

Effects of Two Isolated Compounds from Red Sorghum Bran on p53 and bcl 2 Gene Expression in Human Breast cancer Cell Line (MCF-7)

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Abstract

Aims: The aim of our research was to isolate anthocyanin from red sorghum bran and to study its effects on p⁵³ and bcl₂ gene expressions in Human Breast cancer cell line (MCF-7).

Main methods: Two compounds were isolated from red sorghum bran by using Sephadex LH-20 column chromatography. The antiproliferative activity of isolated compounds were analysed by MTT assay and the expression studies were performed by RT-PCR analysis.

Key findings: Our research focusing on apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside another compound as a potent anticancer agent have demonstrated that the compounds isolated from red sorghum bran inhibited breast cancer cell lines by up regulation of P⁵³ gene expression and down regulation of Bcl₂ gene expression.

Significance: In conclusion, the two compounds isolated from red sorghum bran, were effective in up regulation of P⁵³ and down regulation of Bcl₂ gene expression.

1. Introduction

Consumption of fruits and vegetables that are abundant in flavonoids has been associated with reduced incidence of many forms of cancer (1, 2). These agents are universally present in fruits, vegetables, grains, nuts, tea and fruits. Anthocyanins occur ubiquitously in the plant kingdom and confer the bright red, blue and purple colors to fruits and vegetables. Pure anthocyanins and anthocyanin-rich extracts from fruits and vegetables have exhibited anti-proliferative activity towards multiple cancer cell types in vitro. One of these promising properties is the perturbation of the cell – cycle progression. Research in our laboratory has been focused on apigenin-5-β-D-glucopyranoside (3). Apigenin-5-β-D-glucopyranoside is a flavone that is widely distributed in many fruits, vegetables and grains. The mechanism(s) for the selective effect of the anthocyanins on the growth of cancer cell against normal cells is/are not known. Although anthocyanins have attracted much attention for their possible benefits, little is known regarding their anti-cancer action.

In the present study, the antiproliferative activity of purified anthocyanin compounds isolated from red sorghum bran and its effects on p53 and bcl-2 gene expressions in Human Breast cancer cell line (MCF-7) was determined.

2. Experimental

2.1 Samples

The *Sorghum bicolor* (L.), red sorghum bran was collected from the village area (Coimbatore district, Tamil Nadu, India). The plant was identified and authenticated (No.BSI/SRC/5/23/2011-12/Tech.1486)

by Botanical survey of India (BSI), Tamil Nadu Agricultural University (TNAU), Coimbatore, India. The bran was collected and was stored at -20°C.

2.2 Sample extraction

The bran of red sorghum (200g) was extracted by incubation with 1% Hydrochloric acid in methanol. Overnight at room temperature, followed by a filtration through whatman filter paper no.4. Methanol was removed by a rotary evaporation under 35°C and the pigmented fraction extracts were stored for a further study.

2.3 Isolation of anthocyanin

The concentrated filtrates were then applied onto an amperlite XAD -7 resin column (Sigma Aldrich, Missouri, USA) (1.5 x 40 cm), and washed with distilled water, followed by an elution with 1% Trifluoroacetic acid in methanol (4,5). The elution was evaporated again after being collected by a fraction collector. Isolated anthocyanins were analysed on a UV-Visible spectrophotometer (Genesys 5 Technical trade links pvt.ltd). The fractions with the highest absorbance at 480nm were pooled and evaporated to remove methanol and dried under vacuum at 35°C.

2.4 Purification of Anthocyanin

5 ml of concentrated isolated methanolic pigments were fractionated by Sephadex LH-20 column (100 cm x 2.6 cm, sigma, USA) using water(0.1% TFA)- Methanol(40:60, v/v) as eluent (4,5). The flow rate was 0.3 mL/min. Fractions were collected by a fraction collector. The fractions were evaporated on a rotary evaporator to remove methanol to facilitate the removal of water remaining in the sample. The evaporation temperature was less than 38°C. The homogeneity of individual fractions was checked by HPLC analysis. The lyophilized sample was used for expression studies.

