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CYTOTOXICITY EFFECTS AND CHEMOTHERAPY IMPROVEMENT OF COCOA AGAINST CHRONIC MYELOCYTIC LEUKEMIA CELL LINE (K562)

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Abstract: This study aimed to analyze the anticarcinogenic activity and *in vitro* response to chemotherapy of cocoa in chronic myeloid leukemia (CML) cell line (K562). Cells were exposed to different concentrations of cocoa (30; 100; 500; 750; 1000; 1500 and 2000 **µg/**mL) isolated and associated with ATRA and arsenic trioxide during 24 and 72 hours. Our results indicate that cocoa showed selective cytotoxic action on K562 cells at all concentrations tested in 72 hours. Moreover, cocoa raised oxidative stress by increasing levels of total levels of ROS, nitric oxide, and superoxide. Also, cocoa improved the action of chemotherapy, by reducing cell viability and proliferation. Thus, the results highlight cocoa as a potent anticancer agent, with an excellent selective profile to be used adjuvant therapies for CML.
Keywords: *in vitro*, *Theobroma cacao* L., leukemia, ATRA, arsenic trioxide, anticancer

INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by neoplastic transformation of the hematopoietic stem cell and the pathognomonic presence of the Philadelphia chromosome (Ph) due to a reciprocal translocation between chromosomes 9 and 22 t(9;22)(q34;q11). This chromosomal alteration generates the BCR-ABL1 oncogene. Thus, the BCR-ABL1 oncogene encodes the chimeric BCR-ABL1 protein with constitutive kinase activity (Bonifacio et al. 2019).

As a result of this mutation, the upregulation BCR-ABL1 tyrosine kinase triggers growth factor independence and leukemic cell proliferation in hematopoietic cell lines. CML usually presents in the chronic phase, characterized by the clonal expansion of mature myeloid cells (Bonifacio et al. 2019).

Currently, there are several treatments for leukemias that depend on the clinical condition of each patient. The main therapeutic approach for CML involves the use of molecular-targeted chemotherapy with all-trans-retinoic acid (ATRA) and arsenic trioxide. These drugs differentiate granulocytic cells, facilitating cell apoptosis and decreasing the proliferation of cancer cells (Jambrovics et al. 2020).

However, since there is a great similarity of malignant cells as normal, chemotherapy can cause side effects, such as skin reactions, feeling of tiredness, fatigue, hair loss, bruising, bleeding, anemia, nausea, vomiting, changes in appetite, diarrhea, dysuria and drowsiness (Evangelisti et al. 2018).

Unfortunately, some patients show resistance to treatment. Thus, other treatment options, including arsenic trioxide, have been used to treat patients with disease recurrence after initial therapy with anthracyclines and ATRA (Tomita et al. 2013).

Regarding this, investigations that are looking for new therapies to improve cancer treatment are extremely important. In this scenario, natural products are highlighted in this area. Studies using compounds rich in polyphenols are being carried out due to several health benefits, as they act as important adjuvants in antitumor activity (Schultze et al. 2018).

A functional food rich in polyphenols as well as others important bioactive molecules is cocoa (*Theobroma cacao* L.). Cocoa has been used since past centuries in the production of chocolate, however, it is also being widely explored for its medicinal function, since it has very significant biological properties, such as, DNA protection (Othman et al. 2007; Abbe Maleyki and Ismail 2010; Martin et al. 2013; Pérez-Cano et al. 2013; Martins et al. 2020).

Theobroma cacao is a plant with numerous medicinal activities due to its chemical composition, for instance antioxidant, antitumor, anti-inflammatory action, which have shown important potential in stopping cell growth and proliferation and, thus, provide new challenges in the discovery of anticancer agents of plant origin (Baharum et al. 2014).

In experimental in vitro studies, phenolic compounds in cocoa showed several beneficial effects against platelet aggregation, arterial hypertension, atherosclerosis, hyperglycemia, hypercholesterolemia, inflammation, hepatocarcinogenesis, DNA damage and clastogenic effect (Othman et al. 2007; Abbe Maleyki and Ismail 2010; Martin et al. 2013; Pérez-Cano et al. 2013; Martins et al. 2020).

