



Black Rice Extract Induced Apoptosis and Cell Cycle Arrest on Human Cancer Cell Lines

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Abstract

The purpose of the research is to evaluate the anti-cancer outcomes of aqueous black rice (BR) extract on human cancer cell lines, HeLa, T47D, and U2OS. To begin the investigation, the total phenol and flavonoid content of the BR extract was determined. Additionally, the antioxidant activity of the extract was determined using the ferric reducing antioxidant power (FRAP), total antioxidant capacity (TAC), and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assays. The cell viability, apoptosis and cell cycle on several human cancer cells were determined after BR extract administration in several doses (BR1; 100mg/ml, BR2; 200mg/ml, BR3; 300mg/ml, BR4; 400mg/ml, and BR5, 500mg/ml) using flow cytometry analysis. According to the analysis, the extract had total phenol and flavonoid content are 66.42 mg/g and 11.12 mg/g, respectively. Based on the assay of DPPH (53.19 µg/ml), FRAP (49.86 mg/g), and TAC (96.70 mg/g), BR extract showed strong antioxidant activity. This study also revealed that BR extract significantly reduced human cancer cell lines viability and selectively (shown to have no effect on PBMC cells) ($p < 0.05$) especially BR5 group (500 g/mL). The administration of BR extract was most responsive to induce apoptosis in T47D cells, up to 93.64% in BR5 close to cisplatin-induced apoptosis effect, compared to HeLa and U2OS cells. The study also found BR extract induced the cell (HeLa, T47D, and U2OS) to arrest or in the phase G0/G1. Hence, BR extract was a food-based cancer treatment with a promising effect to inhibit cancer cell growth and progression.



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Introduction

The International Agency for Research on Cancer, reported a continuing rise in the number of cancer patients. According to GLOBOCAN figures for 2020, nearly 10.0 million people died from cancer, and there were 19.3 million new occurrences of the disease. According to reports, breast, lung, colorectal, prostate, and stomach cancers are the main killers of people with cancer.¹ Cancer cells develop when healthy cells lose their ability to control the cell cycle, proliferation, and apoptosis.^{2,3} As a result, these cells multiply continually and quickly without restriction, which is a hallmark of the growth of cancer.⁴ Surgery, radiation, and chemotherapy are the most popular and extensively used cancer treatments, but they are expensive and have a lot of adverse effects.⁵ The use of natural ingredients as anticancer agents offers a solution to these issues. Previous studies have shown that a variety of compounds derived from medicinal plants have anticancer activity by modifying cell cycle and/or apoptosis,⁶ therefore it can be said that this is a highly effective method for treating cancer. Additionally, it has been demonstrated that reducing oxidative stress early on with exogenous antioxidants—most commonly found in plants or herbs like curcumin, resveratrol, catechin, and genistein—as well as eating meals high in vitamins and antioxidants—can help prevent cancer.⁷

One of functional food which has a lot of attention to be used as an alternative for cancer treatment is pigmented rice. A blackish-purple pigmented rice, Keununjami, has anticancer effect against cervical and gastric cell lines due to the high antioxidant activity.⁸ However, one the most popular pigmented rice which is rich in pharmaceutical value is black rice. Black rice is an antioxidant-rich food, and a prior study we conducted in mice with diabetes model indicated that this extract has anti-inflammatory properties.⁹ The high value of antioxidant activity in black rice become one the most concern that link to the anticancer effect.⁸ Besides, anthocyanins, phenols, flavonoids, -oryzanols, and tocol are all present in black rice.¹⁰⁻¹² According to Chen *et al*,¹³ anthocyanin from black rice extract has been shown to inhibit liver cancer cells as well as a breast cancer metastasis-promoting molecule both *in vitro* and *in vivo*.¹⁴ Zhou *et al.* (2017) also observed that black rice anthocyanin inhibited the phosphorylation of molecules that promote metastasis in cell lines for

breast cancer in humans MCF-7 as well as MDA-MB-453 through FAK, cSrc, and p130 Cas. However the comparison effect of black rice in different human cancer cell lines and which pathway that influence the most in the anti-cancer mechanism of this extract are still remain unclear. So that, The the objective of the study is to examine the anti-cancer potential of aqueous black rice extract on the cancer cell lines in humans such as HeLa, T47D, and U2OS.

