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# *Chamaecostus subsessilis* and *Chamaecostus cuspidatus* (Nees & Mart) C.Specht and D.W.Stev as Potential Sources of Anticancer Agents

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## Abstract

Cytotoxicity activities of the crude ethanol extracts from *Chamaecostus subsessilis* and *Chamaecostus cuspidatus* and the fractions obtained by liquid-liquid partition were observed when these species were assayed against a panel of six human cancer cell lineages (HL60, Jurkat, MDA-MB231, MCF-7, HCT, THP-1) and normal (Vero) cells using the MTT colorimetric assay. The cytotoxic effects in the HL60, Jurkat and THP-1 lineages were mediated via an apoptotic mechanism when treated with crude ethanol extracts from these species. The presence of flavonoids, terpenoids and saponins in fractions from both species was observed in the preliminary phytochemical study. These results provide scientific support for the study of the Costaceae family and show that *C. cuspidatus* and *C. subsessilis* are potential sources of compounds with anticancer activities.

**Keywords:** Natural products; *Chamaecostus*; Cancer; Antitumoral agents

## Introduction

The National Cancer Institute estimated that approximately 1,658,370 new cases of cancer will be diagnosed in the United States in 2015 and 589,430 people will die from the disease. The projection is that breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, bladder cancer, melanoma of the skin, non-Hodgkin lymphoma, thyroid cancer, kidney and renal pelvic cancer, endometrial cancer, leukemia, and pancreatic cancer will be the most common cases.

Although research in cancer has advanced significantly in the last few decades and many of the mechanisms of action of cancer are already understood, in special the degenerative process caused for free radicals, cancer is a disease that still kills thousands of people worldwide. According to the statistics regarding cancer in the USA, approximately 1,658,370 new cases will occur in 2015, and 589,430 people will die from the disease [1].

In this context, the discovery of new drugs must be effective because these drugs may present mechanisms of action to prevent the recurrence of cancers.

Natural products (NPs) have been relevant to the development of drugs, particularly in the field of infectious diseases and cancer [2]. The structural complexity of molecules derived from NPs provides selective ligands for disease-related targets, disrupting the normal pathway of the molecular mechanism of the disease [3]. Thus, drugs derived from NPs present potential advantages over purely synthetic forms.

In the study of bioactive compounds, heterocyclic have fundamental importance as source of anticancer molecules, as example, Coumarin-Fused 1,4-Thiazepines, 2H-Chromenes derivatives, and 1-(5-aryl-1,3,4-oxadiazol-2-yl)-1-(1H-pyrrol-2-yl) methanamines [4-6], substances that showed anticancer properties. Because of the extreme level of structural complexity associated, the cost of producing these drugs by purely synthetic strategies would be very high if the pure enantiomeric forms are essential for activity, thus natural products can be a source of new molecules to fulfill this role.

Our research group in NPs has been researching bioactive substances originating from secondary metabolites from several sources, such as plants, fungi, bacteria and marine organisms, in search of active principles against cancer and other diseases. We report here the results of the cytotoxic activities of the plants of the *Chamaecostus* genus - *C. subsessilis* and *C. cuspidatus* - against six cancer cell lines.

Several species of plants of the *Costaceae* family have traditionally been consumed because of their biological activities. The rhizomes of several plant species of the Costaceae family have been traditionally used as antiseptic, diuretic and detoxifying agents, as well as for the treatment of infections and inflammatory diseases [7-10] and the *Costus* genus has been described in folk medicine in Brazil for the treatment of "loose urine", genitourinary disorders and inflammations, hepatitis and pain in the liver, bruises and swelling, hernias, anemia, fever and eye irritation [11-14].

Some of the *Costaceae species* have been used in folk medicine for the treatment of infection diseases in Brazil. The mucilaginous juice of *Costus scaber* Ruiz and Pav (Syn.: *C. anachiri* Jacq.) is used as a febrifuge and for nephritic pain and gonorrhea [15]. Tea prepared from the whole *Costus spiralis* Roscoe plant, popularly known as 'cana-do-brejo' or 'cana-de-macaco', is used as a diuretic in diseases of the urinary tract and as a anti-syphilitic agent [16-19].