Thus, we investigated here a new anticancer agent that can act against drug resistance and present no or reduced side effect. Take this into account, we studied the in vitro effect of cocoa in CML cancer cell line (K562) isolated and in synergism with the chemotherapy, using ATRA and arsenic trioxide.

MATERIALS AND METHODS

COCOA SOLUTION PREPARATION

The cocoa seed powder was obtained from the distribution company Florien®, where information regarding the quality and origin of the plant was assured, with a technical report provided by the company. To produce a cocoa solution, 400 mg of cocoa powder was diluted in 50 mL of PBS, obtaining a concentration of 8000 μg/mL. The sample was placed in ultrasound for 30 minutes and then transferred to a water bath with magnetic stirring at 40 °C for 30 minutes. After this process, the sample was filtered through quantitative filter paper (12.5 cm diameter, black band). Finally, the sample was filtered using 0.22 μm membranes for sterilization and stored in a freezer at -20 °C for later use in the experiments.

In a previous study performed in our research group, the bioactive molecules present in the cocoa solution were analyzed by HPLC (high-performance liquid chromatographic), according to the method validated by (Roggia et al. 2020). Our findings indicated that cocoa solution presented excellent concentrations of catechin, epicatechin, theobromine, theophylline, and caffeine, assuring a formulated with great quality to be used in all the experiments (unpublished data).

CELL CULTURE

CML (K562) cell line was commercially purchased from the Rio de Janeiro Cell Bank and cultivated under ideal cell culture conditions, using *Roswell Park Memorial Institute* (RPMI) and Dulbecco's Modified Eagle Medium (DMEM) respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 U/mL)/ streptomycin (100 mg/mL) antibiotics, until the acquisition of adequate cell numbers and confluence to carry out all the tests proposed.

TREATMENTS

K562 cells, at a concentration of 5x105 cells per well, were exposed to different concentrations of cocoa (30; 100; 300; 500; 750; 1000; 1500 and 2000 μg/mL) during 24 and 72 hours of incubation. In addition, the effect of cocoa, at the same concentrations used previously, was analyzed associated with the chemotherapy drugs ATRA (1 μM) and arsenic trioxide (250 μg/mL). ATRA was diluted in DMSO, and the final concentration of this solvent was determined to be 0.1%.

CELL VIABILITY AND PROLIFERATION MEASUREMENT

MTT Assay

Cell viability and proliferation were measured by the MTT Assay. The supernatant of the treatments was discarded, and the cells were washed and resuspended in a phosphate buffer (PBS, 0,01 M; pH 7.4), to avoid the interference of the polyphenols present in cocoa. The sample treatments were disposed in a 96 well plate. After, MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added (1:10) (dissolved in 5 mg/mL PBS) and incubated for 1 h, at 37 °C. The formazan crystals generated were released from the cells through DMSO added. The quantification was performed colorimetrically by spectrophotometry at a wavelength of 560 nm, the absorbance value being proportional to the number of viable cells (Kang et al. 2010).

Fluorometric Assay for DNA Quantification

The fluorimetric assay for quantification of free DNA in the medium using the DNA-PicoGreen® reagent, brand Invitrogen (Life Technologies), which is a fluorescent dye that binds to minute concentrations of double stranded DNA. This procedure was performed in the medium where cells are treated in order to determine the presence of free DNA due to possible cell disruption and cell death (Ha et al. 2011).

The dye was added to the sample in a black 96-well ELISA plate, with an incubation period of 5 minutes and fluorescence reading in the Spectrofluorimeter device at 480nm of excitation and 520nm of emission.

OXIDATIVE METABOLISM EVALUATION

Reactive Oxygen Species Quantification

To measure the total levels of ROS, 2'-7'-dichlorofluorescein diacetate (DCFH-DA) reagent was used, that can cross the cell membrane, being deacetylated by mitochondrial enzymes, giving rise to 2',7'-dichlorodihydrofluorescein, which reacts with ROS, mainly hydrogen peroxide (H2O2) and produces 2',7'-dichlorofluorescein that emits fluorescence. Therefore, fluorescence was determined based on the wavelengths of 488 nm excitation and 525 nm emission (Degli Esposti 2002).