2.5 High – Performance Liquid Chromatography

The anthocyanin samples was diluted accordingly with methanol and HPLC analysis was carried out using a liquid chromatography system (Shimadzu, Kyoto, Japan) with a Photodiode array detector. The Detection was carried at 480 nm. The flow rate was maintained at 1.0ml/min. A Reverse Phase C₁₈ column having a particle size of 5.0 µm was used for HPLC analysis of anthocyanins. The sample injection was 20 µl. A gradient mobile phase was used for elution : elution A was 10% Formic acid in water and B was Acetonitrile/water/formic acid (5:4:1). Apigenin-5-β-D-glucopyranoside was used as a standard.

2.6 Cell lines and Culture medium

MCF-7 (Human, Breast cancer cell line) was cultured in DMEM supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with

TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

preparation of Test Solutions

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay

Principle: The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used Francis and Rita, (1986).

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 ml of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 ml of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO₂ atmosphere. The supernatant was removed and 100 ml of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC₅₀) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \right)$$

2.7 RT-PCR for p53 and Bcl-2 gene expression.

1 X 10⁶ MCF-7 cells were plated per well of 6 well tissue culture plate and incubated at 37°C/5% CO₂ overnight. These cells were treated with 20 mg/ ml of the test sample dissolved in DMEM containing 10% FBS. As a negative control DMEM containing 10% FBS was used and as a positive control cells were treated with 5µg/ml Doxorubicin hydrochloride. Plates were incubated for further 48 hours. Total RNA was isolated from these cells and mRNA expression levels of p53 and BCL-2 were carried out using semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

2.2. Reverse Transcription (RT)

Reverse transcription of total RNA from MCF-7 control, MCF-7 cells treated with 20 mg/ml samples A (Luteolin-5-β-D-glucopyranoside), B (apigenin-5-β-D-glucopyranoside), and C (Control with out samples, Negative control) and MCF-7 cells treated with 5µg/ml Doxorubicin (Positive control) were performed separately using 200 units of RevertAid™ M-MuLV Reverse Transcriptase (Fermentas Life Sciences).

Polymerase Chain reaction (PCR)

PCR For β-actin:

The sequences of the primers used for β-actin are as follows

Forward: 5'-GTGGGGCGCCCCAGGCACCA -3'

Reverse: 5'- CTCCTTAATGTCACGCACGATTTTC-3'

The amplification was performed in a Master cycler® Thermocycler (Eppendorf, Germany) using the following program

Initial denaturation of 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min and extension at 72°C for 1.2 min and terminated after final extension of 72 °C for 8 min.

Expected size of product: 540 bp

PCR for p53

The sequences of the primers used for p53 are as follows

Forward: 5'-CTGAGGTTGGCTCTGACTGTACCACCATCC -3'

Reverse: 5'-CTCATTGAGCTCGGAACATCTCGAAGCG -3'

The amplification was performed in a Master cycler® Thermocycler (Eppendorf, Germany) using the following program

Initial denaturation of 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 69°C for 1 min and extension at 72°C for 1.2 min and terminated after final extension of 72 °C for 8 min

Expected size of product: 371 bp

PCR for Bcl-2

The sequences of the primers used for Bcl-2 are as follows

Forward: 5'- GTTCGGTGGGGTCATGTGTGTGGAGA-3'

Reverse: 5'- GCTGATTCGACGTTTTGCCTGAAGAC-3'

The amplification was performed in a Master cycler[®] Thermocycler (Eppendorf, Germany) using the following program

Initial denaturation of 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min and extension at 72°C for 1.2 min and terminated after final extension of 72 °C for 8 min.

Expected size of product: 462 bp

Results

1. Isolation and Purification

In red sorghum bran, many components such as sugar, acid and salts occur except for anthocyanins. To better isolate anthocyanins from the red sorghum bran, the crude extract generally was passed through Amberlite XAD – 7 resin column exhibited a high spectral characteristics. The isolated anthocyanins were further purified through sephadex LH 20 column and two compounds were isolated from red sorghum bran anthocyanin. The isolated two compounds luteolin-5-β-D-glucopyranoside and apigenin-5-β-D-glucopyranoside-5-β-D-glucopyranoside was taken for invitro cytotoxicity assay and expression studies in MCF – 7 cell line.

