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IN VITRO CYTOTOXIC AND GENOTOXIC ANALYSIS OF *Garcinia humilis* CRUDE EXTRACT

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Abstract

Garcinia humilis, known commonly as achachairú or bacupari, has great medicinal value. Their fruits have pharmacological, antibacterial, antioxidant, and anticancer properties. Therefore, the objective of the present study was to evaluate the cytotoxicity and genotoxicity of *G. humilis* crude extract in breast tumor cells. Cytotoxicity was determined using the Resazurin reduction assay and genotoxicity by the single cell gel electrophoresis assay (Comet assay) on human MCF-7 cells. Crude extract of *G. humilis* was cytotoxic only when used at high concentrations (IC_{50} = 5.084 mg mL^{-1}). The Comet assay showed that the crude extract did not induce genotoxicity at 1 and 5 mg mL^{-1} but did show signs of DNA fragmentation and DNA fragmentation at 10 mg mL^{-1}. The cytotoxic activity against breast adenocarcinoma cells at high concentrations suggests that this medicinal plant could be used with caution and must be further studied to understand better its therapeutic and toxicological potential in the human body.

Keywords: Achachairú. Anticancer activity. Antioxidant activity. Comet assay.

1. Introduction

Cancer is a chronic multifactorial disease characterized by disordered cell growth, caused by DNA mutations, and presents multiple treatmens depending on the cancer type (Xu et al. 2018; Miller et al. 2019). Current cancer treatments are unavailable to roughly 80% of the people in the world because of their physical distance from treatment centers or lack of economic resources (Munhoz et al. 2016). Many cancer patients seek out Complementary and Alternative therapies (CAT). According to Turan et al. (2020), the reasons for using CAT were appropriateness to the patients' lifestyles, effectiveness against the disease and easier access compared to medical treatment.

Medicinal plants are traditionally used to prevent and treat various diseases in several countries (Santos et al. 2018a). Traditional medicine is used for multiple reasons, such as the ease and availability of preparation methods and pharmacological properties empirically disseminated by the population (Santos et al. 2018b).

Interest in medicinal plants has generated growing international and local initiatives to track and identify compounds with medicinal properties (Volenzo et al. 2020). Medicinal plants or active constituents are present in more than 50% of new drugs developed and approved for sale (Wang et al. 2020). In addition

to health benefits, this market's sustainable expansion can contribute to preserving ecosystem biodiversity while improving incomes in local communities (Teixeira et al. 2019).

The richness and bioactive potential of flora in Brazil are recognized worldwide. *Garcinia humilis* (Clusiaceae family), commonly known as achachairú, is one of these Brazilian plants with great medicinal value (Oliveira et al. 2019). The beneficial antioxidant potential of this plant should translate into significant exports over the next few years (Tome et al. 2019).

Garcinia humilis has been used to treat rheumatism, pain, inflammation, and gastric disorders (Barros et al. 2019), and possesses various structural classes, such as xanthones and bioflavonoids, benzophenones, acylphloroglucinols, depsidones, tocotrienols and biphenyls (John et al. 2018).

Since these treatments are derived from natural sources, it is sometimes assumed that medicinal plants are safe and can be consumed without restriction or criteria. However, high doses or prolonged use of some plants can be harmful because of the mutagenic potential of some compounds (Almeida et al. 2019).

In-depth studies regarding safe doses and duration of use for extracts tinctures and medicinal products are currently lacking. Therefore, in addition to discovering bioactive compounds, it is also important to minimize adverse effects and guarantee desired results by investigating the mechanisms that occur between plant compounds and the organisms that consume them (Ancia and Romão 2016).

Cytotoxic substances need to be analyzed to help control phytopharmaceutical quality and determine how they affect cells (Oliveira and Almeida 2016). Cytotoxicity tests determine cell damage by directly applying active materials to mammalian cell cultures (Rogero et al. 2003).