Materials and Methods

Black Rice Extraction

Wojalaka variety of black rice (*Oryza sativa* L.) from Kepanjen, Malang, East Java, Indonesia, was used in this study. Black rice powder *Simplicia* was formed into samples using was pounded and mashed dry black rice that was using less than 80 mesh size according to the American Standard Test Sieve Series (ASTM). The grounding process affected the simplicial or powder form characteristic which must be considered.¹⁵ After then, *Simplicia* was macerated with Aquadest (1:3, m:v) for an hour while being stirred numerous times. The sample was then filtered to separate the filtrate from the pulp. After that, 4 percent maltodextrin was added, and the filtrate was freeze-dried at -40°C. The yield was then calculated by weighing the extracted material.

Total Phenolic and Flavonoid Content Estimation

Based on Christina *et al* (2021)¹⁶ the total phenolic content was assessed using the Folin-Ciocalteu colorimetric test. 20 µl of BR extract or standard (gallic acid, Sigma®, Germany) were combined and put into a 100 µl batch of freshly prepared 100 % Folin-Ciocalteu reagent (Sigma®, Germany). The combination was given a five-minute incubation period at room temperature before being neutralized with 75 µl of saturated sodium carbonate (Merck®, Germany). It was then given another two hours at room temperature without exposure to light. Next, a wavelength of 740 nm was used to measure the absorbance following a 2-hour reaction.

The measurement of the flavonoid content was based on Christina *et al* (2021)¹⁶ Quercetin (Sigma®, Germany) was used as the standard, and 50 µl of BR extract was added along with 70 µl of distilled water, 15 µl solution of sodium nitrate (Merck®, Germany) (5%) and incubated for 5 min. After incubating for 5 min, the sample received 15 µl of 10% (m/v) AlCl₃ (Merck®, Germany), which was then added.

After that, 100 µl of sodium hydroxide solution (Merck®, Germany) was added to the mixture (1 M). The sample absorbance was then compared to the blank using a 510 nm wavelength analysis.

DPPH Scavenging Ability Assay

The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH, Sigma®, Germany) test was done according to Ablat *et al* (2014)¹⁷ with a slight modification, the effect of BR extract free radical scavenging was investigated. In a nutshell, ethanol was made using a series of BR extract concentrations. The same volume of a 1 ml DPPH solution (0.004 %) in ethanol was then combined with the extract or ascorbic acid (Merck®, Germany) as the standard. The combination was then allowed to stand for 30 minutes at room temperature in the dark. The absorbance value of the scavenging activity was then computed using the following formula after the absorbance at 517 nm was determined.

$$\text{DPPH scavenging activity (\%)} = (A_c - A_t) / A_c \times 100$$

Where A_t denoted the absorbance of the sample, and A_c denoted the absorbance of the control (ethanol and DPPH reaction). The IC₅₀ or 50% of scavenging activity was determined through the graph plotted against percentage inhibition and standard.

Ferric-Reducing/Antioxidant Power (Frap) Assay

The FRAP assay was used done to assess antioxidant activity according to Fernandes *et al* (2016)¹⁸ with slight modification. In a nutshell, the TPTZ (2,4,6-tripyridyl-S-triazine, Sigma®, Germany) and 1.0 mol/l (10 volumes) acetate bauffer (Sigma®, Germany) were combined with 1 volume of 20 mmol/l ferric chloride (CDH®, India) and 40 mmol/l hydrochloric acid (Merck®, Germany) to create the FRAP reagent. Then, the sample was mixed with 3 ml of FRAP reagent, 100 µl of sample, 300 µl of deionized water, and let to sit for 8 minutes. With varied concentrations of FeSO₄.7H₂O (Merck®, Germany) (100–1,000 µmol/l), the absorbance was measured and a standard curve was created.

Total Antioxidant Capacity Determination

The phosphomolybdate technique was done according to Jafri *et al* (2017)¹⁹ and used to determine the total antioxidant capacity (TAC). In a nutshell, 2.0 ml of reagent solution was combined with 0.2 ml (0.5 mg/ml) of BR extract

(600 mM sulfuric acid (Merck®, Germany), 28 mM sodium phosphate (Merck®, Germany), and 4 mM ammonium molybdate (Merck®, Germany)). The mixture was then heated for 60 minutes at 95°C. The absorbance was measured at 695 nm and compared to a control (2 ml reagent solution). Ascorbic acid's antioxidant activity served as a standard, and its equivalent expression was used.