Although the pharmacological activities of *Costus* have been described in the literature, few studies of the properties of the *Chamaecostus* genus exist. Our studies provide support for isolation of the active principles of these species and the mechanism of action of these substances against cancer cells.

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## Materials and Methods

### Collection of plant materials

The Belo Horizonte Zoo-Botanical Foundation (BHZB) at Belo Horizonte, Minas Gerais, Brazil, provided the fresh and healthy rhizomes of the *Chamaecostus cuspidatus* and *Chamaecostus subsessilis* plants. A voucher specimen of each species was deposited at the Herbarium: *Chamaecostus cuspidatus* (Nees & Mart.) C. Specht & D.W.S. (BHZB 5582), *Chamaecostus subsessilis* (Nees & Mart.) C. Specht & D.W.S. (BHZB 8018).

### Preparation of crude ethanol extract from rhizomes

Pure water was used to wash the rhizomes, and they were allowed to dry at room temperature. The fresh plant material was chopped into small pieces of about 5 × 5 $cm^2$ and extracted three times with absolute ethanol at room temperature with three-day periods of rest between extractions (748 g *C. subsesilis* rhizomes and 233 g *C. cuspidatus* rhizomes). The suspensions were filtered through cellulose filter paper, and the ethanol was evaporated under reduced pressure at 40°C using a rotary evaporator, yielding 7.3 g of crude ethanol extract from *C. subsesilis* (1%) and 2.3 g of crude ethanol extract from *C. cuspidatus* (1%) respectively.

### Preparation of fractions by liquid-liquid partition

Crude ethanol extract was dissolved in methanol: water (80:20 v/v) and sequentially partitioned with solvents of increasing polarity: hexane (HEX), dichloromethane (DCM), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and water (AQ). The fractions were evaporated to dryness in a vacuum centrifuge evaporator. The weights and yields based on crude ethanol extracts were: (HEX) - 0.42 g, (5.7%) for *C. subsessilis* and 0.42 g, (18.3%) for *C. cuspidatus*; (DCM) - 1.14 g, (15.6%) for *C. subsessilis* and 0.17 g, (7.4%) for *C. cuspidatus*; ($CHCl_3$) - 2.00 g, (27.4%) for *C. subsessilis* and 0.04 g (1.7%) for *C. cuspidatus*; (EtOAc) - 0.40 g, (5.5%) for *C. subsessilis* and 0.12 g, (5.6%) for *C. cuspidatus*; (AQ) - 3.0 g (41.1%) for *C. subsessilis* and 1.3 g, (56.5%) for *C. cuspidatus*.

## *In-vitro* Cytotoxicity Assays

### Human cancer cell lines

Six cancer cell lines were used; Jurkat (human immortalized line of T lymphocyte), HL60 (human promyelocytic leukemia), THP-1 (acute monocytic), MCF-7 and MDA-MB231 (breast cancer) and HCT-116 (colorectal carcinoma). The Vero lineage (African green monkey kidney cells) was used as a model for normal cells. All lineages were maintained in the logarithmic phase of growth in RPMI 1640 or D-MEM medium supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin enriched with 2 mM of L-glutamine. Leukemia cells were cultured in RPMI and 10% foetal bovine serum. The adherent cells were maintained in D-MEM enriched with 5% foetal bovine serum. All cultures were maintained at 37°C in a humidified incubator with 5% $CO_2$ and 95% air. The media were changed twice weekly and the cells were examined regularly. All cell lines were used for 20 passages.

### Evaluation of cytotoxic effect against human cancer cell lines

Cell lineages were inoculated using 1 × $10^4$ cells (MCF-7, HCT, MDA-MB-231 and Vero), 5 × $10^4$ cells (HL60) and 1 × $10^5$ cells (Jurkat and THP-1) per well. The plates were pre-incubated for 24 h at 37°C to allow adaptation of cells prior to the addition of the test compounds. All extracts (crude ethanol extract and partitioned fractions) were dissolved in DMSO prior to dilution. The half-maximum inhibitory concentration ($IC_{50}$) was determined over a range of concentrations (eight nonserial dilutions from 100 to 1.5 µg/mL). All cell cultures were incubated in a 5% $CO_2$/95% air humidified atmosphere at 37°C for 48 h. The negative control included treatment with 0.5% DMSO. Cell viability was estimated by measuring the rate of mitochondrial reduction of tetrazolium dye (MTT). All the samples were tested in triplicate in three independent experiments [20]. The selectivity index (SI) was defined as the ratio of the $IC_{50}$ observed in Vero cells to the $IC_{50}$ observed in the experiment with cancer cells for the same substance tested. Etoposide was evaluated under the same experimental conditions as the positive control.