Genotoxic evaluations of plant extracts are used to detect damage to the genetic material of individual cells via the Comet assay (Alvarez-Moya et al. 2011). This technique is widely used in studies on biological responses to dosages and exposure times to substances that can cause DNA damage, oxidative stress, and mutagenic effects (Neri et al. 2015; Mangalampalli et al. 2018; Liman et al. 2020).

According to Demenciano et al. (2020), the leaves of *Garcinia gardneriana*, which belongs to the same genus of the species here studied, showed cytotoxic activity in the hexane fraction against a breast carcinoma line. Thus, the objective of the current study was to evaluate cytotoxicity and genotoxicity in MCF7 human breast adenocarcinoma cells caused by crude *Garcinia humilis* extract.

2. Material and Methods

Botanical material and extract preparation

The extract was prepared from *G. humilis* leaves. The plant material was collected (-17.721944º, -48.159722º), identified (Lorenzi and Matos 2008), confirmed (Souza and Lorenzi 2012), and then washed and dried at 50º until reaching a constant weight. Afterward, the leaves were ground in a Willey mill with 0.75 mm circular sieves to increase surface area and optimize extraction.

Thirty grams of dried *G. humilis* extract was boiled in deionized water (300 mL) for 15 minutes in an Erlenmeyer (500 mL) and qualitative filter paper (40x40cm 80g) to reduce pressure. Finally, the extract volume was adjusted to 300 mL with deionized water and stored at 4° C for further dilution.

Evaluating cytotoxicity by the Resazurin reduction assay

The MCF7 cells (1 x 10^5 cells/well) were maintained *in vitro* in a chamber at 37ºC and 5% CO_2 to determine cell viability via the resazurin assay. The cells were then incubated in 200 µL of RPMI 1640 culture medium (Roswell Park Memorial Institute) supplemented with fetal bovine serum (10%) and antibiotics (1%) in a sterile 96-well microplate. The treatments were serially diluted (1:2) and added to a microplate that had been incubated at 37ºC with 5% CO_2 for 24 hours. Afterward, 15 µL of the resazurin developing agent was added to each well of the microplate, and a microplate reader was used to determine the absorbance at 570 and 600 nm after 6 hours of incubation (Higuchi et al. 2007). Cell viability was calculated by comparing the wells' optical densities (OD) with treated monolayers to the average OD of the control wells (cells incubated without treatments), according to the formula recommended by the test protocols.

GraphPad Prism software was used to evaluate the ratio of the inhibition percentage to the logarithm of the tested concentrations at the 95% confidence interval ($p < 0.05$).

Comet Assay: Cell culture

MCF7 cells were cultured in a complete RPMI-1640 medium and then plated ($5x10^4$ cells/well) in 12-well plates. After 24 hours, the cell cultures were treated with various extract concentrations (T1 – 10 mg mL^{-1}; T2 - 5 mg mL^{-1}; T3 – 1 mg mL^{-1}), a negative control (culture media), and a positive control of methyl methanesulfonate (MMS 0.36 mM). After 24 hours of exposure, the plate supernatant was discarded, and each well was washed with 500 µl of phosphate-saline buffer (PBS). Afterward, each well was treated with 500 µl of trypsin for five minutes. The trypsin was then deactivated using a complete medium and centrifuged for another 5 minutes at 1800 rpm. After centrifuging, the supernatant was discarded, and the cell pellet was resuspended in 500 µl of inactivated and filtered bovine fetal serum (BFS).

Slides were prepared by adding 180 µl of cell solution (60 µl of cells + 120 µl low melt agarose) to slides coated with standard agarose. Autoclaved applicators were used to apply the solution in a straight line. After application, a coverslip was added to spread the solution over the slide. The slides were then placed in a refrigerator for 40 minutes. Next, the coverslips were carefully removed by pulling sideways to avoid removing cell content. The slides were then soaked in lysis solution for 24 hours. Afterward, the slides were placed in a horizontal electrophoresis tank, occupying as much space as possible, and an electrophoresis buffer was gently added until the slides were covered. The slides and buffer vat were placed in a refrigerator for 30 minutes to denature the DNA in an alkaline medium. Afterward, the remaining buffer was added, and electrophoresis was carried out for 25 minutes at 25 V and 300 mA.

