Original Article

Evaluation of Anti-cancer Activities of Cranberries Juice Concentrate in Osteosarcoma Cell Lines (MG-63)

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ABSTRACT

Aim/Background: Osteosarcoma is one of the prevalent cancers occurring mostly in adolescents and has a high risk of malignancy. With complications involved in the current treatment strategies, alternates including the use of phytochemicals have gained fame. Cranberries are known for their exceptional health benefits and have been explored for their effective activities in various cancers. The current study aimed at evaluating the anti-cancer properties of cranberry juice concentrate (CJC) on MG-63 cell line for human osteosarcoma, by investigating its apoptotic activity through changes in cell viability and mitochondrial membrane potential. **Materials and Methods:** Cranberry juice concentrate was obtained by pulverization and lyophilization. The MG-63 cells were treated with 12.5-800 μg/mL of the CJC and incubated for 24, 48, and 72 hr. The percentage cell viability and IC_{50} values were obtained. The mitochondrial membrane potential and nuclear changes were examined. The induction of apoptosis was studied by flow cytometer using BD cell Quest 7.5.3 software. GraphPad Prism was used for statistical analysis with significant p-value at <0.05. **Results:** The IC_{50} values obtained for CJC were 847.9, 637.4, and 440.6 μg/mL for 24, 48, and 72 hr respectively. Change in the mitochondrial membrane potential and nuclear morphology was observed following incubation with CJC. Flow cytometric analysis shows cells detected at early and late apoptoic stages after treatment with CJC. **Conclusion:** Our result suggests that CJC has significant effects on MG-63 osteosarcoma cells and can be considered to supplement conventional therapeutic strategies.

Keywords: Apoptosis, Cranberry, MG-63, Osteosarcoma, Phytochemicals.

INTRODUCTION

Osteosarcoma is a common type of pediatric bone cancer with a high metastatic rate as well as drug resistance.[1] The American Cancer Society (ACS) statistics and Surveillance, Epidemiology, and End Results Program (SEER) put the diagnosis rate of osteosarcoma in teens and children at 56%. In 2021, 3,610 new cases and 2,060 deaths due to bone and joint cancer were estimated. The five-year average survival rate in heavily metastasized cases of osteosarcoma drops from 60% to 27%.[2] Cancer of bone mesenchymal cells, hence known as sarcoma,[3] often metastasizes to various body parts, predominantly to the lungs and blood. In terms of treatment, surgical amputation was the first line of therapeutic setting before the 1970s, followed by chemotherapy.[4] The National Cancer Institute (NCI) lists surgery, chemotherapy, radiation therapy, the use of samarium, and targeted therapy as standard routes of treatment, nevertheless coming with side effects and complications.

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Conventionally, DNA damage of cancer cells brought by doxorubicin and apoptosis induced through DNA adducts by cisplatin are useful in chemotherapeutic treatments. Other drugs and combinations are also used and are being studied for clinical intervention.

Chemotherapeutic drugs are added to treatment complications due to side effects and increased recurrence rates while developing drug resistance,[1] which are prompting researchers to develop alternate therapies.[5] As traditional Indian and Chinese medicine are not new to the use of natural compounds for treatment, many researchers worldwide are directing their strategies to compounds and extracts from natural sources.[6-7] With more than half of the anti-cancer drugs originating from natural products the phytochemicals present in these, exhibit an eclectic array of activities right from anti-microbial to anti-cancer and neuroprotective effects.[7-8] Of anti-cancer effects, studies ranging from *in-vitro* assays to clinical trials have shown radical scavenging, anti-tumor, angiogenesis, anti-proliferative and apoptotic activities.[7]

Looking at osteosarcoma alone, studies reporting the anti-cancer activities of plant-derived metabolites show various mechanisms and pathways involved in inducing these effects. A study recently showed that caspase 3/9 directed apoptosis and mitochondrial dysfunction in osteosarcoma cell lines through the activity of corosolic acid. Interestingly, it also prevents metastasis to the lungs by acting on signal transducer and activator of transcription -3 (STAT3) expression and exhibiting, tumor suppression and immune-suppression activities in murine models.[9] Phytochemicals are also preferred in combination therapies along with conventional drugs and treatment strategies as they amplify their therapeutic actions and act on various apoptotic pathways.[10] Recommendations for a change in dietary habits are made for a cost-effective chemoprevention strategy. These are encouraged in clinical trials as well, to evade drug toxicity and the subsequent complications of drug resistance.[11]

