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ORIGINAL ARTICLE

CYTOTOXIC ACTIVITY OF A BLACK BEAN (*PHASEOLUS VULGARIS* L.) EXTRACT AND ITS FLAVONOID FRACTION IN BOTH *IN VITRO* AND *IN VIVO* MODELS OF LYMPHOMA

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ABSTRACT

Background: Black bean (*Phaseolus vulgaris* L.) is a very common legume seed in Mexican diet. Flavonoids and crude extracts from different plants have been reported as effective agents for chemoprevention and cytotoxicity in several cancer cell lines. We investigated the effects of black bean hulls extract (BBE) and its flavonoid fraction (FF) on lymphoma cells. **Methods:** BBE and FF were characterized by high-performance liquid chromatography. Viability and flow cytometry assays were carried out. Finally, a mouse model was generated to test the *in vivo* effect of both fractions. **Results:** Both BBE and FF inhibited cell proliferation in a dose-dependent way. In addition, cells underwent apoptosis, and the cellular population at S-phase increased after exposure to these fractions. Furthermore, mice treated with BBE or FF increased the overall survival by 5 or 6 days, respectively, in comparison with a placebo group (p = 0.056). **Discussion:** BBE and FF had cytotoxic action by driving OCI-Ly7 cells into apoptosis as well as blocking progression to G2/M phase. In addition, BBE and FF treatments were effective in xenograft models.

Key words: Flavonoids. Black bean extract. Lymphoma. *Phaseolus vulgaris*.

INTRODUCTION

Diffuse large-B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma (NHL) in adults, representing 30–40% of all cases[1]. In Mexico, lymphoma is the third and fourth cause of death in males and females of economically productive age (25–45 years), respectively[2].

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With respect to survival rates (SR) for this entity, the initial response to treatment is a key factor. Patients with partial response or refractoriness to cyclophosphamide, hydroxydaunorubicin, vincristine (oncovin) and prednisone (CHOP) had a short SR (median: 10 months)[3]. In patients receiving treatment regimens with the monoclonal antibody rituximab (R-CHOP), the SR increased by 10%[4]. Therefore, research of new therapeutic molecules to improve the initial response and, consequently, the SR is mandatory.

Several drugs obtained from natural sources have been added to chemotherapy schemes of DLBCL. Vincristine, a molecule isolated from an endemic plant of Madagascar named Madagascar periwinkle (*Catharanthus roseus*), is currently used to treat leukemia and Hodgkin's lymphoma, among other tumors. Another molecule, doxorubicin, was obtained from *Streptomyces peucetyus* and is part of regimens for breast cancer, leukemia, and lymphomas[5]. In this context, several reports have suggested that diet-derived phenolic compounds may have potential antineoplastic effects by reversing, inhibiting or delaying tumor development[6]. Black bean (*Phaseolus vulgaris* L.), a commonly consumed seed in the Mexican diet, is extremely rich in flavonoids, polyphenols, tannins, saponins, phytosterols, and other antioxidants[7]. Some studies have reported that the black bean hulls extract (BBE) is a protective agent against chemically-induced DNA damage in bone marrow and peripheral blood cells *in vivo*[8], as well as providing protection against colon carcinogenesis in mice[9]. Its cytotoxic effect has been tested in several tumor cell lines such as breast (MCF-7), hepatic (HepG2), colon (CaCo$_2$), and cervix (HeLa)[7,10].

Several authors have fractioned the BBE to isolate its molecules and characterize its antineoplastic properties. One study showed that the saponin-rich fraction of BBE stimulates the immune system against cancer cells[11,12]. Black bean tannins inhibited cellular proliferation of colon, prostate, and breast cell lines[13,14]. In addition, flavonoid fractions (FF) and flavonoid single molecules inhibited cell growth and triggered apoptosis in human prostate cancer cells (PC-3)[15], as well as having proapoptotic effects against CCRF-CEM, Daudi, HeLa and lung adenocarcinoma cell lines[16-18].