2. Hplc Analysis

The purified anthocyanins were checked for homogeneity with the standard apigenin-5-β-D-glucopyranoside. The second fraction matches with the standard apigenin-5-β-D-glucopyranoside with the same retention time. (Figs. 1 & 2). The first fraction doesn't match with the standard.(Fig. 3) Thus the data indicate that two compounds was able to isolate from red sorghum bran by the combination of Amberlite XAD – 7 and Sephadex LH – 20 column chromatography.

2. invitro Cytotoxicity Assay

There was no significant cytotoxicity observed even at 20 mg/ml concentration by MTT assay. This could be due to the presence of color to the sample which interferes with the reading at 570 nm.

3. expression Of P Gene In Mcf-7 Cell Line

P⁵³ protein is essential for check point control that arrests Human cells with damaged DNA in G1 phase of cell cycle. P⁵³ also leads to induction of proteins that promotes apoptosis.

In this study of P⁵³ gene expression, the MCF-7 cells were treated with 20mg/ml of apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside isolated from red sorghum bran. Our results indicated that the expression of P⁵³ increased after one hr incubation, and were maintained at a high level over 48 hr of treatment with 20 mg/ml of apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside isolated from red sorghum bran.

Expression of P⁵³ gene was investigated by RT-PCR in untreated and treated MCF-7 cells. Quantitation of each band was performed by densitometry analysis software and the results expressed as the ratio of P⁵³/β – actin in percent to the medium (control). Doxorubicin hydrochloride (DOX) was used positive control. As shown in Fig. 4 and table 1 in comparison to the medium control, the P⁵³ gene expression is increased in the presence of apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside.

In table 1, the % to medium in P⁵³ (medium)(-ve control) is 100, in P⁵³ (DOX) (+ ve control) is 112.32, in P⁵³ (sample A) is luteolin-5-β-D-glucopyranoside the % to medium is 111.7, but in P⁵³ (sample B) Apigenin-5-β-D-glucopyranoside, the % to medium is 131.36. In such cases the apigenin-5-β-D-glucopyranoside value was higher than the + ve control Doxorubicin hydrochloride, which shows the P⁵³ gene expression was upregulated when treated with Apigenin-5-β-D-glucopyranoside in MCF-7 cells than luteolin-5-β-D-glucopyranoside. So, Apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside significantly upregulates the P⁵³ gene expression.

4. expression Of Bcl Gene In Mcf-7 Cell Line

Bcl₂ protein function is to suppress apoptosis or promotes cell survival. In our study Bcl₂ gene expression, MCF – 7 cells were treated with 20 mg/ml.

In this study of Bcl₂ gene expression, the MCF-7 cells were treated with 20mg/ml of apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside isolated from red sorghum bran. Our results indicated that the expression of Bcl₂ markedly increased after one hr incubation, and were maintained at a high level over 48 hr of treatment with 20 mg/ml of apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside isolated from red sorghum bran.

In table 1, the % to medium in Bcl₂ (medium)(-ve control) is 100, in Bcl₂ (DOX) (+ve control) is 9.15, in Bcl₂ (sample A) is luteolin-5-β-D-glucopyranoside the % to medium is 17.64, but in Bcl₂ (sample B) Apigenin-5-β-D-glucopyranoside, the % to medium is 4.7. In such cases the apigenin-5-β-D-glucopyranoside value was lower than the +ve control Doxorubicin hydrochloride, which shows the Bcl₂ gene expression was downregulated when treated with apigenin-5-β-D-glucopyranoside in MCF-7 cells than luteolin-5-β-D-glucopyranoside. So apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside significantly down regulates the Bcl₂ gene expression by at least 50–70% .

The expression of p53 and bcl-2 gene in MCF-7 cells was first determined by RT-PCR after incubation with luteolin-5-β-D-glucopyranoside and apigenin-5-β-D-glucopyranoside. An increase in p53 gene expression was observed after incubation with luteolin-5-β-D-glucopyranoside and apigenin-5-β-D-glucopyranoside when compared with the control. In the case of effect of bcl-2 gene expression on MCF-7 cells reduced bcl-2 gene expression was observed after incubation with luteolin-5-β-D-glucopyranoside and apigenin-5-β-D-glucopyranoside. The relative gene expression level; REL was normalized and compared to the expression of β-actin (Fig. 5).