Of late, berries are famed for their anti-oxidant and anti-inflammatory properties and vibrant health benefits. Rich with flavonoids and phenols, they have attracted the attention of those in search of potent anti-cancer phytochemicals. The anti-cancer properties of cranberries encompass a reduction in cancer cell proliferation, viability, inflammation, and oxidative stress and extend to the modulation of signal transduction pathways, adhesion, and migration.[12] A study performed on cranberry juice extract revealed polyphenols to be the major phytochemicals followed by proanthocyanidins, flavonoids, anthocyanins, and organic acids and has shown anti-cancer effects in urinary bladder cancer.[13]

Many of these extracts impart certain activities in cancer cells and tumors, which may be attributed to specific phytochemical compounds present. For instance, of the major triterpenoids found across all cranberry species, ursolic acid imparts anti-cancer activities by inhibiting cancer cell proliferation, migration, and metastasis.[14] A recent study showed cell cycle arrest and induction of apoptosis by ursolic acid in human osteosarcoma cells.[15] Various studies on cranberry extracts have shown their potential ameliorating activities in many cancer cells and tumors. These extracts have the added advantage of acting on multiple apoptotic pathways, thereby effectively inducing the inhibition in cancer cells.[16] With a plethora of phytochemicals found in cranberries,[8] and the wide array of functions displayed by its various phytochemicals,[15] there seems to be a need to establish studies exploring more uses for osteosarcoma treatment strategies. Although studies involving certain compounds found in phytochemical fractions of cranberries have been reported in osteosarcoma,[17] the activity of cranberry juice concentrate (CJC) as a whole, in the same has not been reported to the best of our knowledge. In this study, we concentrated the juice extracted from cranberries after pulverizing them and evaluated its anti-cancer activities in the human osteosarcoma cell line, MG-63 by investigating its effects on the viability of the cancer cells, changes in membrane potential, and induction of apoptosis.

MATERIALS AND METHODS

Materials

Frozen cranberries were procured from an authorized distributor (Peony Food Products, Pune, India), Dulbecco`s Modified Eagle Media (DMEM) with low glucose, Antibiotic – Antimycotic (100X) solution, and Fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, MA, USA. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and DAPI (4',6-diamidino-2-phenylindole) were purchased from HiMedia Laboratories, India. Dimethyl sulfoxide (DMSO) and paraformaldehyde were procured from Qualigens, Mumbai, India. Annexin V-FITC Apoptosis Detection Kit was purchased from R&D Systems, Inc. USA.

Methods

Preparations of cranberries juice concentrate (CJC)

The procured cranberries were washed with double distilled water and stored at -80°C until use. The CJC was obtained by pulverizing the cranberries in a commercial mixer to form a homogenous puree and subjecting it to rotary evaporation (R-210, Buchi, Switzerland) under reduced pressure at 55 ± 2°C for 2 hr. The obtained CJC was filtered through Whatman filter paper and subjected to lyophilization to get the dry concentrate. Frozen CJC (-20°C) was pre-frozen at -80°C for 24 hr in an ultra-low-temperature freezer (U410-86, New Brunswick, Eppendorf India Private Ltd., Chennai, India). Christ freeze dryer (Alpha 1–2 LD plus, Martin Christ Freeze Dryers, Germany) was used to lyophilize at two phases (main drying - 0.011 mbar pressure and -60°C for 24 hr, and final drying -0.0054 mbar pressure and -65°C for 24 hr).

Cell Culture

Human osteosarcoma cancer (MG-63) cell lines were procured from National Center for Cell Science (NCCS), Pune, India,were maintained in a CO_2 incubator (New Brunswick galaxy 170R, Eppendorf India Private Ltd., India) at 37°C in a humidified environment of 5% CO_2. Dulbecco's Modified Eagle Medium (DMEM) was used as the standard culture medium along with 10% heat-inactivated fetal calf serum (FBS) and 1% Antibiotic – Antimycotic 100X solution.