Cell Culture and Flow Cytometry Analysis

Five dosages of an anticancer test were performed on selected samples, Control (C; 0 µg/mL), dosage I (BR1; 100 µg/mL), dose II (BR2; 200 µg/mL), dose III (BR3, 300 µg/mL), dose IV (BR4; 400 µg/mL), dose V (BR5; 500 µg/mL), and Cisplatin (PT. Ferron Pharmaceuticals, Indonesia) (Cis, 1.5 µg/mL). Several cancer cell lines, including human breast cancer (T47D), sarcoma (U2OS) cells, and servical cancer (HeLa), were used in *in vitro* anti-cancer tests. Besides, this study also used human peripheral blood mononuclear cells (PBMC) as normal or control cell which was obtained from healthy female donor aged 45 years old who was fulfilled the inform consent before conducting the study. The protocol was approved by the ethics commission of University of Brawijaya with ethical clearance number 474-KEP-UB. The PBMC isolation from whole blood was done according to Puleo *et al* (2017)²⁰ with modification. The blood was collected and mixed with PBS (1:1) (Biowest) and put in 15 ml centrifuge tube filled with Ficoll-Hypaque 1077. The sample was then formed 2 separated layer and centrifuged at 1500 rpm 20° C for 30 min. The sample was then formed 3 layers and PBMC in interphase part was collected and washed with PBS 5 ml in centrifuge tube. The PBC was then centrifuged again for 5 min at 2500 rpm and 10 °C and the pellet was collected as PBMC. In addition, U2OS cells were grown in DMEM complete medium (Gibco®, USA) whereas PBMC, HeLa and T47D cells were grown in RPMI 1640 complete medium (Gibco®, USA). The cells were kept alive in a cell culture incubator with 5% CO₂ and temperature 37 °C. A 10% fetal bovine serum/FBS (Gibco®, USA) and 1% penicillin-streptomycin (Gibco®, USA) were added to each basal medium, DMEM and RPMI. After one day of cell culture, the viability, apoptosis, and cell cycle of the treated cells were examined. The cells were then treated with various doses of BR extract.

Cell Viability Analysis

The cell viability analysis was done using WST-1 reagents (Roche Diagnostics GmbH, Germany) according to Christina *et al* (2022)²¹ with modification. The PBMC, T47D, U2OS, and HeLa cells were cultured in 96-well plate and several doses of BR extract (BR1, BR2, BR3, BR4, BR5) were given and compared to cisplatin-treated cells (100µl). Then, the treatment medium was discarded and added by 100µl medium containing 5µl WST-1 reagent in the dark. After 30 min incubation, a wavelength 450 nm used to measure the absorbance.

Cell Cycle Analysis

The cell cycle analysis was done by flow cytometry analysis using PI staining (BioLegend®, USA). With a slight modification, the cell cycle analysis was referred to Pumiputavon *et al* (2017).²² The administration of BR extract and cisplatin was done as the cell viability and apoptosis analysis. After treatment, the cells were harvested using trypsin solution (Gibco®, USA). After trypsination, the cells were added with the same volume of culture medium and centrifuged at 2500 rpm, 10°C and 5 min. The pellet was then stained with 50µl PI antibody and incubated in 4°C for 30 min. After staining, the flow cytometry analysis was done.

Cell Apoptosis Analysis

The analysis of cell apoptosis was done through flow cytometry analysis with Annexin V/PI staining²² (BioLegend®, USA). The antibody (50µl) was added after the cells were harvested and incubated for 30 min. After incubation, the samples were analyzed using flow cytometer.

Statistical Data Analysis

The flow cytometry data was analyzed using BD Cell Quest Pro™. The data showed as the mean + SD or SEM (?) results where the statistical data were analyzed using One-way ANOVA using SPSS software ver. 16 for windows.

Results

According to this investigation, the BR extract contained 11.12 mg QE/g of total flavonoids and 66.42 mg GAE/g of total phenolic content. The extract contained higher level of phenolic than flavonoid which strongly correlated to the antioxidant activity. This study analyzed the antioxidant activity through DPPH, FRAP, and TAA assays. The analysis showed the antioxidant value based on the IC50 of DPPH assay was 53.19 µg/ml, while the value based on FRAP and TAA assay were 49.86 mg/ml and 96.70 mg/ml, respectively (Table 1).

Table 1: Yield percentage (%), water content (%), total phenol content (TPC as mg gallic acid/g extract), total flavonoid content (TFC as mg of quercetin/g extract), DPPH % inhibition (IC50),

Yield in percentage (%)	Water Content (%)	TPC (mg/g)	TFC (mg/g)	DPPH (µg/ml)	FRAP (mg/g)	TAC (mg/g)
69.35	5	66.42 ± 0.23	11.12 ± 0.19	53.19	49.86 ± 0.04	96.70 ± 0.30

FRAP % inhibition, and TAA % inhibition of BR extract.