The slides were carefully removed from the vat and neutralized with 5 mL of solution for five minutes when finished. The neutralization process was repeated twice more. The slides were then dried in an inclined position, fixed with absolute ethyl alcohol (100%) for 5 minutes, and refrigerated. Next, the slides were stained with 25 µl of ethidium bromide and recovered with a coverslip. Afterward, the slides were immediately analyzed using an Evos fluorescence microscope (Thermo Fisher Scientific®, Massachusetts, USA) with PE filter (Absorption 521 nm and emission 602 nm) setting to 1 and 1.5x magnification, and nucleoids images were captured of 300 cells for each concentration which were evaluated with the help of the software “Comet score” version 1.5.

The resazurin viability test is commonly used as an indicator of redox. Resazurin usually is blue. However, when this dye crosses the membranes of viable cells with an active metabolism it turns fluorescent pink (detectable by spectrophotometry) and is reduced to resorufin by mitochondrial dehydrogenases (Riss et al. 2016).

Statistical Analysis

In the cytotoxicity assay, IC50 (the concentration that inhibits 50% of cell growth relative to a control) was determined using non-linear regression (GraphPad Prism 6.01), where cell viability was determined as a logarithm of the tested concentrations, ($p < 0.05$). The graph was expressed as mean ± standard error of the mean. The data from the comet assay were analyzed using one-way analysis of variance (ANOVA) and the Kruskal-Wallis Multiple Comparison Test ($p<0.05$), followed by Dunn’s multiple comparison test. The distribution of the results was checked for normality utilizing the Kolmogorov–Smirnov test (GraphPad Prism version 6.01). Error bars were used to represent the standard error of the mean.

3. Results and Discussion

Cell viability – Resazurin reduction

The cell viability assay indicated an $IC_{50}$of 5.084 mg mL^{-1} for the crude *G. humilis* extract (Figure 1). IC_{50} represents the concentration of extract needed to reduce initial cell activity by 50% (Bighetti et al. 2018).

The present study showed cytotoxic effects in MCF7 tumor cells with a relatively high IC_{50} value (5.084 mg mL^{-1}), underscoring the importance of determining appropriate doses for safe consumption. It is also essential to decide on species-specific dosages since different species may behave differently, even from the same family. The present study is the first to study the cytotoxicity and genotoxicity of crude *G. humilis* extracts. Nunes et al. (2019) used Trypan blue and MTT exclusion methodologies to test cytotoxicity caused by the methanolic fraction of *G. humilis* on neutrophils, fibroblasts, and the RAW 264.7 strain but did not test for toxicity.