In vitro Cell Viability Assay

Trypsinized MG-63 cells seeded in a 96-well plate (5×10^3 cells per well) and incubated overnight in a CO_2 incubator at 37°C in a humified environment of 5% CO_2 were treated with CJC at a concentration range of 12.5 - 800 µg/mL and incubated for 24, 48, and 72 hr. The untreated cells with culture medium were employed as control. After incubation, the culture media from each well was discarded and washed twice with phosphate-buffered saline (PBS). Following this, 20 µL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the cells and incubated at 37°C for 4 hr, in dark. After aspirating this, 100 µL of dimethyl sulfoxide (DMSO) was added and agitated to dissolve the insoluble formazan crystals. The absorbance was recorded at 570 nm using a microplate reader (Lisa plus microplate reader).[18] The experiment was repeated three times mean ± standard deviation (SD) of the results was recorded. The percentage of cell viability was determined by using the following formula. Further, the Half-maximal inhibitory concentration (IC_{50}) value was calculated by using Graph Pad Prism software (version 5; GraphPad Software, San Diego, CA).

Surviving cells (%) = Mean Optical Density of test compound /Mean Optical Density of Negative control ×100

Determination of mitochondrial membrane potential

MG-63 cells grown overnight on coverslips (HiMedia) inside a 24-well plate at a density of 5×10^4 cells per well) were treated with the obtained IC_{50} concentrations (at 24, 48, and 72 hr) of CJC from cell viability assay for 48 hr and followed up with a protocol as mentioned by Dai J-Q *et al.*,[19] with little modification. Following incubation, PBS-washed cells were fixed with 4% paraformaldehyde for 30 min. These were then subjected to staining with Rhodamine-123 (Rh-123) dye for 30 min and fluorescence microscopy at 20X magnification (Olympus BX41, Olympus, Tokyo, Japan) and visualized using RES®CapturePro software (Jena, Germany). The untreated cells were used as control.

Assessment of nuclear changes by DAPI staining

MG-63 cells cultured and fixed in a similar way as mentioned previously were incubated with 20 µL of DAPI (0.1 µg/mL in PBS) in dark at room temperature for 5 min. After a PBS wash, the cells were observed under a fluorescence microscope at 20X magnification and visualized using Pro RES® Capture Pro software (Jena, Germany).[20]

Determination of Apoptosis by Flow Cytometry

The MG-63 cells seeded (5×10^5cells per well) in a 24-well plate were treated with 200 µg/mL of CJC for 48 hr. The PBS-washed cells were centrifuged (ROTEK Laboratory centrifuge) for 5 min at 500 x g at 4°C. The pellet was collected and resuspended in an ice-cold 1X binding buffer at 1×10^5 cells per mL. To this, 1 µL of Annexin V-FITC solution and 5 µL propidium iodide were added while maintaining the cold temperature on ice. After incubation for 15 min in the dark, 400 µL of ice-cold 1X binding buffer was added and mixed gently. The cells were analyzed by flow cytometer (BD FACSCalibur™) within 30 min and analyzed with BD CellQuest 7.5.3 software.[21]

Statistical Analysis

The data for each experiment in triplicates was expressed as ± standard error of the mean (SEM). Statistical analysis of data was done by using

Graph Pad Prism software (Version 5; GraphPad Software Inc., CA, USA). "One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test for MTT analysis and Bonferroni test for apoptosis assessment were used to compare the significance difference between the groups. Significant p values (significant probability value) at less than 0.05.

RESULTS

CJC Inhibited the Proliferation of MG-63 cells

The cytotoxicity of CJC in MG-63 cells was investigated using MTT. Figure 1 depicts the percentage of viable MG-63 cells after treatment with different concentrations of CJC for various durations displaying a concentration-dependent cytotoxicity. Further, the cytotoxicity increased in a time-dependent manner with 72 hr treatment showing slightly higher inhibition at all the tested concentrations as compared to 24 and 48 hr. The IC_{50} values of CJC were 847.9, 637.4, and 440.6 μg/mL respectively for 24, 48, and 72 hr incubation period.