In this study, we investigated the cytotoxic effect of methanolic BBE and its flavonoids fraction against the aggressive lymphoma cell line. In addition, we evaluated their potential apoptotic effect *in vitro* as well as their capability to disrupt the cell cycle. Then, we tested their effect on a xenograft model and compared with placebo and cyclophosphamide (used as reference control) by oral administration.

MATERIALS AND METHODS

Preparation of the BBE and FF

The BBE was prepared as described by Gutiérrez-Uribe et al.[10], whereas the FF was obtained by dissolving 2 g of dried BBE in 20 mL of methanol (DEQ Monterrey, Mexico) followed by sonication (Branson® 8510) for 5 min, then 20 mL of tri-distilled water was added and homogenized by sonication for 5 more min. The blend was centrifuged at 800 g for 5 min (Centrifuge Allegra and Beckman Coulter®) and the supernatant was recovered. The flavonoids were purified using a C$_{18}$ Solid Phase Extraction cartridge 20 cc/5 g (Waters®, Milford, and MA) followed by a washing step with 10 mL of 25% MeOH, and the flavonoid-rich fraction was recovered by eluting 10 mL of 60% MeOH. The flavonoid solution was then dried by lyophilization (Freezone Benchtop 20.5 L, Labconco®).

High-performance liquid chromatography (HPLC) identification and quantification of flavonoids

Both BBE and FF were analyzed according to the procedure described by Guajardo et al.[19]. Briefly, the extracts (BBE or FF) were dissolved in 80% methanol to a concentration of 1 mg/mL before their injection into the HPLC system. HPLC analysis was achieved with gradient elution using (A) water adjusted to pH 2 with trifluoroacetic acid (Sigma, St. Louis, MO), and (B) HPLC-grade acetonitrile (Merck, Darmstadt, Germany) as the mobile phase at a flow rate of 0.5 mL/min. The column employed was Zorbax SB-Aq 4.6 × 150 mm and 3.5 µm (Agilent, USA). Fractionation was achieved with gradient conditions at room temperature as follows: 20% B for the first 6 min, increasing the B concentration to 50% at 12 min and to 100% at 30 min. At the end of the chromatographic separation, initial conditions were reacquired to achieve accurate equilibration of the column. The injection volume was 5 µL

of either BBE or FF. Each chromatogram was recorded at 360 nm (bandwidth-16) and 280 nm. BBE and FF compounds were analyzed with HPLC LC/MSD-TOF (Agilent Technologies, Waldbrunn, Germany) to confirm the identity of flavonoids. On both analyses, standards of myricetin, quercetin, and kaempferol (Sigma, St. Louis, MO) were used to quantify the flavonoids present in the BBE and the FF as reported previously[10,19].

Cell culture

The DLBCL-derived cell line (OCI-Ly7) was kindly provided by Professor Ricardo Dalla Favera from Columbia University, (New York, NY). NIH-3T3 and VERO cells were obtained from ATCC (CRL-1658 and CCL-81). Cells were cultured in IMDM, (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, (Invitrogen, Carlsbad, CA), 1% pen-strep; and maintained at 37°C in 5% of CO_2 atmosphere.

Cytotoxicity assays

OCI-Ly7 cells were cultured in a white opaque 96-well plate (Corning, Pittston, PA), at a density of 2×10^4 cells/well. Cells were exposed to different concentrations of BBE, FF or CTX (90% pure per USP reference standards, Laboratorios Sanfer S.A. De C.V., Mexico), used as positive control. After 24 h, cellular viability was monitored using CellTiter-Blue reagent (Promega, Madison, WI). To evaluate the effect over normal cells, NIH-3T3 and VERO cell lines were exposed to the extracts under the same conditions.