Discussion

Suganyadevi et.al., (7), already reported the presence of apigenin and leutolin in crude sorghum bran anthocyanin extract. Apigenin was an antimutagenic against nitropyrene – induced genotoxicity in Chinese hamster ovary cells (16) and inhibited mitogen – activated protein kinase and the down stream oncogenes in \sqrt - H – ras – transformed NIH 3T3 cells (9). Suganyadevi et al., (8), reported that the crude red sorghum bran anthocyanin upregulates the expression of p53 and also down regulates the expression of bcl-2 gene in MCF-7 cell lines. Dietary apigenin inhibited tumour necrosis factor – induced intercellular adhesion molecule – 1 upregulation (10).

The mechanisms by which apigenin-5-β-D-glucopyranoside protected against skin carcinogenesis in animal models seem to be related to the induction of cell cycle arrest at the G2/M and /or Go/G1 phase (11, 12). Recently we demonstrated that apigenin-5-β-D-glucopyranoside inhibited the growth of three Human colon carcinoma cell lines in association with a reversible G2/M cell – cycle arrest (13). That arrest was associated with decreased activity of P³⁴ cdc2 kinase and reduced accumulation of P³⁴ cdc2 and cyclin B1 proteins (14).

Conclusion

Flavonoids are a major class of non nutritive components of plant – derived diets, and they are widely distributed in plant – based foods and have been suggested to play a role in cancer prevention. Current studies in our laboratory focusing on apigenin-5-β-D-glucopyranoside and luteolin-5-β-D-glucopyranoside as a potent anticancer agent have demonstrated that the compounds isolated from red sorghum bran inhibited breast cancer cell lines by upregulation of P⁵³ gene expression and down regulation of Bcl₂

gene expression. Among the two compounds isolated from red sorghum bran, the apigenin-5- β -D-glucopyranoside was more effective in upregulation of P⁵³ and down regulation of Bcl₂ gene expression.

Declarations

Acknowledgments

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Tables

S.No	Gene (treatment)	Ratio to β -actin	% to Medium
1	p53 (Medium)	0.8526	100
2	p53 (Dox)	0.9576	112.32
3	p53 (Luteolin-5- β -D-glucopyranoside)	0.9524	111.7
4	p53 (Apigenin-5- β -D-glucopyranoside)	1.112	131.36
5	bcl-2 (Medium)	0.306	100
6	bcl-2 (Dox)	0.028	9.15
7	bcl-2 (Luteolin-5- β -D-glucopyranoside)	0.054	17.64
8	bcl-2 (Apigenin-5- β -D-glucopyranoside)	0.0144	4.7