CJC Decreased the Mitochondrial Membrane Potential (MMP)

Untreated MG-63 cells and CJC treated cells at different concentrations for 8 hr as visualized by fluorescence microscopy are shown in Figure 2. The cells in the untreated group (Figure 2, A) showed a strong green fluorescence intensity, whereas cells treated with different concentrations of CJC (Figure 2, B-D) presented a weak green fluorescence

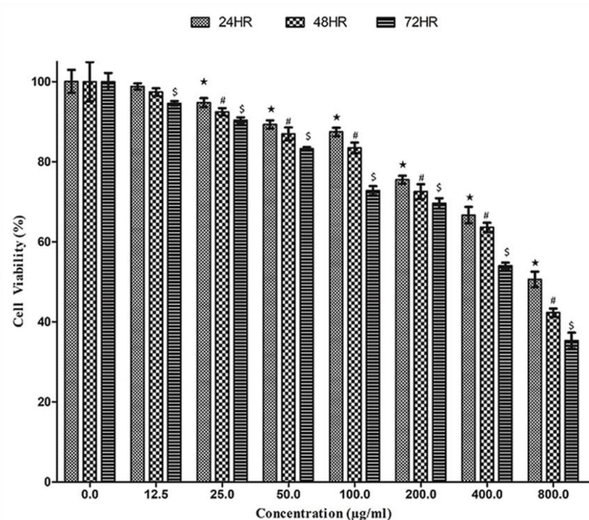

Figure 1: The *in vitro* cytotoxicity effect of CJC on MG-63 cancer cell line following 24, 48 and 72 hr incubation.

The data is expressed as mean ± SEM (n = 3). The significance difference indicated as p < 0.05 between untreated control cells vs. *cells treated with CJC for 24 hr; # cells treated with CJC for 48 hr and $ cells treated with CJC for 48 hr.

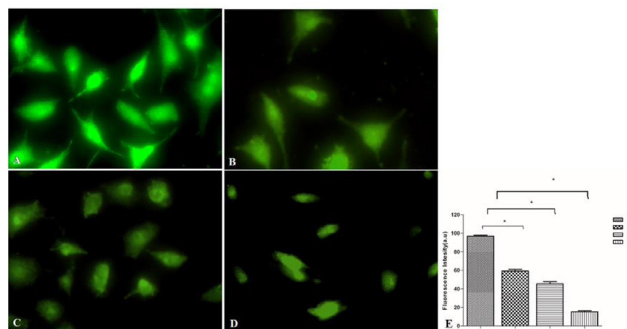

Figure 2: Analysis of mitochondrial membrane potential in MG-63 cells after treatment with CJC by fluorescence microscope.

The images above represent: (A) untreated control cells, (B) cells treated with 440.60 μg/mL of CJC, (C) cells treated with 637.46 μg/mL of CJC, and (D) cells treated with 847.90 μg/mL of CJC. (E) Percent fluorescent intensity of different groups. Data are expressed as mean ± S.D. *p< 0.05 vs. untreated control.

intensity suggesting loss in mitochondrial membrane potential. Furthermore, the fluorescence intensity decreased with increasing CJC concentration. The quantification of fluorescence intensity demonstrated that the changes in mitochondrial membrane potential induced by CJC were significant (Figure 2E).

CJC Induced Changes In Nuclear Morphology

Fluorescence microscopic images depicting the apoptotic nuclear changes in MG-63 cancer cells after treatment with CJC for 24 hr are shown in Figure 3. The untreated cells with normal intact nuclei showed weak blue staining, while the CJC treated cells displayed small nuclei with bright chromatin condensation. In addition, blebbing of cell membrane, nuclear fragmentation, and formation of apoptotic bodies were observed in the cells treated with high concentrations of CJC. These results indicate that CJC induced apoptosis in MG-63 cells.