Determination of superoxide production (O_{2^-})

To quantify the production of O2-, an assay based on the formation of formazan salt was applied via the reaction between Nitrobluetetrazoliun Chloride (NBT) and O2-. The levels of O2- production were measured colorimetry, using a wavelength of 550 nm (Noh et al. 2015).

Determination of nitric oxide (NO•)

The nitric oxide (NO•) assay detects the presence of organic nitrite (NO2-) in the sample. The nitrite is detected and analyzed by the formation of a pink color when the Griess reagent is added to the sample containing NO2-. The sulfanilamide of the Griess reagent is responsible for the formation of diazonium salts from the sample NO2-. When N-1-naphthylethylenediamine-dihydrochloride interacts with the diazonium salts, the pink color appears in the sample, that is detected using a wavelength of 540 nm (Choi et al. 2012).

STATISTICAL ANALYSIS

The results obtained were submitted to statistical analysis using a one-way ANOVA test, followed by Dunnet's post hoc test, using the statistical graphing program Graph Pad Prism 5.0. Treatments were expressed as a percentage of control (%). Results were considered statistically significant when p is equal to or less than 0.05.

RESULTS

COCOA DECREASED THE VIABILITY AND PROLIFERATION OF K562 CELLS

Cocoa was able to decrease the viability levels of K562 cells exposed to higher concentrations (1000; 1500 and 2000 **µg**/mL) during 24 hours, while in 72 hours of exposure, cocoa reduced proliferation levels at all concentrations tested (Figure 1).

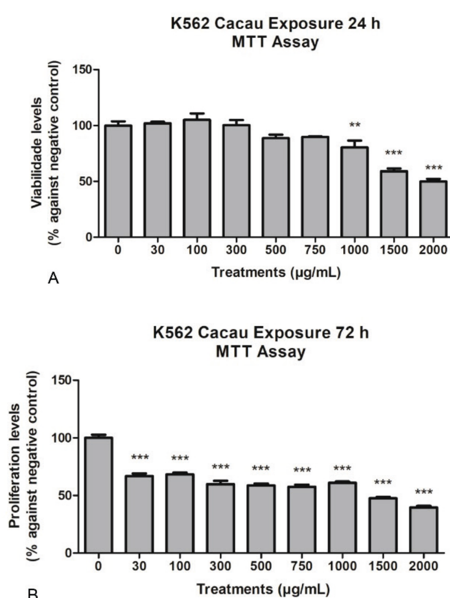
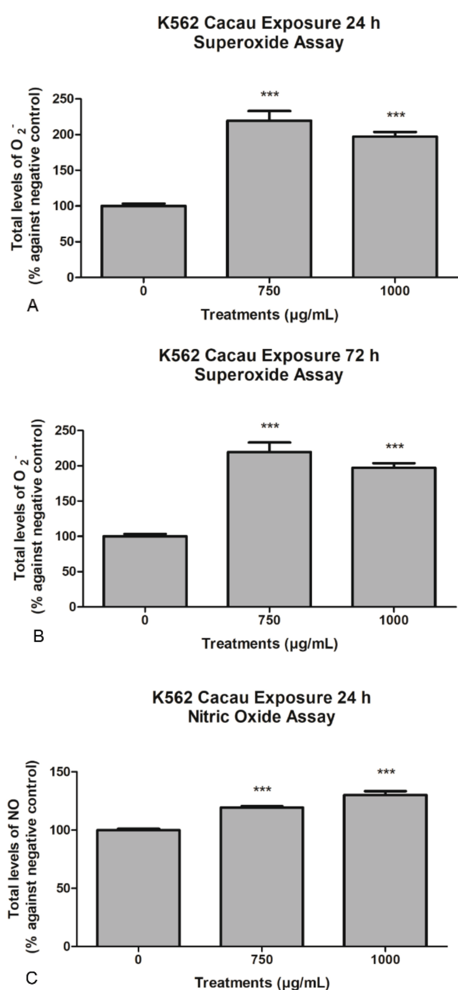


Figure 1. K562 cells exposed to different concentrations of cocoa extract (30; 100; 300; 500; 750; 1000; 1500; 2000 **µg**/mL) for 24 (A) and 72 (B) hours. Cell viability and proliferation was determined by the MTT Assay. The results were compared with the percentage of the negative control (cells and medium only). N = 3, ** (p <0.001), *** (p <0.0001).