### DNA fragmentation assay

Propidium iodide (PI) staining was employed to determine the cell cycle status and for quantification of DNA fragmentation (hypo diploid DNA-content) to determine the mechanism of cellular death by apoptosis [21]. Cells were treated with the samples (crude ethanol extract and partitioned fractions) at a concentration of 20 µg/mL in a humidified 5% $CO_2$/95% air atmosphere at 37°C for 24 h. After incubation, the cells were centrifuged and ressuspended in hypotonic fluorochrome solution (HFS; 50 µg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100). The samples in HFS were incubated at 4°C during 4 h and immediately analyzed by flow cytometry. The PI fluorescence of 20,000 individual nuclei was measured using a FACScalibur flow cytometer. Data were analyzed using FlowJo® software (TreeStar Inc.).

The $IC_{50}$ values for cytotoxicity activity were determined using Prism 5.0® (GraphPad Software Inc.). Data were presented as medians and 95% confidence intervals. Statistical differences between the treatments and the control were evaluated by ANOVA. To express the DNA content, each data point represented the mean ± SD from at least two independent experiments performed in duplicate. Statistical differences between the treatments and the control were evaluated by ANOVA, followed by the Bonferroni test (P>0.05).

### Phytochemical screening

All the fractions from *C. cuspidatus* and *C. subsessilis* were analyzed for the presence of the principal natural products classes by Thin Layer Chromatography (TLC) on Silica gel G-60/$F_{254}$ plates (0.25 mm, Merck, Darmstadt, Germany) using the appropriate solvents for elution and visualization by UV absorbance or by spraying with developing reagents. The presence of alkaloids, antraquinones, cardiotonic heterosides, cumarins, flavonoids, terpenoids and saponines was determined using the specific reagents [22].

## Results and Discussion

### Cytotoxic activity of the crude ethanol extracts and partitioned fractions

Analyzing the behavior of the Vero cell (used as the control for toxicity) it was noticed that this cell was a little more sensitive against crude ethanol extract from *C. subsessilis* than crude ethanol extract from *C. cuspidatus* ($IC_{50}$ 40.8 ± 5.6 vs. 52.5 ± 6.4). This cell was more sensitive against fractions from *C. subsesseilis* than *C. cuspidatus*, $IC_{50}$ ranging from 18.8 ± 1.4 to 48.6 ± 9.7 µg/mL and 52.5 ± 6.4 to 96.4 ± 14.9 µg/mL, respectively. Aqueous fractions (AQ) did not show activity against this cell.

Crude ethanol extract from *C. subsessilis* was active against all cancer cell lines tested, $IC_{50}$ ranging from 8.2 ± 0.2 (Jurkat) to 53.8 ± 0.7 (MCF-7) and the lines Jurkat, HL-60 and THP-1 were the most sensitive. The crude ethanol extract from *C. cuspidatus* was not active

against MCF-7 nor HCT-116 ($IC_{50}$>100 µg/mL). This observation segregates the species on the basis of potency against this lineage.

In general, all fractions were active against the lines tested, exception AQ fraction from both species, that was inactive. The MDA-MB231 cell line was sensitive against no fraction from *C. cuspidatus*.

The main parameter in the biological evaluation of a natural product is the toxicity that the substance presents for normal cells. Active substances will no longer be good targets for the development of new drugs when they present toxicity levels for normal cells of the same order of magnitude as that presented for the therapeutic targets. In other words, good candidates for the development of new drugs will be those that present high selectivity indices (SI) and low values of $IC_{50}$.