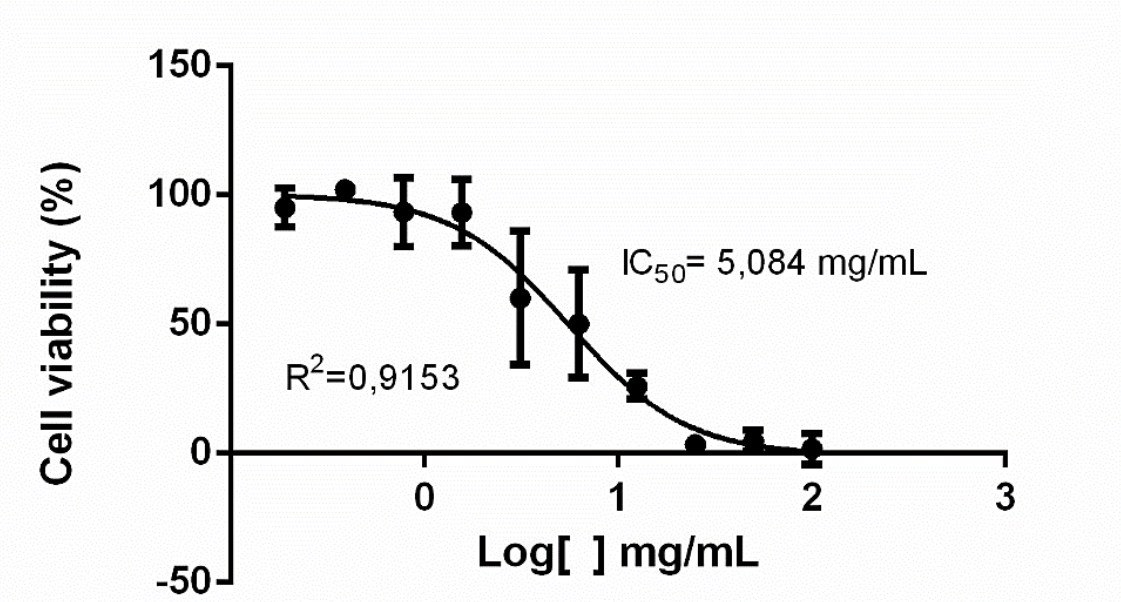

Figure 1. Sigmoidal dose-response curves after treating MCF7 cells with crude *G. humilis* extract. Each point represents the mean ± the standard deviation for n = quadruplicates.

DNA damage – Comet assay

DNA damage induction from unprocessed *G. humilis* was measured by the Comet assay parameters: DNA content, tail moment, and comet length. The length and percentage of DNA in the tail are directly proportional to the extent of DNA damage, while the tail moment is determined as the product of tail length multiplied by the percentage of DNA in the tail (Stefani et al. 2020). These three parameters showed that the concentration used in the T1 treatment (10 mg mL^{-1}) increased DNA concentration in the tail, indicating greater DNA damage, relative to the negative control (Figure 2 and Figure 3). In addition, the DNA damage caused by treatments T2 and T3 was significantly lower than in the negative control.

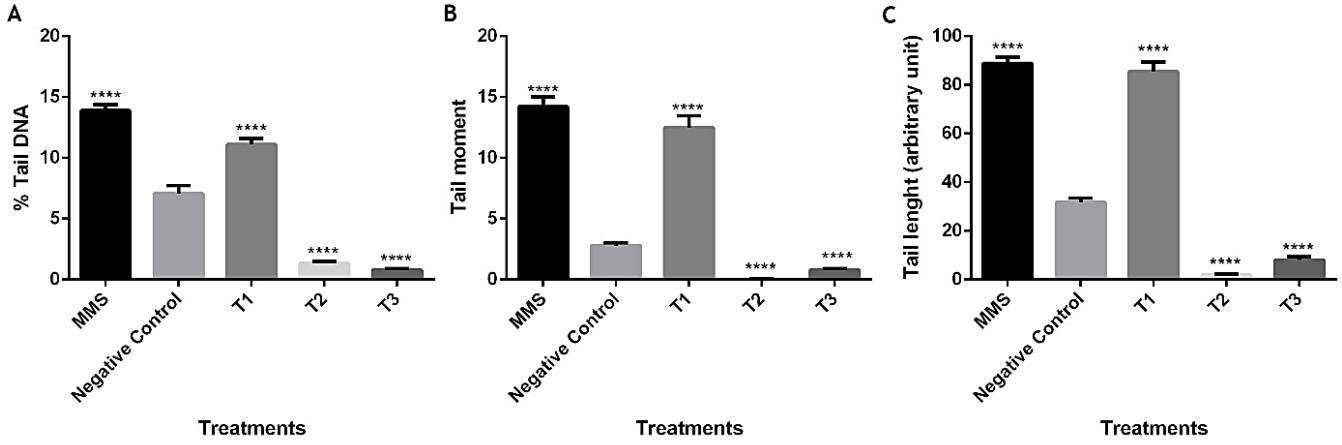

Figure 2. DNA strand breaks in MCF7 cells shown as A) % of DNA in tail; B) tail moment and C) tail length, according to the comet assay. The bars show the mean ± SEM of quadriplicates and significant differences compared to the control are indicated by asterisks (ANOVA). * P-value < 0.05, ** P-value <0 .01, *** P-value < 0.001 and **** P-value < 0.0001. T1: 10 mg mL^{-1}; T2: 5 mg mL^{-1}; T3: 1 mg mL^{-1}.