CJC Induced Apoptotic Cell Death

The flow cytometric analysis of MG-63 cells following the treatment with CJC was shown in Figure 4. The untreated cells in the control sample were mostly live (99.10%) with only 0.50% and 0.39% of the cells in the late apoptotic and dead/necrotic phases respectively. However, post-treatment with CJC, there was a decreasing percentage of viable cells, while that of early and late apoptotic cells increased. The percentage of viable cells decreased from 99.10% of control cells to 74.70% of CJC treated groups. 19.0% of the CJC treated cells were in early apoptotic phase and 5.82% of the cells were in the late apoptotic phase. However, a neglectable percentage of untreated cells were in the apoptotic phases. A similar observation was made for cells in necrotic stage in the treated and untreated

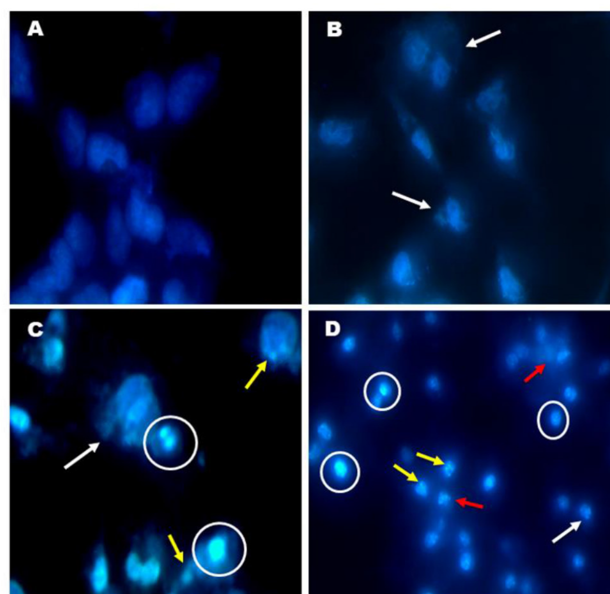

Figure 3: Apoptotic nuclear changes detected in MG-63 cancer cells treated with different concentrations of CJC by DAPI staining.

(A) untreated control cells, (B) cells treated with 440.6 µg/mL of CJC, (C) cells treated with 637.46 µg/mL of CJC, and (D) cells treated with 847.9µg/mL of CJC The white arrows show membrane blebbing, yellow arrows represent apoptotic bodies, red arrows show nuclear fragmentation and white circles represent the chromatin condensation.

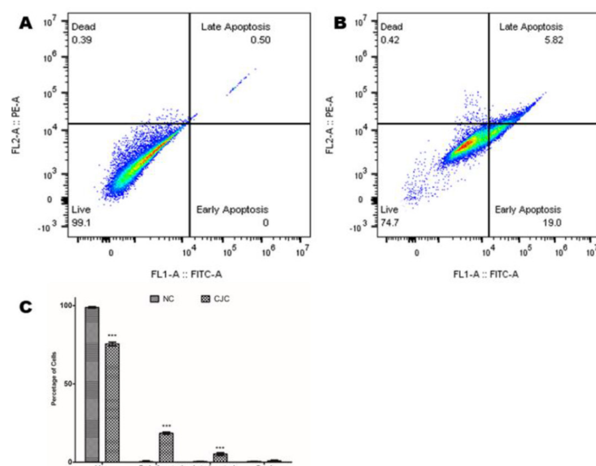

Figure 4: Assessment of apoptosis in MG-63 cells by flow cytometry.

The images represent (A) Untreated cells and (B) cells treated with 200 µg/mL for 48 hr. (C) Shows the percentage of cells undergoing apoptosis and necrosis in untreated and CJC treated cells after 48 hr.

cells. These findings suggest that a significantly ($p < 0.05$) higher proportion of cells were detected in the early and late apoptotic stages after treatment with CJC (Figure 4, C).

DISCUSSION

The sarcoma of bones is generally treated with chemotherapy,[4] and other strategies like surgeries and radiation therapies are often faced with complications (NCI data). National Cancer Institute's data lists infertility, mood swings, and complications in learning and memory along with risks of developing other cancers like breast or leukemia as some of the side effects. In an alternate strategy to tackle such complications, researchers are focusing on phytochemicals having anti-cancer and chemo-sensitizing properties.[22] Previously reported studies on plant-based extracts have shown the presence of high-performance bioactive compounds like phenols and flavonoids in cranberries.[8] Focusing on their anti-oxidant activities, these compounds have also been explored for their anti-cancer properties.