## Preliminary phytochemical analysis

It is important to mention that the TLC technique is only predictive on the existence or not of a compound/chemical class, the results indicate possible presence. All the fractions from *C. cuspidatus* and *C. subsessilis* were screened for alkaloids, antraquinones, cardiotonic heterosides, cumarins, flavonoids, terpenoids and saponins. Flavonoids were present in chloroform- and ethyl acetate-soluble fractions from *C. subsessilis* and ethyl acetate-soluble fraction from *C. cuspidatus*. Terpenoids were found in hexane- and dichloromethane-soluble fractions, whereas saponins were detected in water-soluble fractions from both plant species (Tables 1 and 2). According to the TLC profile, the activity was greatest in fractions of medium and low polarity, whereas no activity was detected in aqueous fractions. No alkaloids, antraquinones, cardiotonic heterosides, or cumarins were detected in any of all the fractions tested in preliminary phytochemical studies.

The hexane and dichloromethane fractions from both species could be used as good sources of compounds in the bio guided studies for the isolation of lead compounds. Terpenes and flavonoids may be the active constituents responsible for the antiproliferative activity in nonpolar fractions. However, there are few reports regarding the isolation of flavonoids and terpenes from Costaceae species. The triterpenes 31-norcycloartanone, cycloartanol, cycloartenol and cycloaudenol were isolated from *Costus speciosus* roots. Flavonol diglycosides and flavonoids were isolated from the leaves of *Costus spicatus*. In this same study, quercetin 3-*O*-neohesperidoside exhibited moderate inhibitory activity against nitric oxide production ($IC_{50}$ value of 55 µM) [23].

## Apoptosis evaluation of cytotoxic extracts from *C. cuspidatus* and *C. subsessilis* rhizomes by flow cytometry

Crude ethanol extracts from *C. cuspidatus* and *C. subsessilis* were evaluated with respect to the pro-apoptotic activity against HL60, Jurkat and THP-1, the cell lines most susceptible to these samples. The extracts induced DNA fragmentation in all the lines at the concentration of 20 µg/mL, with higher apoptotic activities than etoposide in these cell lines (Figure 1).

Cancer lines (HL-60, Jurkat and THP-1) were observed to be more sensitive to the treatment *C. cuspidatus* and *C. subsessilis* crude ethanol extracts. They had $IC_{50}$ values in the same 20 µg/mL range, an SI greater than three and could be considered good candidates for the isolation of natural products [24-26]. In addition, these extracts presented pro-apoptotic activities, characterized by chromatin condensation and nuclear shrinkage, in the assays using PI, which binds to DNA after the plasma membrane, resulting in fluorescence that can be detected by flow cytometry.

Apoptosis is a programmed cell death that is disrupted in the tumorigenesis. At the concentration of 20 µg/mL, pro-apoptotic effects of the crude ethanol extracts from *C. cuspidatus* and *C. subsessilis* could be observed in all the cell lines. These results support previous cytotoxic effects in the screening assays, suggesting that their anticancer properties can be mediated by apoptosis induction. Nadumane et al. (2011) demonstrated that the ethanol extract of *Costus pictus* is cytotoxic for HT-1080 fibrosarcoma cells at 120 µg/mL using the MTT assay, but it was nontoxic to the normal human lymphocytes

