4.6 | 0.9–2.2 | |

* $p < 0.05$ as compared by Student's t test with kaempferol.

kaempferol inhibited the first cleavage of the sea urchin egg development, and the inhibiton at this level is related to DNA and/or protein synthesis or microtubule assembly, once RNA synthesis is very slow or absent after fertilization. At this time, the rapid increase in the rate of protein synthesis is largely due to the recruitment of maternal mRNA into polysomes (Brandhorst, 1985). However, when a substance blocks microtubule assembly, clear spots corresponding to nucleus duplication can be observed in the cytoplasm. Since cells treated with isokaempferide and kaempferol presented a homogeneous cytoplasm, this process appears not to have been affected (Jacobs and Wilson, 1986). Hence, these compounds might affect DNA and/or protein synthesis.

The antimitotic effect of isokaempferide and kaempferol was also observed using tumoral cell lines. Both flavonoids inhibited the proliferation of the five cell lines analysed by the MTT assay. However, the efficacy of isokaempferide seemed to be higher than that of kaempferol as observed through the $IC_{50}$ values (Table II, $p < 0.05$). The other tested compounds, amburoside A and protocatechuic acid, did not present a strong cytotoxicity.

In order to verify whether the observed cytotoxicity is related to membrane disruption, the compounds were tested for their ability to induce lysis of mouse erythrocytes. The protocatechuic acid induced hemolysis with an $IC_{50}$ of 242.7 (234.3–251.4) μg/ml, while none of the other compounds were hemolytic even at the highest tested concentration (250 μg/ml). This result suggested that the mechanism of cytotoxicity is not a result of membrane damage. It is worthwhile to mention that previous work with protocatechuic acid showed that this compound retarded the hemolysis induced by 2-2′-azobis(2-amidinopropane)dihydrochloride, a hydrophilic free radical initiator, instead of causing lysis even in concentrations 3 times higher than the ones used in this present work (Kitagawa *et al.*, 1996).

The cytotoxicity of kaempferol and isokaempferide was already described in previous studies

Table II. Cytotoxic activity of compounds isolated from *Amburana cearensis* on tumoral cell lines. Data are presented as $IC_{50}$ values and 95% confidence interval obtained by non-linear regression for leukemia (HL-60 and CEM), breast (MCF-7), colon (HCT-8) and skin (B-16) cancer cells. Experiments were performed in triplicate.

| Substances | CEM μg/ml [μM] | HL-60 μg/ml [μM] | HCT-8 μg/ml [μM] | MCF-7 μg/ml [μM] | B-16 μg/ml [μM] |
|---|---|---|---|---|---|
| Isokaempferide | 2.6* (8.7) | 3.0* (10.0) | 5.4* (18.0) | 5.5* (18.3) | 3.6* (12.0) |
| | 2.1–3.2 | 2.4–3.7 | 4.1–7.2 | 3.5–8.6 | 3.1–4.1 |
| Protecatechuic acid | > 25.0 (162.0) | 20.7 (134.1) | > 25.0 (162.0) | > 25.0 (162.0) | > 25.0 (162.0) |
| | | 11.0–38.9 | | | |
| Amburoside | > 25.0 (59.0) | > 25.0 (59.0) | > 25.0 (59.0) | > 25.0 (59.0) | > 25.0 (59.0) |
| Kaempferol | 13.4 (46.8) | 22.7 (79.4) | 15.2 (53.1) | 21.2 (74.1) | 11.5 (40.2) |
| | 12.2–14.6 | 22.4–23.1 | 13.4–17.1 | 20.0–22.6 | 9.6–13.8 |

* $p < 0.05$ as compared by Student's t test with kaempferol.

(Banskota *et al*., 2000a; Cos *et al*., 2001). As mentioned before, isokaempferide demonstrated a strong cytotoxicity towards murine colon 26-L5 carcinoma cells (Banskota *et al*., 2000a), while kaempferol was only weakly cytotoxic to human fibroblasts (Banskota *et al*., 2000a). It is interesting that these two flavonoids differ only by the presence of a methoxyl group on carbon 3 of the C ring in isokaempferide instead of a hydroxyl present in many flavon-3-ols like kaempferol. In fact, many authors suggested that the presence of methoxyl substituents modify the cytotoxicity of flavonoids (Ducki *et al*., 1998; Middleton Jr *et al.*, 2000; Cunha *et al*., 2003). Cunha *et al*. (2003) compared the cytotoxicity of two chalcones derricin and lonchocarpin isolated from *Lonchocarpus sericieus* in the same assays used in the present work. The authors concluded that derricin is the most active and its stronger activity could be related to the presence of a methoxyl group. Our results corroborated this theory at least when the cytotoxicity is measured in tumoral cell lines. In the sea urchin assay, kaempferol and isokaempferide presented the same activity. Studies are in progress to elucidate the mechanisms of cytotoxicity exhibited by these two flavonoids.

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