Figures

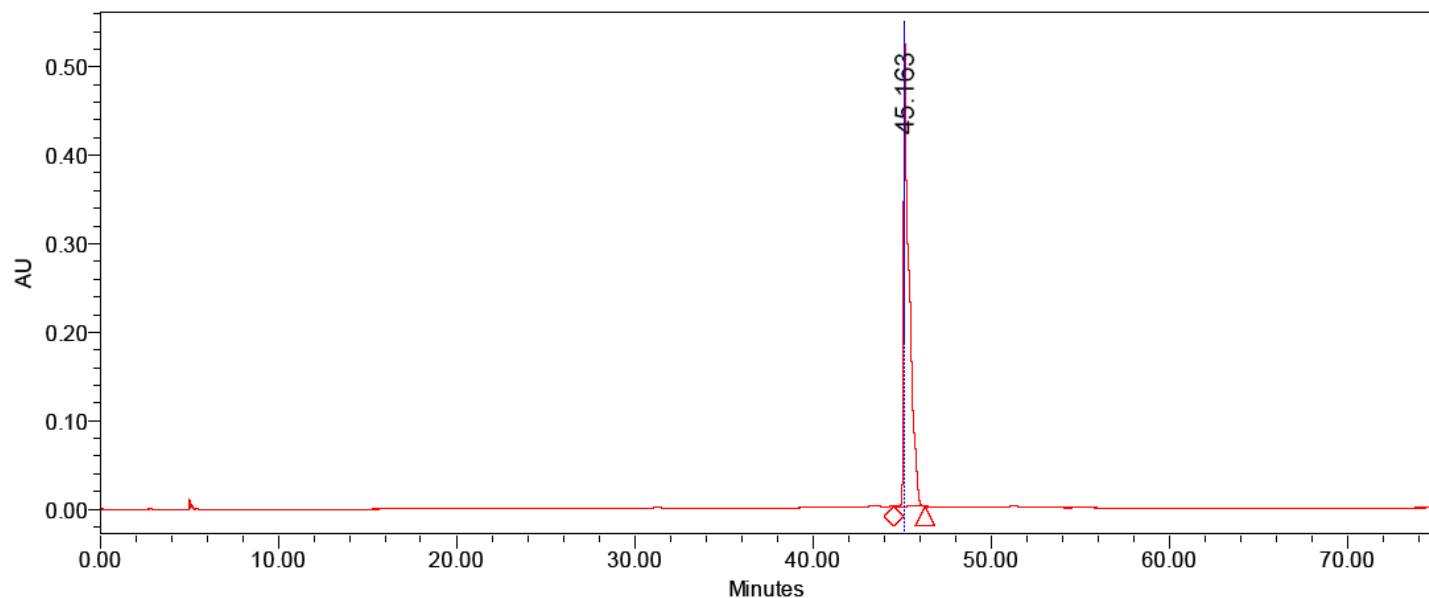


Figure 1

HPLC analysis of standard apigenin

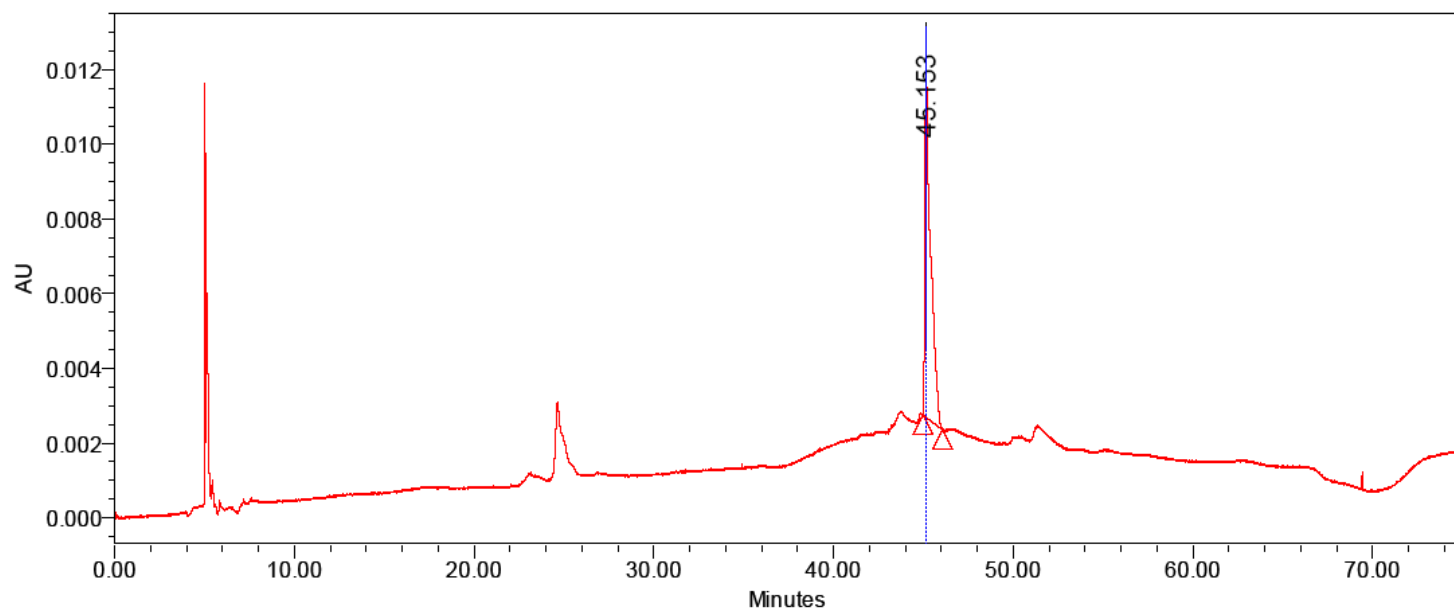


Figure 2