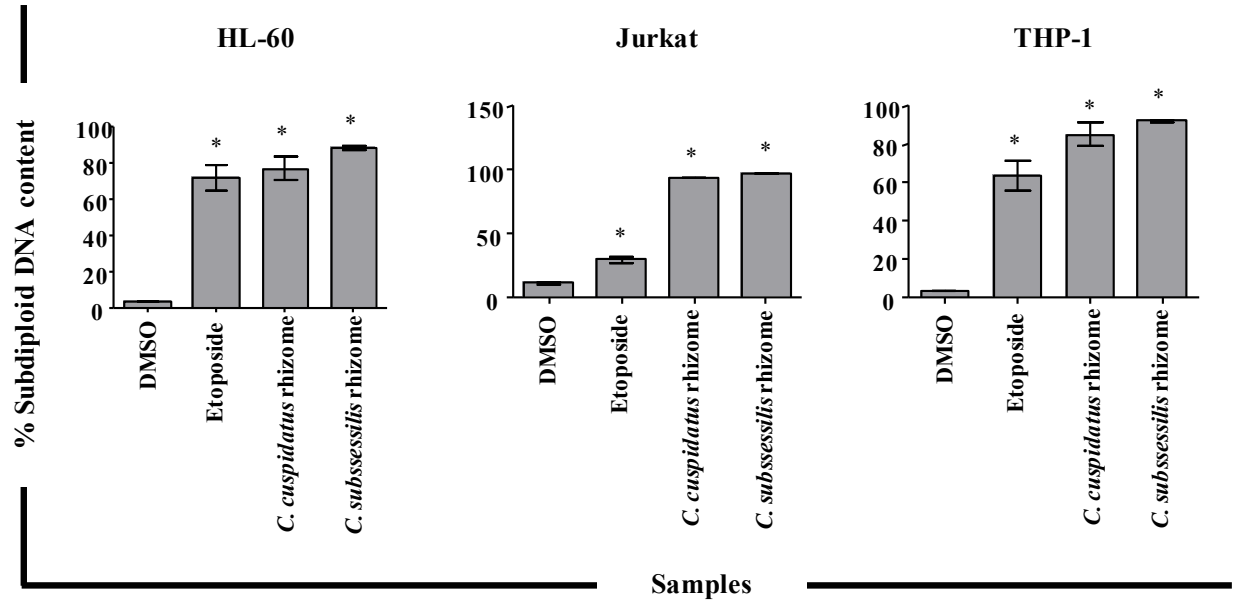


**Figure 1:** Effect of the extracts on the induction of fragmentation in leukemic cells. The cells were incubated with the compounds at the concentration of 20 µg/mL at 37°C in a 5% $CO_2$ atmosphere for 24 h. DNA content was assessed by flow cytometric analysis of cells labeled with propidium iodide. Each data point represents the mean ± SD from two independent experiments (*P<0.05 Student T-test). Etoposide and ethanol extracts of *C. cuspidatus* and *C. subsessilis* rhizome were tested at a concentration of 20 µg/mL.

| Plant extract | Test strain $IC_{50}$ (ug/mL)/ SI | | | | | | |
|---|---|---|---|---|---|---|---|
| | HL-60 | Jurkat | MDA-MB231 | MCF-7 | HCT-116 | THP-1 | VERO |
| ***C. cuspidatus*** | | | | | | | |
| Crude ethanol extract | 15.4 ± 1.3/3.4 | 16.3 ± 0.4/3.2 | 98.9 ± 5.8/0.5 | >100.0/nd | >100/n.d. | 11.3 ± 0.7/4.6 | 52.5 ± 6.4 |
| HEX | **4.7 ± 2.6/>21.3** | **2.9 ± 1.1/ >34.4** | >100.0/n.d. | 45.7 ± 18.1/>2.2 | **8.3 ± 1.7/>12.0** | 36.3 ± 9.8/>2.7 | >100.0 |
| DCM | **7.3 ± 2.6/13.2** | **2.2 ± 0.2/43.8** | >100.0/n.d. | 37.2 ± 15.2/2.6 | **7.3 ± 4.3/13.2** | 18.5 ± 11.4/5.2 | 96.4 ± 14.9 |
| $CHCl_3$ | **6.1 ± 1.9/>16.4** | 22.7 ± 15.1/>4.4 | >100.0/n.d. | 22.5 ± 7.1/>4.4 | 11.9 ± 4.2/>8.8 | 11.7 ± 4.2/>8.5 | >100 |
| EtOAc | 25.4 ± 9.4/>3.9 | 35.3 ± 11.5/>2.8 | >100.0/ n.d. | >100.0/ n.d. | 27.9 ± 4.7/>3.6 | >100.0/ n.d. | >100.0 |
| AQ | >100.0/n.d. | >100.0/n.d. | >100.0/n.d. | >100.0/n.d. | >100.0/n.d. | >100.0/n.d. | >100.0 |
| ***C. subsessilis*** | | | | | | | |
| Crude ethanol extract | **8.3 ± 1.5/4.9** | **8.2 ± 0.2/4.9** | 32.6 ± 0.3/1.3 | 53.8 ± 6.7/0.7 | 18.4 ± 1.4/2.2 | 11.9 ± 0.2/3.4 | 40.8 ± 5.6 |
| HEX | **9.3 ± 0.5/5.2** | 10.6 ± 1.3/4.6 | **5.5 ± 2.8/8.8** | 43.6 ± 6.2/1.1 | 17.3 ± 1.2/2.8 | 16.6 ± 1,3/3.0 | 48.6 ± 9.7 |
| DCM | **4.7 ± 0.2/6.8** | **6.0 ± 0.1/5.3** | **9.2 ± 1.9/3.4** | 23.1 ± 0.9/1.4 | **9.6 ± 1.0/3.3** | **8.0 ± 0.1/4.0** | 31.9 ± 5.9 |
| $CHCl_3$ | **2.6 ± 0.1/7.2** | **4.2 ± 0.1/4.5** | **6.5 ± 0.8/2.9** | 14.5 ± 0.2/1.3 | >100.0/ n.d. | **4.8 ± 0.1/3.9** | 18.8 ± 1.4 |
| EtOAc | **4.7 ± 0.8/6.0** | 19.1 ± 2.5/1.5 | 13.8 ± 2.7/2.1 | >100.0/ n.d. | >100.0/ n.d. | **9.1 ± 2.5/3.1** | 28.4 ± 0.9 |
| AQ | >100.0/ n.d. | >100.0/ n.d. | >100.0/ n.d. | >100.0/ n.d. | >100.0/ n.d. | >100.0/ n.d. | >100.0 |
| Control (Etoposide) | 1.8 ± 0.7/>55.6. | >100.0/ n.d. | >100.0/n.d. | >100.0/ n.d. | >100.0/ n.d. | 2.1 ± 1.2/n.d | >100.0 |