Using the WST-1 reagent, the viability of cancer cells (HeLa, T47D, and U2OS) was assessed in comparison to peripheral blood mononuclear cell (PBMC) as normal or non-cancer cells. The findings demonstrated that BR extract decreased cell viability specifically in cancer cells (Tab. 1). Following the administration of cisplatin (31.35%), the viability of PBMC dramatically decreased (p<0.05), however it had grown along with BR extract. The study also

demonstrated that, in a dose dependent manner, BR extract dramatically decreased the viability of HeLa, T47D, and U2OS cells (p<0.05). When compared to BR extract, cisplatin therapy, however, had the lowest effect on cancer cell viability.

According to the study, BR extract significantly and dose-dependently increased apoptosis in HeLa and T47D cells (p<0.05). In addition, U2OS cells

saw a substantial increase in apoptosis in a dose-independent manner ($p < 0.05$) when exposed to the effects of BR extract. BR extract was more capable of inducing T47D cell apoptosis than any other cancer cell lines. Compared to cisplatin, the number T47D apoptosis cells of BR extract groups were

similar to low dose of cisplatin treatment (1.5 $\mu\text{g}/\text{mL}$). Cisplatin stimulated T47D cells apoptosis to a level of 95.15 %, whereas BR extract was 93.64 % (BR5). However, untreated cells group displayed 0.82, 1.19, and 1.21 % of cell death (HeLa, T47D, and U2O S, respectively).

Table 2: BR extract effect on several cancer cell lines viability

Group	Cell viability (%)			
	PBMC	HeLa	T47D	U2OS
Untreated	61.91 \pm 0.28 ^b	100 \pm 0 ^g	100 \pm 0 ^g	100 \pm 0 ^f
BR1 (100 $\mu\text{g}/\text{mL}$)	65.366 \pm 0.55 ^c	68.55 \pm 0.55 ^f	71.47 \pm 0.61 ^f	61.43 \pm 0.54 ^e
BR2 (200 $\mu\text{g}/\text{mL}$)	76.21 \pm 0.61 ^e	63.58 \pm 0.53 ^e	67.57 \pm 0.54 ^e	60.26 \pm 0.72 ^e
BR3 (300 $\mu\text{g}/\text{mL}$)	69.5 \pm 0.5 ^d	57.75 \pm 0.68 ^d	62.3 \pm 0.16 ^d	46.61 \pm 0.26 ^d
BR4 (400 $\mu\text{g}/\text{mL}$)	85.49 \pm 0.45 ^f	50.3 \pm 0.31 ^c	55.56 \pm 0.57 ^c	47.9 \pm 0.27 ^c
BR5 (500 $\mu\text{g}/\text{mL}$)	89.22 \pm 0.25 ^g	37.83 \pm 0.61 ^b	41.76 \pm 0.28 ^b	45.35 \pm 0.48 ^b
Cis (1.5 $\mu\text{g}/\text{mL}$)	31.35 \pm 0.47 ^a	14.83 \pm 0.50 ^a	15.19 \pm 0.3 ^a	25.73 \pm 0.19 ^a

*Black rice extract treatment on several cancer cell lines. Untreated group as control cells without BR extract treatment. BR1 to BR5 (Black rice extract dose 1 to dose 5), and Cis group as cells group treated with Cisplatin. Value was the mean of data \pm sd. Superscript notation showed a significant difference by Tukey test ($p < 0.05$).

Table 3: BR extract effect on several cancer cell lines apoptosis

Group	HeLa	T47D	U2OS
Untreated	0.82 \pm 0.032 ^a	1.196 \pm 0.135 ^a	1.21 \pm 0.149 ^a
BR1 (100 $\mu\text{g}/\text{mL}$)	20.66 \pm 0.085 ^b	83.29 \pm 0.305 ^b	56.12 \pm 0.2 ^f
BR2 (200 $\mu\text{g}/\text{mL}$)	24.06 \pm 0.16 ^c	90.83 \pm 0.745 ^c	42.86 \pm 0.29 ^e
BR3 (300 $\mu\text{g}/\text{mL}$)	24.57 \pm 0.6 ^c	90.17 \pm 0.244 ^c	39.79 \pm 0.9 ^d
BR4 (400 $\mu\text{g}/\text{mL}$)	30.64 \pm 0.52 ^d	93.11 \pm 0.615 ^d	35.15 \pm 0.8 ^c
BR5 (500 $\mu\text{g}/\text{mL}$)	36.33 \pm 0.53 ^e	93.64 \pm 0.449 ^d	30.74 \pm 0.42 ^b
Cis (1.5 $\mu\text{g}/\text{mL}$)	50.5 \pm 0.087 ^f	95.19 \pm 0.871 ^e	67.1 \pm 0.19 ^g