COCOA SHOWED CYTOTOXIC ACTION ON K562 CELLS BY INCREASING OXIDATIVE STRESS

Intermediate concentrations of cocoa were selected for the investigation of the cytotoxic causal mechanism, 750 and 1000 µg/mL. The results found suggested that cocoa increasing free DNA at a concentration of 1000 µg/mL in 24 hours (Figure 2A) and at all concentrations in 72 hours (Figure 2B) of incubation, confirming the cytotoxicity measured by MTT previously. Furthermore, cocoa was able to increase the production of ROS at a concentration of 1000 µg/mL in 24 hours (Figure 2C) and at both concentrations in 72 hours of incubation (Figure 2D).



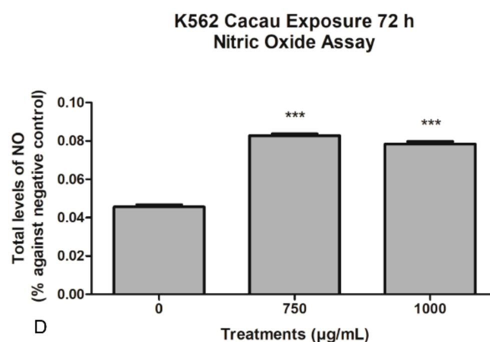
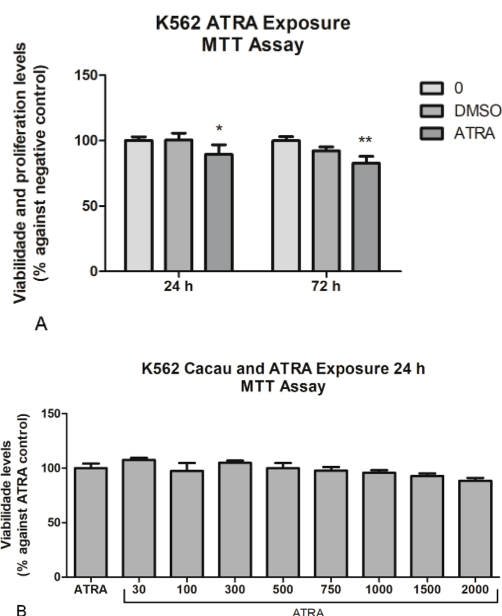


Figure 2. K562 cells exposed to intermediate concentrations of cocoa extract (750 and 1000 µg/mL) for 24 (A and C) and 72 (B and D) hours. Free DNA levels were measured by the PicoGreen Assay (A and B) and the total ROS rate by the 2'-7'-dichlorofluorescein diacetate (DCFH-DA) test (C and D). The results were compared with the percentage of the negative control (cells and medium only). N = 3, * (p <0.005), ** (p <0.001), *** (p <0.0001).

Furthermore, additional analyzes to investigate the causal mechanism of cocoa indicated that cocoa increased levels of superoxide (Figure 3A and 3B) and nitric oxide (Figure 3C and 3D) at both concentrations (750 and 1000 µg/mL) and tested periods (24 and 72 hours).



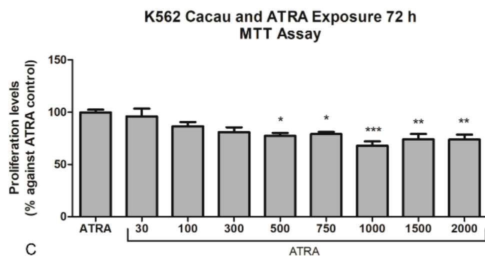


Figure 3. K562 cells exposed to intermediate concentrations of cocoa extract (750 and 1000 µg/mL) for 24 (A and C) and 72 (B and D) hours. Superoxide (A and B) and nitric oxide (C and D) levels were measured. The results were compared with the percentage of the negative control (cells and medium only). N = 3, ** (p <0.001), *** (p <0.0001).