Apoptosis and cell cycle assay

Cells were plated in a 12-well plate (Costar, Wilkes Barren, PA) at a density of 2×10^5 cells/well and exposed to either BBE or FF using the IC_{50} obtained in the cytotoxicity assays. Following an incubation of 24 h, cells were trypsinized and washed with cold phosphate-buffered saline (PBS); the number of apoptotic cells was determined using the annexin V (AV)-FITC/PI apoptosis assay kit (Miltenyi Biotech, Auburn, CA). A minimum of 1×10^4 events was recorded using FACS Canto™ II system (BD, San Jose, CA). Fluorescence intensities for both AF and PI were obtained and analyzed in FACSDiva software (BD Biosciences). Double positive (AF and PI) or single

positive (AF) were set as apoptotic cells. For the cell cycle analysis, the Cell CycleTEST™ plus DNA reagent kit BD (San Jose, CA) was used. A total of 1×10^4 events were captured and histograms of PI fluorescence intensity were used to determine the distribution of cells in three major phases within the cycle (G1 vs. S vs. G2/M). The percentage of cells in each cycle was determined using the ModFit LT 3.2, Verity Software House (Topsham, ME). All experiments were run in triplicate.

Mice strain and housing conditions

The protocol was approved by the Institutional Committee for laboratory animal welfare of the Escuela de Medicina y Ciencias de la Salud at Tecnológico de Monterrey (approval 2010-011, September 07, 2010), and the animals were treated under institutional guidelines for the care and use of animals. Male C.B-17/IcrHsd-Prkdc SCID mice, 6–8 weeks old, were obtained from Harlan Laboratories Inc., (Indianapolis, IN) and housed at sterile conditions in the clean room conditioned with positive pressure air flow at the vivarium of the Escuela de Medicina y Ciencias de la Salud at Tecnológico de Monterrey. Environmental conditions were: 21°C ± 2°C and 55% ± 15% relative humidity and light/dark cycles of 12 h. Mice were held individually in sterile ventilated cages (Tecniplast, Italy). Before the experiments, mice were kept under standard animal housing conditions for 2 weeks with free access to sterile water and food (Global 19% Protein Extruded Rodent Diet, Teklad Diets, Madison, WI). Sterile water was supplemented with trimethoprim and sulfamethoxazole as a prophylactic antibiotic as part of the protocol for avoiding opportunistic infections. At the end of the experimental protocol, animals used as negative controls underwent anesthesia and euthanasia with a mixture of Ketamine (100 mg/mL, Laboratorios Aranda, Qro, Mexico) and Xylacine (20 mg/mL, 25 mL Laboratorios Aranda, Qro, Mexico). The doses were 200 mg of ketamine and 16 mg of xylacine per kg of body weight administered into the anterolateral region of any caudal limb muscle, followed by cervical dislocation once the mice presented absence of motor reflexes and periorbital signs.

Xenografting model and treatment

To generate the animal model, a suspension of lymphoma cells (1×10^7 cells suspended in 300 µL of

media) was administrated intraperitoneally as previously described by Schimdt et al.[20]. Cells used for injection were maintained in log phase with viability around 98%. The animal model for lymphoma was characterized by histopathological analysis using hematoxylin and eosin staining, along with immunohistochemistry for CD79a and BCL-6 to confirm the tumor type. Following the characterizations, mice underwent treatment and were divided into four groups: The placebo group which received only PBS, one group receiving the FF (15 mg/mouse/day, using the rodent pellets as the vehicle), another group receiving the BBE (20 mg/mouse/day, using the rodent pellets as the vehicle), and the control group which received CTX (i.p. At 150 mg/kg of body weight); the administration schedule was one injection every 3 days for a total of 7 applications[21]. Body weight and mice behavior were monitored during the experiment. After animal death, tumors were extracted and underwent pathological analysis. The survival time was registered since tumor implantation.

Statistical analysis

Matlab® 2010 software was used for the statistical analysis. All *in vitro* assays were performed in triplicate reporting data as a mean and standard deviation. IC_{50} was calculated by a variation of the Hill equation[22]. For apoptosis and cell cycle, statistical differences were assessed using the two-tailed *t*-test to test the null hypothesis of no difference between populations. The differences between tumor volumes and weights were evaluated through ANOVA analyses. In the animal model, statistical differences between treatments from Kaplan–Meier curves were evaluated using the log-rank test (SPSS Statistics 19 was used in this test). $p \leq 0.05$ was considered significant.