HPLC analysis of purified apigenin-5- β -D-glucopyranoside

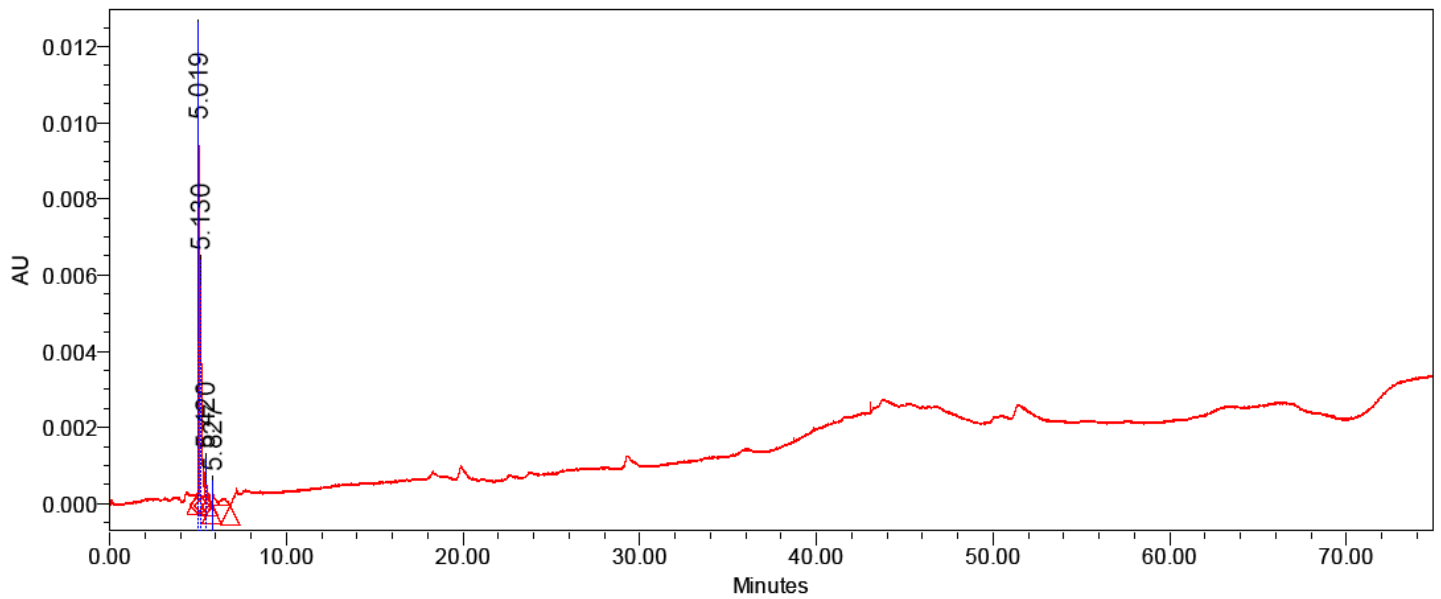
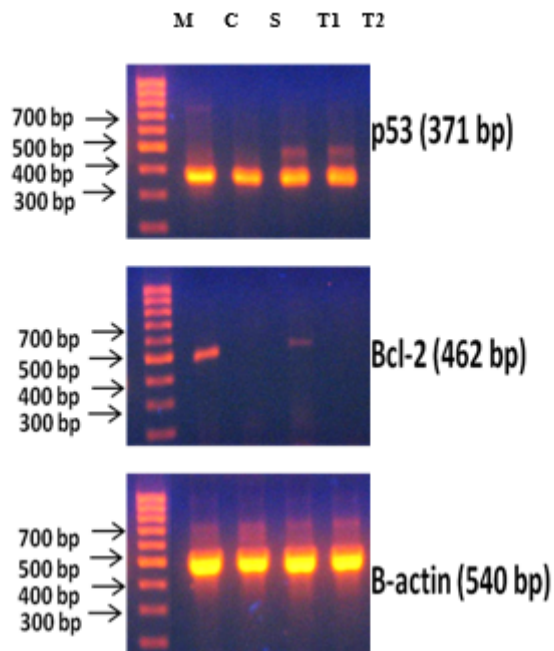