COCOA IMPROVED THE CHEMOTHERAPY ACTIVITY

ATRA decreased the viability and proliferation of K562 cells for 24 and 72 hours, respectively. DMSO, which was used as a vehicle to dilute ATRA, at a concentration of 0.1%, did not change these parameters when compared to the negative control (only cells and medium) (Figure 4A).

When ATRA was associated with different concentrations of cocoa during 72 h, there was a reduce of levels of cell proliferation at concentrations of 500, 750, 1000, 1500 and 2000 µg/mL of cocoa, when compared to the control of cells treated only with ATRA (Figure 4C). However, during 24 h there was no change in cell viability (Figure 4B).

In addition, arsenic trioxide was able to reduce the viability and proliferation of K562 cells for 24 and 72 hours, respectively (Figure 5A). Also, when arsenic trioxide was associated with different concentrations of cocoa, in 72 hours lower levels of cell proliferation were found at concentrations of 1500 and 2000 µg/mL of cocoa, when compared with the control of cells treated with chemotherapy alone (Figure 5C). On the other hand, during 24 hours there was no change in cell viability (Figure 5B).

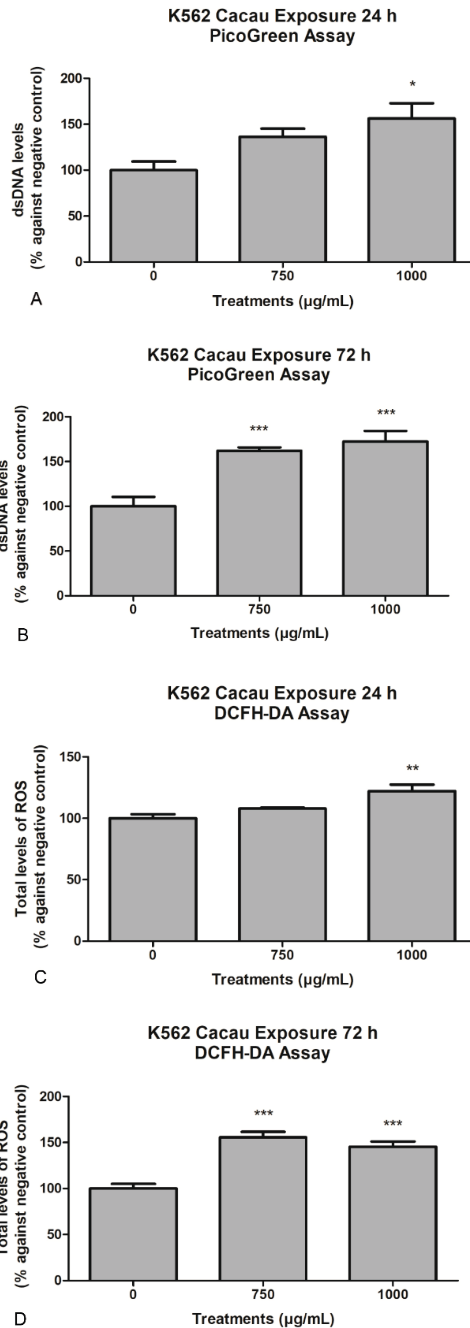


Figure 4. K562 cells exposed to different concentrations of cocoa extract (30; 100; 300; 500; 750; 1000; 1500; 2000 µg/mL) associated with ATRA for 24 and 72 hours. Cell viability and proliferation were determined by the MTT Assay. The results were compared with the percentage of the negative control (cells and medium only) in "A" and compared with the chemotherapeutic control (cells exposed only to the drug) in "B" and "C". N = 3, * (p <0.005), ** (p <0.001), *** (p <0.0001).