*Black rice extract treatment on several cancer cell lines. Untreated group as control cells without BR extract treatment. BR1 to BR5 (Black rice extract dose 1 to dose 5), and Cis group as cells group treated with Cisplatin. Value was the mean of data \pm sd. Superscript notation showed a significant difference by Tukey test ($p < 0.05$).

Through the use of PI staining and flow cytometry analysis, the cancer cell cycle was identified. The graph's orange and grey lines, which correspond to the S and G2/M phases, respectively, and the blue line, which represents the G0-G1 phase (Fig. 2). On HeLa cells, BR extract dramatically

inhibited G2/M in a dose-dependent manner ($p < 0.05$) and significantly inhibited S phase in a dose-independent manner ($p < 0.05$). Additionally, the study showed that BR extract strongly promotes cell cycle arrest to the G0/G1 phase, with BR5 showing the highest percentage of cells in this

phase (73.58%). Cisplatin treatment suppressed the cells in the G0/G1, S, and G2/M phases, we hypothesized that this was because the medication mostly triggered apoptosis in the cells (Fig. 1).

On the other hand, the percentage of untreated T47D cells in S and G2/M phase have been relatively low. BR extract maintained the cell to not increase the S and G2/M phase that showed by the flat of orange

and grey line on the graph. But the G0/G1 phase increased along with the treatment of BR extract significantly in dose independent manner ($p < 0.05$). The treatment of BR extract induced G0/G1 phase of U2OS cells significantly ($p < 0.05$) and inhibited S and G2/M phase significantly in dose dependent manner ($p < 0.05$). BR extract and cisplatin treatment showed the same inhibition of cell cycle to S and G2/M phase.

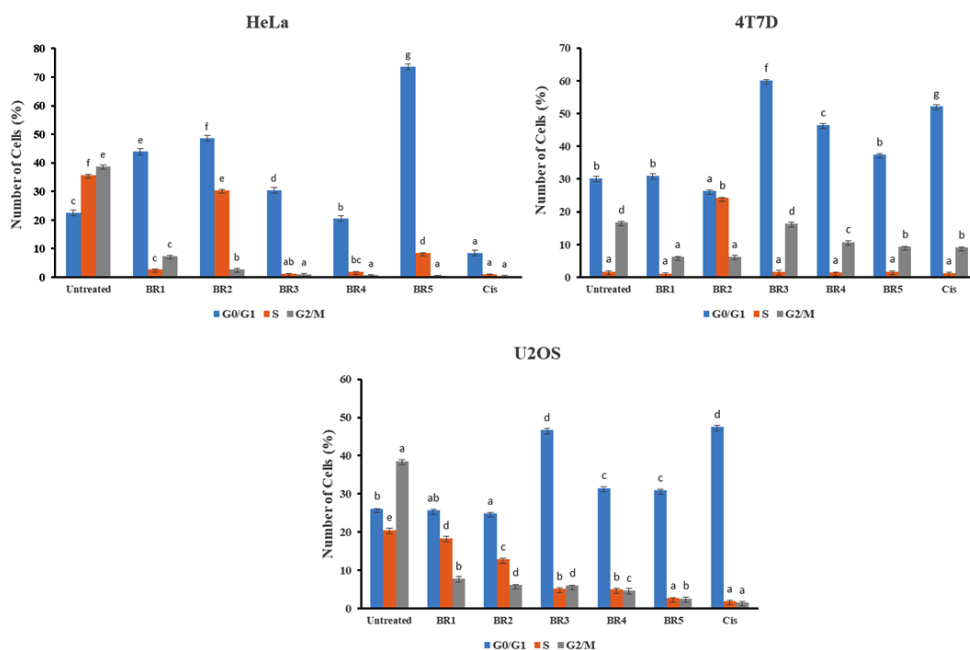


Fig. 1: Cancer cell cycle modulation of BR extract. Untreated (0 µg/mL), BR1 (100 µg/mL), BR2 (200µg/mL), BR3 (300 µg/mL), BR4 (400 µg/mL), BR5 (500 µg/mL), and Cis (1.5 µg/mL). Value was the mean of data ± sd. Superscript notation showed a significantly difference by Tukey test ($p < 0.05$).