RESULTS

Characterizations of flavonoids content

The chromatographic analysis of FF identified three glycosylated flavonoids: myricetin 3-O-glucoside at a concentration of 11.29 mg/g, while quercetin 3-O galactoside and kaempferol 3-O glucoside were present at concentrations of 97.68 and 1.00 mg/g, respectively[19,23].

Cytotoxic properties

BBE showed a dose-dependent cytotoxic effect on a culture of OCI-Ly7 with a calculated IC_{50} of 0.131 ± 0.004 mg/mL. A similar response was observed in cells exposed to the FF (IC_{50}: 0.154 ± 0.007 mg/mL), while the IC_{50} for CTX was 0.745 ± 0.035 mg/mL, showing that both fractions (BBE and FF) were more cytotoxic than CTX ($p < 0.0005$) (Fig. 1).

Specificity for tumor cells is one of the most-wanted characteristics in drugs for cancer therapy; therefore, we exposed non-cancer cells (NIH-3T3 and VERO) to our extracts. For this purpose, we used a high concentration of BBE (1 mg/mL), and the percentages of living cells were 95.31% ± 6.38% and 90.17% ± 7.33% in NIH-3T3 and VERO cells, respectively, both being highly different to the viability found in OCI-Ly7 cells (1.17% ± 0.18). Regarding the FF, with a final concentration of 1.32 mg/mL, the viabilities observed were 65.74% ± 10.25% and 80.42% ± 4.36% in NIH-3T3 and VERO cells, respectively. These results showed that the tumor cells tend to be significantly more sensitive to the fractions obtained from black bean than non-cancer cells.

Flow cytometry assay

After exposing the tumor cells to BBE using the IC_{50} obtained in cytotoxicity assays, we observed that 41.6% of cells were positive for both AV, a molecule expressed in cell surface during early stages of apoptosis, and propidium iodide (PI), a molecule that binds DNA in cells with damaged membranes or late stage of apoptosis (Fig. 2B). While 44.6% were positive for both markers in the cells exposed to FF (Fig. 2C), only 7.4% of cells were AV and PI positive when they were exposed to the vehicle (PBS) (Fig. 2A) ($p < 0.0001$ and $p < 0.001$, respectively) almost similar was observed in cells exposed to cyclophosphamide (CTX) (Fig. 2D).

Cell cycle analysis

Regarding the cell cycle stages, the normal distribution of log phase OCI-Ly7 cells (treated with vehicle) was 24.51% ± 0.63% in G1, 65.16% ± 0.88% in S,

Figure 1. Cell viability assays. OCI-Ly7 cells were exposed to several concentrations of either black beans hulls extract (BBE), its flavonoid fraction (FF), cyclophosphamide (CTX), or Vehicle (DMSO) for 24 h.
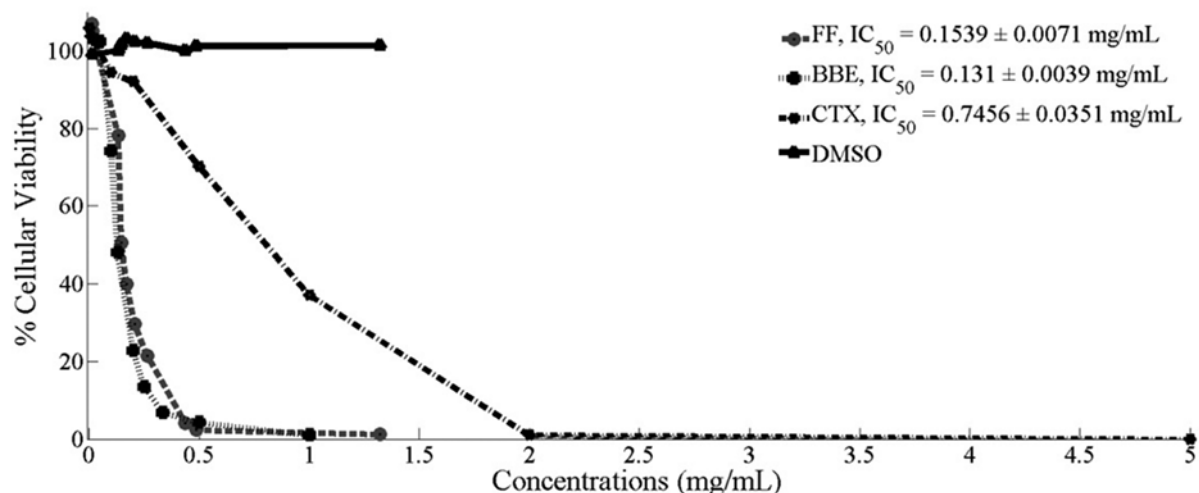
and 10.33% ± 1.23% in G2/M (Fig. 2E). The addition of BBE to the cell culture changed the distribution to 21.4% ± 0.91% in G1, 77.15% ± 0.93% in S, and 1.53% ± 0.88% in G2/M (Fig. 2F), while in the cells exposed to FF, the distribution was 21.5% ± 0.82% in G1, 77.91% ± 0.87% in S, and 0.59% ± 0.27% in G2/M (Fig. 2G). Interestingly, the treatment with CTX resulted in similar distributions in G1 and S phases (21.69% ± 0.76% and 62.62% ± 0.52%, respectively) compared to control, but more cells (15.4% ± 0.43%) were arrested in G2/M (Fig. 2H).