Cranberries, belonging to the genus *Vaccinium* come in various species depending on their geographical distribution and have been equally praised for their health benefits and anti-oxidant activities. Thanks to their phytochemical profile with flavonoids, terpenoids, catechins, and organic acids as they have found a wider application in cancer treatment studies as well.[23] Cranberries have rich flavonoids like quercetin that inhibit proliferation and tumor growth,[24] making them a suitable candidate for anti-cancer studies. Specific phytochemical compounds of cranberry contributing to such activities in different cancers have also been determined using techniques like Mass spectroscopy.[13] For instance, ursolic acid inhibits the growth of MG-63 cells and arrests the cell cycle at the phases G2/M and sub-G1. It collapses the mitochondrial membrane permeability and activates caspases to induce apoptosis without inducing toxicity in non-cancer cells.[17] Studies evaluating subjects' urine and plasma content after cranberry consumption also indicate the presence of flavonoids and phenolic acids, that improve anti-oxidant performance.[12] Since the effects of whole CJC have not been priorly evaluated in osteosarcoma (MG-63) cells, we attempt to do so, by extracting the juice from procured cranberries.

Evaluation of MG-63 cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), after exposing them to varying concentrations of the cranberry juice extract shows a concentration-dependent and time-dependent inhibition. This cell viability evaluation is based on the fact that metabolically active cells break down these tetrazolium salts into formazan crystals.[12] To facilitate the optical density measurements, these crystals are solubilized using Dimethyl Sulfoxide (DMSO). Half-maximal inhibitory concentration (IC_{50}) determines the appropriate concentration required to kill about 50% of cells. Considering these concentrations obtained through IC_{50} for CJC treated MG-63 cells at 24, 48,

and 72 hr respectively, further experiments were carried out with 847.9 **μg/mL**, 637.46 μg/mL, and 440.6 μg/mL. Other studies evaluating inhibitory properties of cranberry extracts in other cancers have reported IC_{50} values at 40-50 μg/mL (organic extract) in oral cancer cells like CAL27 and 70 μg/mL (organic extract) in SCC25 cells, while other studies showed this at 150 μg/mL (cranberry juice extract),and 50-180 μg/mL (whole cranberry extract). 38 to 113 μg/mL cranberry juice extract inhibited 50% of LS-513 colon cancer cells and 10 mg/mL of the same showed LS-513 stomach cancer cells inhibition.[12] Another study showed IC_{50} values of cranberry juice extracts on PC-3 (prostatic adenocarcinoma) cells at 25 μl/mL and 46 μl/mL in MDA-MB-231 (mammary gland adenocarcinoma) cells.[25] With all the varying extraction methods and units of quantifications, it is somewhat difficult to effectively compare these results.

Triterpenoids like ursolic acid have shown activities like DNA fragmentation with enhanced cytochrome c release due to a change in the mitochondrial membrane potential (ΔΨM), leading to apoptosis.[24] In this study, we assessed the early stage of apoptosis induced by CJC on the ΔΨM of MG-63 cells using fluorescent microscopy. Rhodamine 123, a cationic fluorescent dye used in this method generally accumulates the mitochondria of intact cells with highly polarized mitochondria which brightly stains the control cells. In CJC treated MG-63 cells, the mitochondria are de-polarized leading to leakage of this dye into the cytosol. This opening of mitochondria leads to the release of pro-apoptotic factors triggering the apoptotic pathway.[26] The cells treated with an increasing concentration of CJC at 440.60 μg/mL, 637.46 μg/mL, and 847.90 μg/mL show a significantly decreasing pattern of the fluorescent intensity imparted by Rhodamine 123.