Dicussion

Numerous bioactive substances found in black rice which has been throughly documented, such as phenolic and flavonoid content. Phenolic substances have the potential to treat cancer and other oxidative stress-related disorders. Phenolic compounds are frequently claimed to have anti-carcinogenic potential due to a variety of properties including cell cycle arrest induction, cell proliferation suppression, ROS level modulation, protein tumor suppressor modulation, and cell repair augmentation.²³ Promising substances such as flavonoids are utilized to control reactive oxygen species (ROS), induce cell cycle arrest and death,

autophagy, and reduce the growth and invasiveness of cancer cells.²⁴

Based on this study, an active value of antioxidant activity (50–100 µg/ml) was assigned to the BR extract's DPPH assay which was categorized as a strong class of antioxidant activity.²⁵ The antioxidant activity of black rice extract was the highest when compared to red and brown rice. In agreement with that, red and brown rice extracts were contrasted with BR extract, which showed the greatest antiproliferative action, with IC50 values of 148.6 and 119.2 mg/mL against the MCF-7 and MDA-MB-231 cell lines, respectively.²⁶ The presence of phenolic

and flavonoid chemicals, which are responsible for the suppression of cancer growth, contributes to the strong antioxidant activity of black rice extract.

In line with Hui *et al* (2010),²⁷ BR extract has a reductive effect on the viability of cancer cell types. Black rice extract high in anthocyanins decreased the viability of the cell lines MCF-7, MDA-MB-231, and MDA-MB-453 from human breast cancer and activated the caspase cascade to cause cell death in MDA-MB-453 cells. When compared to T47D (BR5, 41.76 percent) and U2OS, the cytotoxic activity of BR extract was shown to be most effective on HeLa cells, reaching up to 37.38 percent viable cells on BR5 (BR5; 45.35 %). However, there was inconsistent data between viability and apoptosis cells. The study found BR5 induced the T47D cells for 94% cells, where the viability cells in previous analysis showed 41.76%. This was possible because there were a lot of factors that can influence the cells health, including passage number, medium, incubator, etc. Nevertheless, the BR extract's ability to induce apoptosis was consistent with that of Pratiwi *et al*.²⁸ Because cyanidin 3-glucoside and peonidin 3-glucoside were present, it was believed that the black rice bran fraction caused apoptosis in HeLa cells. Pratiwi discovered that the apoptosis-induced capability of black rice fraction extract was superior to doxorubicin in terms of effectiveness. Compared to doxorubicin, an Indonesian black rice methanolic extract was observed to increase apoptosis in T47D cells.²⁹ The study discovered that T47D cells were more sensitive to the BR extract's ability to induce apoptosis than HeLa or U2OS cells, which is similar with Pratiwi *et al*'s²⁹ research.

Unregulated cell cycle and unchecked cell proliferation are characteristics of cancer. Controlling cell proliferation and growth is mostly governed by the cell cycle and cyclin-dependent kinases (CDKs) and cyclin.³⁰ Unchecked proliferation is caused by dysregulation of genes involved in the cell cycle machinery, which is frequently detected in cancer.

According to the study, HeLa, T47D, and U2OS cells showed a significant percentage of cells in the S and G2/M phases when left untreated. G0/G1 were elevated along with the BR extract, however S and G2/M were considerably decreased. One promising method of treating cancer was the triggering of cell cycle arrest.

Conclusion

This study reported that the total phenolic and flavonoid content of BR extract were 66.42 and 11.12 mg/ml. Besides, the antioxidant activity of BR extract based on different analysis such as DPPH, FRAP, and TAC analysis were strong with value of 53.19 µg/ml, 49.86 mg/ml, and 96.70 mg/ml, respectively. According to the findings of an *in vitro* research, the high antioxidant content of the BR extract was expected to display anti-cancer action. Human cancer cells' viability was selectively decreased by BR extract. However, T47D showed the greatest sensitivity to BR extract-induced apoptosis in range of 83 to 95%. In cell cycle analysis, it was discovered that BR extract caused HeLa, T47D, and U2OS cells to enter the G0/G1 phase of the cell cycle.

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Conflict of Interest

Authors declare that there is no conflict of interest in this study.

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