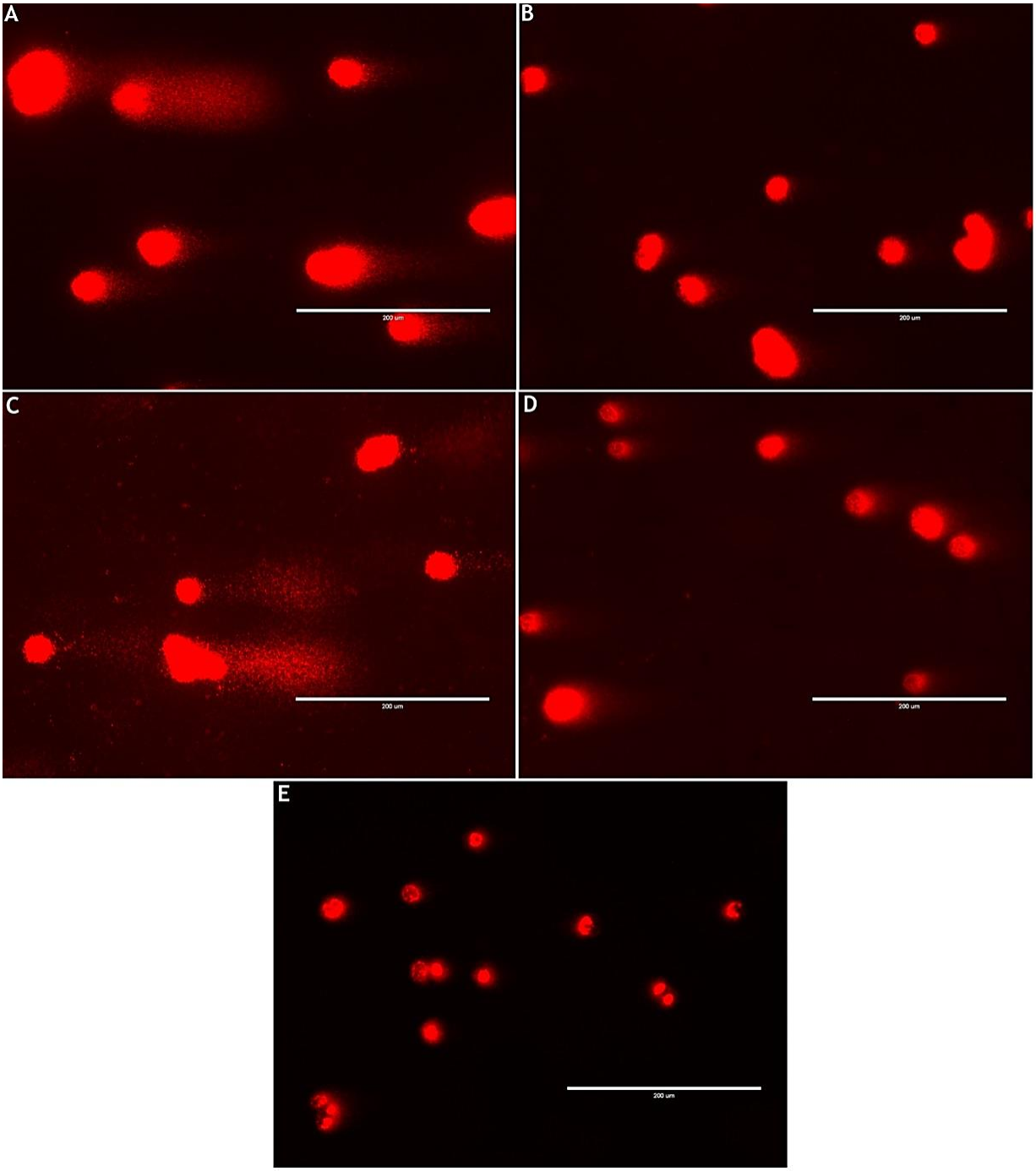

Figure 3. Representative photomicrography images of comet assays performed to measure DNA damage in MCF7 cells treated with: A - MMS (positive control); B - untreated (negative control); C - T1; D - T2 and E - T3. The white bars represent 200 µm.