In vivo analysis of cytotoxicity

All inoculated mice presented characteristics of tumor growth in nodes compared to non-inoculated mice. Histopathological analysis confirmed that neoplastic tissue corresponded to diffuse proliferation of large lymphocytes showing positive nuclear expression of BCL-6 and positive membrane expression for CD79a (Fig. 3A-C). Interestingly, oral administration of either BBE or FF increased the SR compared with the group of mice treated with placebo (p=0.056); surprisingly, the response was similar to the group treated with CTX (Fig. 4). The SR for the FF group was 33.3 days (confidence interval [CI]: 95%: 28.1–38.5), while for BBE and CTX were 33.8 (CI 95%: 28.3–39.5) and 33.1 (CI 95%: 28.4–37.8) days, respectively. The SR in the placebo group was 26.7 days (CI 95%: 23.7–29.8).

DISCUSSION

DLBCL represents the most common subtype of NHL, accounting for 30–40% of all newly diagnosed cases. In the United States, 8000 deaths per year are caused by this disease. The current therapeutic schemes for NHL include the administration of doxorubicin and vincristine, two molecules obtained from natural sources.

In this respect, 60% of all approved anticancer drugs in the USA during 2010, were obtained from natural sources[24]. It is well-known that natural dietary compounds have chemoprevention effects by blocking, inhibiting, reversing, or retarding the process of carcinogenesis. In this context, BBE has been widely used for its antineoplastic properties[8]. The phytochemical content (tannins, flavonoids, saponins, and phytosterols) of this extract has been linked to the inhibition of proliferation of cancer cells and the scavenging of free radicals[7,9].

In this study, chromatographic analysis of the FF obtained from the BBE of P. vulgaris L., identified three molecules (myricetin 3-O glucoside, quercetin 3-O glucoside, and kaempferol 3-O glucoside). In this context, Cheng et al. described an FF from Gynostemma pentaphyllum composed of 8 molecules, two of them quercetin derivatives, and showed a cytotoxic effect over prostate cancer cell line (PC-3)[15]. Furthermore, Jeganathan et al. reported the same three flavonols contained in Camellia sinensis L. (Myricetin 3-O glucoside, Quercetin 3-O glucoside, and Kaempferol 3-O glucoside), in a widely used