Further assessment of apoptotic changes to the nuclei was done using 4',6-diamidino-2-phenylindole (DAPI). The dye binds to adenosine-thymidine regions in the nuclear DNA and enables its visualization using a fluorescent microscope. The concentration-dependent investigations of CJC on osteosarcoma cells done in this study show that it induces blebbing, formation of apoptotic bodies, nuclear fragmentation as well as condensation. The condensation and apoptotic bodies observed could be as a result of a change in membrane potential, caspase-3 activation, and the subsequent apoptotic pathway,[27] as has been previously observed in the activities of ursolic acid.[24]

Flow cytometry was used to quantify apoptosis induced in CJC treated MG-63 cells by staining the damaged cells with FITC. In these cells, the phosphatidylserine from the inner membrane is exposed and allows its interaction with Annexin V-FITC indicating the early apoptosis.[19] Further damage opens the door for propidium iodide to enter the cells and stain the DNA which indicates late apoptosis or necrosis.[28] The four quadrants in the graph indicate the percentage of cells alive, the ones that are in the early and late apoptotic phase along with the ones entering a necrotic stage. This quantification is seen in CJC treated MG-63 cells at 19.0% early apoptotic, 5.82% late apoptotic, and less than 1% necrotic cells, indicating a beginning stage of apoptosis-induced cell death.

With about ten thousand phytochemicals,[23] to choose from, cranberries provide a wider pallet for experimentation for cancer treatment. These phytochemicals induce apoptosis in cancer cells through various pathways, thereby supplementing the conventional therapeutic strategies and helping overcome drug resistance.[1] Their anti-cancer application as seen in other studies,[23,29] gives a ray of hope for osteosarcoma applications as well.

CONCLUSION

Our study involving juice concentrates extracted from cranberries shows that it has significant effects on osteosarcoma cells. The inhibitory effect of cranberry juice concentrate can be seen with the IC_{50} values (847.9 μg/mL, 637.46 μg/mL, and 440.6 μg/mL) derived from the MTT assay at 24, 48, and 72 hr respectively. Staining the CJC treated cells with DAPI showed changes in nuclear morphology including condensation, blebbing, fragmentation, and induction of apoptotic bodies. CJC induces changes in the mitochondrial membrane potential and significant apoptosis in the human osteosarcoma, MG-63 cells. These investigations leave us with the potential anti-cancer activities imparted by phytochemicals cranberry juice concentrate as a whole. Its efficacy, bioavailability, and knowledge of the mechanism of action and pathways involved remain to be explored.

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CONFLICT OF INTEREST

The authors declare no competing or conflict of interests.

ABBREVIATIONS

CJC: Cranberry Juice Concentrate; **ACS:** American Cancer Society; **SEER:** Surveillance, Epidemiology, and End Results Program; **NCI:** National Cancer Institute; **STAT3:** Signal transducer and activator of transcription -3; **DMEM:** Dulbecco`s Modified Eagle Media; **MTT:** (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); **DAPI:** (4',6-diamidino-2-phenylindole); **DMSO:** Dimethyl sulfoxide; **NCCS:** National Center for Cell Science; **FBS:** Fetal Calf Serum; **PBS:** Phosphate-buffered saline; **SD:** Standard deviation; **IC_{50}:** Half-maximal inhibitory concentration; **SEM:** Standard Error of the mean; **MMP:** Mitochondrial Membrane Potential.

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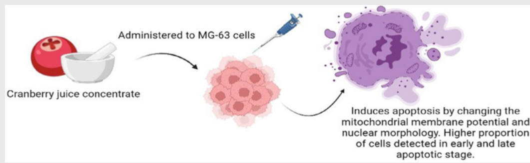
PICTORIAL ABSTRACT



SUMMARY

Various studies on cranberry extracts have shown their potential ameliorating activities in many cancer cells and tumors. These extracts have the added advantage of acting on multiple apoptotic pathways, thereby effectively inducing the inhibition in cancer cells. Although studies involving certain compounds found in phytochemical fractions of cranberries have been reported in osteosarcoma, the activity of cranberry juice concentrate (CJC) as a whole, in the same has not been reported to the best of our knowledge. Our study involving juice concentrates extracted from cranberries shows that it has significant effects on osteosarcoma cells. The investigations leave us with the potential anti-cancer activities imparted by phytochemicals cranberry juice concentrate as a whole.

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