Fractions and extracts from *Allanblackia gabonensis* (the same family as *G. Humilis* also performed well against cancer cells, with the most significant cytotoxicity caused by extracts. Jagtap et al. (2017) found considerable potential for antioxidant and cytotoxic activity in *Garcinia indica*. These authors encouraged further study on phytochemical and pharmacological components that could be developed into novel herbal anticancer treatments (Fankam *e*t al. 2017).

Costa and Cavalcante (2018) studied the effects of *P. juliflora* stem bark on breast and ovarian cancer cells and found toxicity in tumorous and non-tumorous cells. The authors also stressed the importance of studying the chemical profile of the plant extract to understand the relationship between the active compounds and their effect on cells.

Some studies have shown that extracts from various plants such as *Centaurea bruguierana* (Ostad et al. 2016) and *Croton urucurana* (Viera et al. 2017) produce cytotoxicity in human tumor cells.

Synthetic drugs are associated with relatively inconsistent clinical responses, drug resistance, and recurring and worsening pathology (Khwairakpam et al. 2018; Rocha et al. 2020). Thus, plant extracts have

potential as cancer treatments, even when used in combination with other drugs to improve treatment, decrease costs, and reduce side effects (Sharma and Zafar 2015; Goyal et al. 2017; Raeisi et al. 2018; Ahuja et al. 2018).

The comet assay showed that high doses (10 mg mL^{-1}) of *Garcinia humilis* extract induced DNA damage in the current study. Santos et al. (2018c) found that different doses of *Paullinia cupana* (guarana powder) induced cytotoxicity and genotoxicity and had the potential to change cell cycles. This species is widely used as a natural energy booster and is supplied with information on toxicity and guidance regarding the moderate and responsible use of medicinal plants.

Lower doses (T2: 5 mg mL^{-1} and T3: 1 mg mL^{-1}) of the *Garcinia humilis* extract exhibited a protective effect that reduced DNA damage relative to the untreated control group. Antioxidant metabolites probably caused this protective effect in the crude extract that neutralized pro-oxidant molecules and reduced DNA fragmentation relative to the control. Tome et al. (2019) reported that some fractions of *G. humilis* extract contain high concentrations of phenolic compounds and flavonoids.

John et al. (2018) found that the main bioactive constituents of *G. humilis* bark are type B procyanidins and citric acid, which, in addition to their potential antioxidant benefits, improved cardiovascular parameters in rats fed on a high-carbohydrate and fat diet. The authors also emphasized the importance of additional studies on *G. humilis* to understand its therapeutic potential better.

Nunes et al. (2019) also described the anti-inflammatory potential of *G. humilis* extracts and concluded that in vivo treatment inhibited neutrophilic functions, such as secretion and migration, and reduced the perception of inflammatory pain, suggesting that these extracts could be used to treat acute inflammatory diseases.

Brida et al. (2019) did not observe genotoxic effects from diluted extracts of *Ardisia elliptica* at 2000, 1000, 500, 250, and 50 µg mL^{-1}. These authors suggested that their results should be complemented by in vivo tests to determine pharmacological potential better.

4. Conclusions

The IC_{50} (half maximal inhibitory concentration) value of a crude *G. humilis* extract was 5.084 mg mL^{-1} for MCF7 human breast adenocarcinoma cells.

A T1 concentration (10 mg mL^{-1}) of *G. humilis* extract also induced DNA damage in these cells.

In Brazil, medicinal plants can provide alternative health care treatments; however, most of these plants, including *G. Humilis*, require further study to understand better their therapeutic and toxicological potential in the human body. Finally, even though plant extracts are natural, they should still be used sparingly.

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