Figure 2. Flow cytometry analysis. Apoptosis results (Annexin-V/PI) of cells exposed to vehicle (A), to black beans hulls extract (BBE) (B), to flavonoid fraction (FF) (C), and cyclophosphamide (CTX). In addition, DNA content was analyzed in cells treated with vehicle (E), BBE (F), FF (G), and CTX (H).
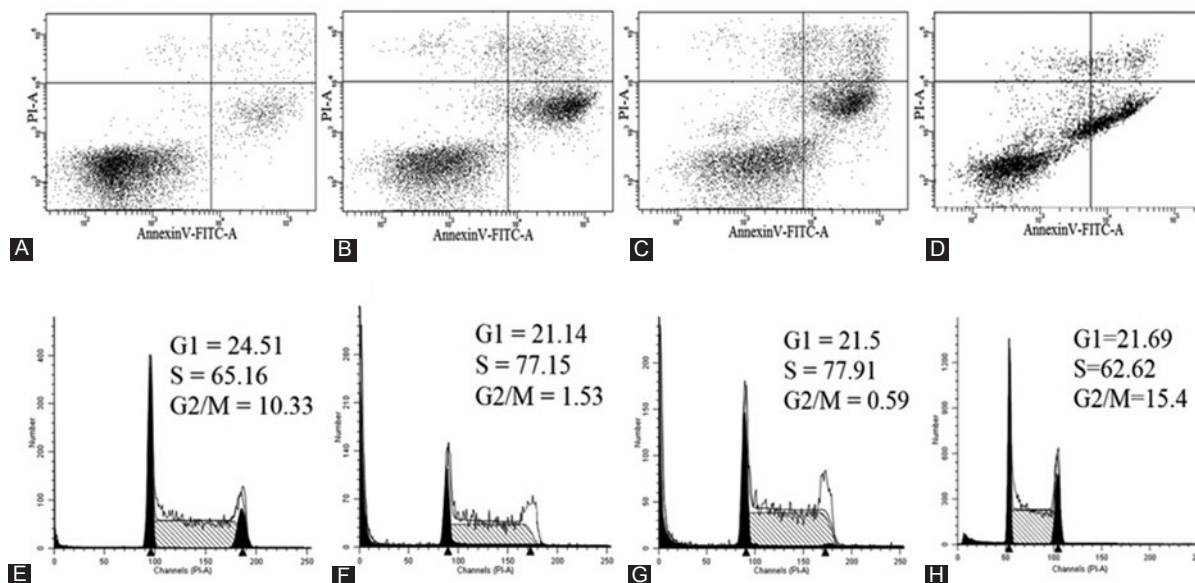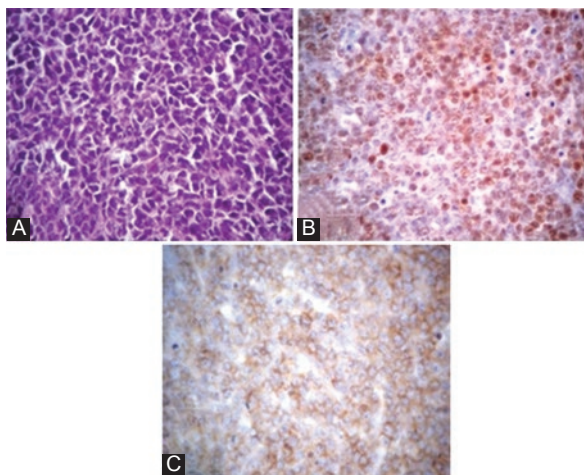

Figure 3. Characterization of the *in vivo* model. (A) Hematoxylin and Eosin staining of the tumor excised from mice injected with the OCI-Ly7 cell suspension resulted in a classical aggressive non-Hodgkin's lymphoma cell population. In addition, both nuclear expression of positive BCL-6 (B) and membrane expression of CD79a (C) were detected.

tea plant (*C. sinensis* L.) from Sri Lanka but at different concentrations (0.94 mg/g, 1.5 mg/g, and 1.31 mg/g, respectively)[25]; this plant has been reported to have antioxidant, antimicrobial, anticancer, anti-atherosclerotic, and anti-proliferative properties.

In this study, we describe the anti-proliferative effect of both BBE and FF over a lymphoma cell line (OCI-Ly7) in a dose-dependent manner. In addition, we found that both FF and BBE were capable of inducing apoptosis and arresting the cell cycle in S-phase, which also suggests that the glycosylated flavonoids from BBE were responsible for its effect. In terms of selectivity, our experiments demonstrated that the tumor cell line was more sensitive to both extracts than non-cancer cells (NIH-3T3 and VERO), which can be explained by the growth rate of tumor cells compared with non-cancer cells.