Figure 3

HPLC analysis of purified luteolin-5- β -D-glucopyranoside

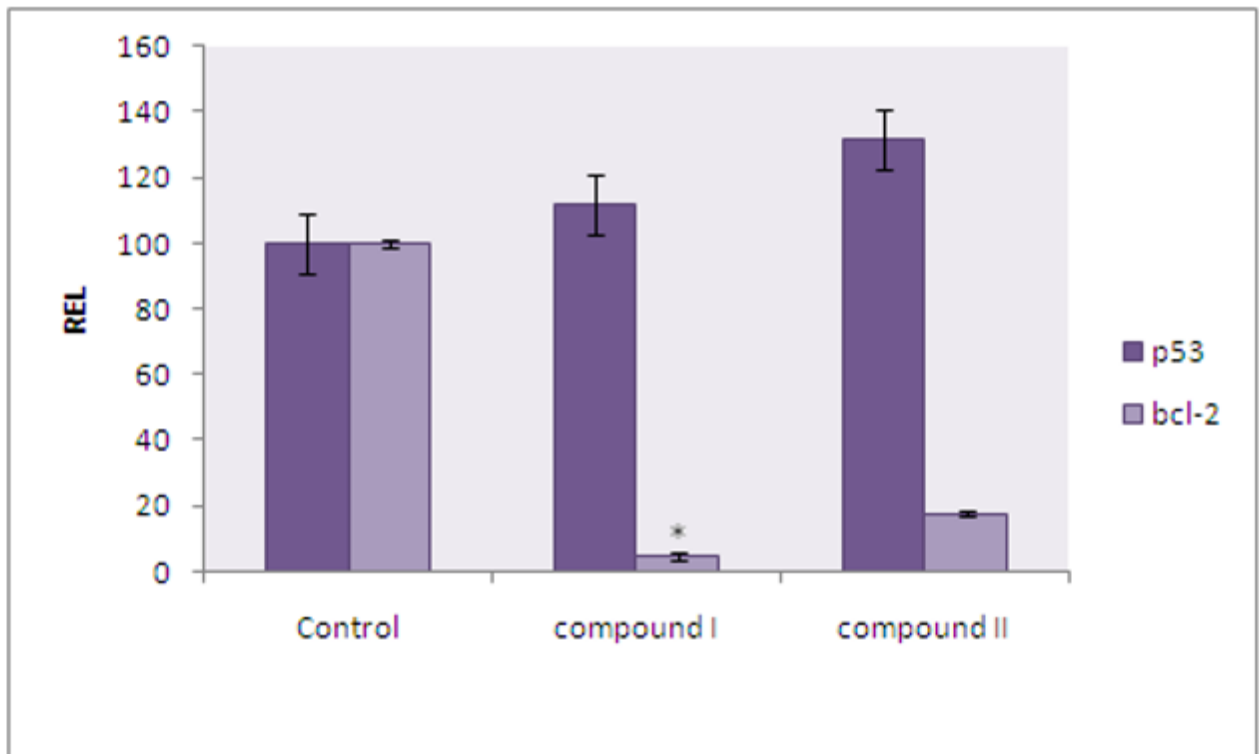


M – 1000 bp ladder
 C – Cell Control
 S – Cells treated with std. drug
 Doxorubicin
 T1 – Cells treated with test luteolin-5- β -D-
 glucopyranoside
 T2 – Cells treated with test apigenin-5- β -
 D-glucopyranoside

The effects of Sorghum anthocyanins on the expression of p53, bcl-2 and β -actin in MCF-7 cells. The cells were incubated with sorghum anthocyanins for 48 hrs and the total RNA was isolated and reverse transcribed. The resulting cDNAs were subjected to PCR with the indicated primers and the reaction products were subjected to electrophoresis in 1% agarose gel and visualized by EtBr staining. Doxorubicin was used as the internal control.

Figure 4

Agarose gel electrophoresis of RT-PCR products



Error bars represent as Mean \pm standard error (n=3). * indicates Significantly different at ($p < 0.05$) from the control analyzed by Duncan's multiple range test.

Figure 5

Expression of p53 and bcl-2 in MCF -7 cells