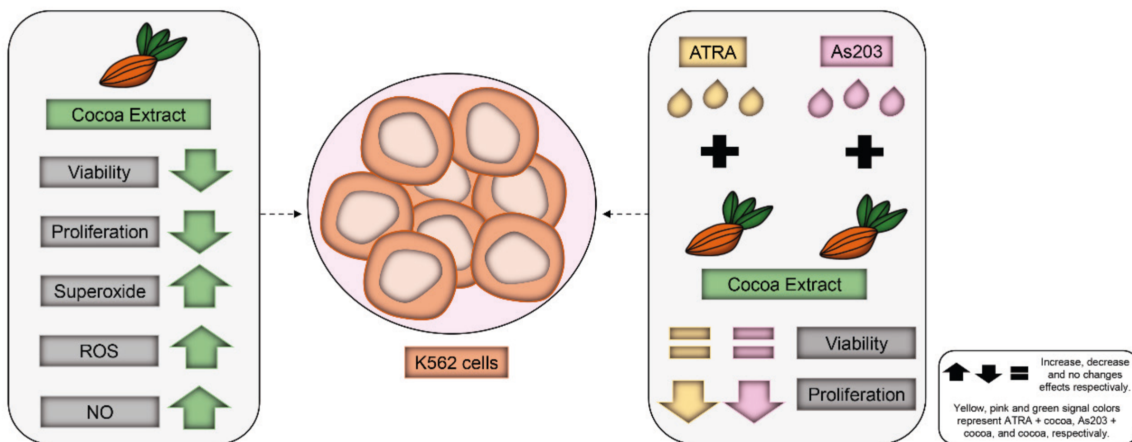
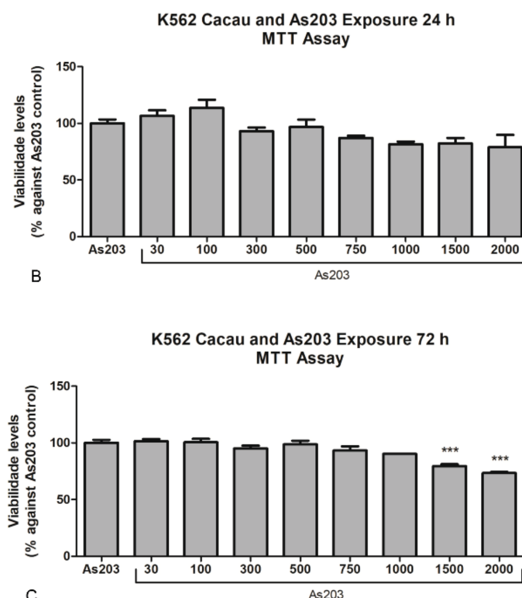


Figure 5. K562 cells exposed to different concentrations of cocoa extract (30; 100; 300; 500; 750; 1000; 1500; 2000 µg/mL) associated with As203 for 24 and 72 hours. Cell viability and proliferation were determined by the MTT Assay. The results were compared with the percentage of the negative control (cells and medium only) in "A" and compared with the chemotherapeutic control (cells exposed only to the drug) in "B" and "C". N = 3, * (p <0.005), ** (p <0.001), *** (p <0.0001).

DISCUSSION

Our results found in this study suggested that the cocoa showed selective cytotoxic action on K562 cells at all concentrations tested in 72 hours. In addition, the anticarcinogenic mechanism of cocoa can be explained through the production of oxidative stress, generated by increasing levels of total levels of ROS, nitric oxide and superoxide. Moreover, cocoa improved the action of chemotherapy, by reducing cell viability and proliferation (Figure 7).



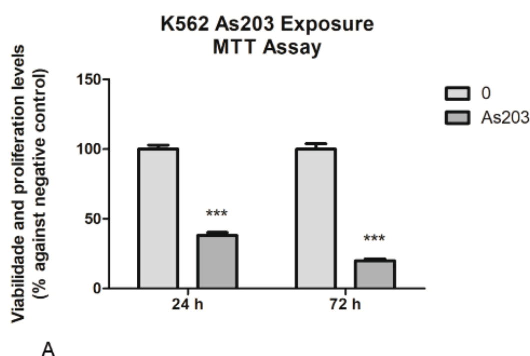


Figure 6. Schematic representation of the results found in the present study. Cocoa showed a cytotoxic effect on K562 cells tested, by reducing cell viability and proliferation and increasing oxidative stress. Cocoa potentiated the action of the chemotherapy drug ATRA and As203 on K562 cells, via viability and proliferation reduction.