The results of cytotoxicity assays using the BBE and FF were similar to other studies. For instance, the FF obtained from *Rhus verniciflua* Stokes induced apoptosis in B-cell lymphoma (BJAB) and T-cell lymphoma (Jurkat)[26]. A similar apoptotic effect and arrest of G2/M phase in human B lymphoma cells (BJAB) were observed by other researchers using the same component at 100 µg/mL[27].

Regarding the cell cycle arrest, our results suggest that both treatments (BBE and FF) blocked the entrance to the G2/M phase since an increase in duration of the S phase was observed. In this context, Cheng et al. published that both flavonoids and saponins obtained from *Gynostemma pentaphyllum* were capable of inducing apoptosis and arresting in S and G2/M phases in prostate cancer cells (PC-3)[15]. In addition, they found that both fractions modulated the expression of cyclin A, a molecule part of the complex needed for this transition

Figure 4. Kaplan–Meier lymphoma survival curves. Survival population was plotted since day 20 after inoculation and 1 day before the first subject decease was reported. Statistical differences between groups were calculated using chi-square test according to the log-rank test. Black beans hulls extract (BBE) group and flavonoid fraction (FF) group were not statistically different from CTX group, with p values of 0.42 and 0.8, respectively. All treated groups, BBE, FF, and CTX, obtained a trend p = 0.056.
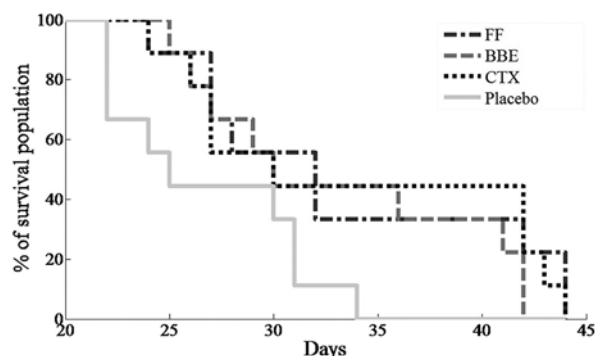
(cyclin-A/cdk2)[28] and the expression of caspase-3, which is an important key in the apoptotic process.

To test the effect of these fractions *in vivo*, we developed a xenografting model and assessed it using histological analysis. The results of biopsies taken from the grafted mice showed positive cells for CD79a and BCL-6 (two commonly used markers for DLBCL). With the animal model well characterized, the group of mice under placebo treatment died around day 26.7, while the group under CTX treatment (150 mg/kg i.p.) died days later (33.1 days), which showed the expected outcome. Surprisingly, the cytotoxic effect observed *in vitro* was observed *in vivo* as well, since the groups of mice receiving BBE or FF survived around 33.3 and 33.8 days, respectively.

In addition, oral administration through food is an advantage and a straightforward way of treatment using these fractions. A similar observation was reported using either alkaloid or terpenoid fractions administered in the food of mice grafted with either lymphoma (DAL) or lung cancer cells (L-929); in this study, the fractions increased the SR around 35.64 ± 1.05 days and a reduction in tumor volume were observed[29].

Data showed in our study addresses new questions about the components in both BBE and FF, specifically, the effect of the sugar residue on the glycoside forms isolated from this plant, and the outcome of different concentrations and/or ratios among the flavonoids.

Finally, in this study, we found that both BBE and FF from *P. vulgaris* L. Had cytotoxic properties comparable to cyclophosphamide, a widely-used molecule for cancer therapy. In addition, these extracts seemed to be specific for tumor cells, since they were almost innocuous to non-cancer cells (NIH-3T3 and VERO cells). Another important observation was that the administration of the extracts in a xenografting model of lymphoma increased the SR, similar to that was observed with cyclophosphamide treatment. Further studies are needed to identify specific components of the fractions that exhibited the observed cytotoxic properties. For example, Oncamex, the myricetin-derived flavonoid, has shown antitumor activity in a preclinical model[30].

ACKNOWLEDGMENTS

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