**Table 1:** Results of the cytotoxicity experiments of the crude ethanol extracts and partitioned fractions from *C. cuspidatus* and *C. subsessilis* against cancer cell lines. Highlighted results present the $IC_{50}$ lower than 10 µg/mL.

| Chemical class | Phytochemical result | | | | | | | | | | Reagent | Solvent mixtures |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *C. cuspidatus* | | | | | *C. subsessilis* | | | | | | |
| | HEX | DCM | $CHCl_3$ | EtOAc | AQ | HEX | DCM | $CHCl_3$ | EtOAc | AQ | | |
| Alkaloids | - | - | - | - | - | - | - | - | - | - | Dragendorff | $CHCl_3$MeOH:Diethylamine (50:40:10 v/v) |
| Antraquinones | - | - | - | - | - | - | - | - | - | - | Potassium hydroxide | DCM (100%) |
| Cardiotonic heterosides | - | - | - | - | - | - | - | - | - | - | Kedde | EtOAc:MeOH:$H_2O$ (80:20:5 v/v) |
| Cumarins | - | - | - | - | - | - | - | - | - | - | Potassium hydroxide | DCM (100%) |
| Flavonoids | - | - | - | + | - | - | - | + | + | - | NP/PEG | EtOAc:MeOH:$H_2O$:AcOH (80:20:5:0.1v/v) |
| Terpenoids | + | + | - | - | - | + | + | - | - | - | Liebermann-Burchard | DCM: MeOH (95:5 v/v) |
| Saponins | - | - | - | + | + | - | - | - | - | + | Vanillin-sulphuric acid | EtOAc:MeOH:$H_2O$ (80:20:5 v/v) |

**Table 2:** Phytochemical investigation of the crude ethanol extracts and fractions from *C. cuspidatus* and *C. subsessilis*.

by the trypan blue dye exclusion method [8]. This extract also induced apoptosis in the fibrosarcoma cells. In another study, the methanol extract of the from *Costus pictus* furnished chloroform- and methanol-soluble and methanol-insoluble fractions that were toxic to HT29 and A549 cells at concentrations ranging from 125 to 200 µg/mL [7].

## Conclusion

To the best of our knowledge, this is the first time that anticancer studies for these plant species have been reported. The antiproliferativie effects of these plants can be linked to the induction of apoptosis. The antiproliferative effects of some fractions from *C. cuspidatus* and *C. subsessilis* were superior to those of the crude extract. Terpenes and flavonoids may be the active constituents of these species. The isolation and identification of the active metabolites in these extracts are currently under investigation in our laboratory.

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