Corroborating these results, (Baharum et al. 2014) determined the antiproliferative activity of methanolic extracts from parts of cocoa plants (leaf, fermented and unfermented husk, kernel and root) by the MTT assay in breast cancer cells (MCF- 7) and normal liver cells (WRL-68). The assay revealed that the leaf extract had the highest antiproliferative activity against MCF-7 cells, however, caused no damage in normal cells. Subsequent to this study, in 2016 the same authors evaluated the cytotoxic activity of cocoa in several cancer strains by the MTT test. The lines evaluated were MCF-7 and MDA-MB-231 from breast cancer, HepG2 from liver cancer, HT-29 colon carcinoma, A549 from lung cancer, HeLa from cervical cancer and from normal human liver WRL-68, which were exposed to various cocoa extracts. The lowest cell viability was observed in MCF-7 breast cancer cells.

Moreover, some studies have reported that cocoa and its bioactive molecules are associated with anticarcinogenic activity (Pick et al. 2011; Williamson 2017; Perez-Vizcaino and Fraga 2018). Among their various actions and benefits to human health, flavonoid compounds, such as (-)-epicatechin and procyanidins, have a range of biological activities related to antiproliferative effects, which are found in cocoa and other functional foods (Williamson 2017; Perez-Vizcaino and Fraga 2018). In this regard, a previous study reported that polyphenols can be used to inhibit the growth of cancer cells due to their ability to modulate the activity of multiple targets involved in carcinogenesis through interaction simultaneous direct or modulation of gene expression (Benvenuto et al. 2013).

In a study described by (Ramljak et al. 2005), this cytotoxic effect was also observed in breast cancer cell lines (MDA MB-231, MDA MB-436, MDA MB-468, SKBR-3 and MCF-7) evidencing that breast cancer cells are selectively susceptible to the cytotoxic effects of pentameric procyanidin and suggesting that inhibition of cell proliferation by this compound is associated with site-specific dephosphorylation or negative regulation of various cell cycle regulatory proteins.

Natural flavonoids have antioxidant, anti-inflammatory and anticancer activities through multiple pathways, and have great potential against various types of cancers, contributing to the induction of apoptosis in breast, colorectal and prostate cancers, reducing the activity of nucleoside diphosphate kinase-B, in the lung, bladder and colon, inhibiting cell proliferation (Hazafa et al. 2020). Different types of therapies are being investigated for chemoprevention in the treatment of cancer, bioactive compounds play an important role in this field and have been explored due to their main role in the prevention of carcinomas, being able to delay, reverse or completely inhibit the process of carcinoma (Cragg and Pezzuto 2016).

According to (Kim et al. 2014), in some studies carried out *in vitro*, it was reported that polyphenols in cocoa liquor have antimutagenic and anticlastogenic activity and procydinins in cocoa liquor reduced the incidence and multiplicity of lung carcinomas and rodent adenomas developed in male mice. Furthermore, cocoa consumption may be beneficial in inhibiting complex molecular processes that lead to cancer, highlighting that angiogenesis plays an important role in cancer growth and metastasis formation.

Evidence has shown that cocoa consumption may be able to inhibit complex molecular processes that lead to cancer, emphasizing that angiogenesis plays an important role in cancer growth and metastasis formation. In some *in vitro* studies, cocoa liquor polyphenols were reported to have antimutagenic and anticlastogenic activity and cocoa liquor procyanidins reduced the incidence and multiplicity of pulmonary and

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rodent-like adenomas developed in male rats (Yamagishi et al. 2003).

In the study carried out by(Rodríguez-Ramiro et al. 2011) *in vivo* in rats, the effect of a diet rich in cocoa in the prevention of colon cancer was investigated, reducing oxidative stress and cell proliferation via activation of apoptosis, induced by azoxymethane. (AOM) and aberrant crypt foci (ACF) used to detect the early stage of carcinogenesis. The results showed that after a cocoa-rich diet there was a significant reduction in the formation of new foci of aberrant colonic crypts induced by AOM and in the multiplicity of crypts in colon cancer, demonstrating antiproliferative effects. The inhibitory effect of cocoa-derived phenolic compounds on colon cancer development can be explained through the regulation of genes involved in cancer development, such as oncogenes, tumor suppressor genes (Kampa et al. 2007).

A previous study reported that cocoa beans subjected to different processing conditions have cytotoxicity against human lung cancer cells (A549) by decreasing cell proliferation via stimulation of apoptosis and cell cycle arrest. The results suggested that the cocoa extract promoted arrest in the G1 phase and increased the number of apoptotic cells when compared to the negative control (Bauer et al. 2016).

The oxidative stress generated by cocoa as an anticarcinogenic causal mechanism found in the present study was also reported by the investigation carried out by (Kozikowski et al. 2003), where they observed that procyanidin-rich fractions prepared from cocoa seeds inhibit growth *in vitro* in human breast cancer cell lines. Using the cell line MDA-MB-231, they indicated that the growth inhibition caused by procyanidin is a result of cell cycle arrest in G0/G1, through the promotion of oxidative stress, mainly by increasing the production of H_2O_2.

Corroborating our findings, the review study carried out by (Aboelella et al. 2021) elucidated the role of oxidative stress and ROS production in the tumor microenvironment. ROS have many signaling pathways with opposing effects and diverse roles in cancer progression. When the accumulation of ROS exceeds the levels beneficial to cancer cells, in order to stimulate cell proliferation and survival pathways, such as the Mitogen-activated Protein Kinases (MAPKs) pathway, for example, which are ROS dependent, their carcinogenic roles in proliferation and invasion are changed to antitumor effects through the induction of programmed cell death, mainly apoptosis.

Additionally, several drugs that are used in chemotherapeutic treatments, act in the process of tumor destruction through the formation of ROS, such as, for example, the drug adriamycin (ADR), used in studies in the treatment of various neoplasms. In the study conducted by Li et al. (2000) it was shown that, in rats treated with ADR, there was an increase in oxidative stress.

Furthermore, the present study suggested that cocoa was able to increase the action of ATRA and arsenic trioxide. These results can be explained by the fact that cocoa has numerous bioactive molecules in its chemical matrix, which have already been associated with an increase in chemotherapeutic activity, in different drugs and cancer cell lines.

Cocoa is a natural product rich in catechins, which are known to have biological activities, such as antioxidant, antiangiogenic, antitumor and have been relevant for cancer prevention. Regarding this, the study of Mayr et al. (2015) reported the synergism of catechin with the chemotherapy drug cisplatin. This association generated a blockade of the tumor cell cycle, decreasing cell viability and thus, improving the response to the treatment of biliary tract cancer.

As in another study, carried out by Bimonte et al. (2015) it was indicated that catechin in association with the chemotherapy drug bleomycin generated an efficient synergism for the inhibition of cell growth in the pancreatic cancer cell line (MiaPaCa-2). The combination of catechin with bleomycin was able to decrease cell proliferation by blocking the S-phase cycle and depolarization of the plasma membrane, and there was an increase in apoptosis and DNA damage.

Another highlighted bioactive molecule in cocoa is caffeine, which was investigated in the study conducted by Nakata et al. (2015). The results found suggested that caffeine was able to potentiate the chemotherapy treatment against osteosarcoma.

Moreover, the effect of caffeine in association with cisplatin is described in the literature. This synergism was able to increase the promotion of apoptosis in lung cancer cells and thus reducClique ou toque aqui para inserir o texto.e the resistance of tumor cells to chemotherapy. The presence of caffeine increased the expression of caspase 3 by cisplatin in the lung cancer strains HTB182 and CRL5985. Also, this synergism generated a reduction in the levels of cell proliferation and changes in the cell cycle (Wang et al. 2015).

The synergistic potentiating effect of caffeine with some chemotherapeutic drugs could be explained by the fact that this bioactive molecule is able to inhibit the activity of two protein kinases, Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia Rad-3-related (ATR), which are essential for protection of the genome and checking its integrity during the cell cycle. Thus, caffeine could potentiate the genotoxic action of the chemotherapeutic agent, increasing its antitumor activity (Tsabar et al. 2015).

Therefore, since cocoa is a natural product rich in bioactive molecules in its chemical matrix, our study suggested that the synergism of these bioactive compounds could result in cytotoxic action on CML cells and increase the action of chemotherapy selectively, without affecting the normal cells.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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