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# Anti-proliferative Effect of Isoflavones Isolated from Soybean and Soymilk Powder on Lymphoma (DG 75) and Leukemia (CEM) Cell Lines

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author CA experimental design, isoflavone extraction, data and statistical analyses, carried out some of the in vitro assays and drafted the manuscript; author CL conceived of the study, and participated in its design, statistical analysis and writing of manuscript, author RAO participated in the design of study, coordination, data analysis and helped to draft the manuscript, authors FKNA and RAA participated in the design of study, coordination, and helped draft the manuscript, author IT performed the cell culture experiments, some of the in vitro assays and data analysis. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** Isoflavones in soybean have been linked to reduced risk of some cardiovascular diseases as well as some type of cancers. Extensive epidemiological, *in-vitro* and animal data suggests lower incidence of cardiovascular disease, hormone-dependent cancers of the breast and prostate, for consumers of soy and soy products. In this study, isoflavones were isolated from Soybean (SB) and soymilk powder (SMP) and the anti-proliferative effects of these isoflavones on Burkitt's lymphoma (DG-75) and Leukemia (CEM) cell lines were investigated.
**Place of Study:** Department of Clinical Pathology, Noguchi Memorial Institute of Medical

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Research, University of Ghana, Accra, Ghana between June and December, 2014.
**Methodology:** Isoflavanoids were isolated and HPLC done to confirm the isolate. Isoflavanoids were isolated form SB and SMP and the anti-proliferative effect of isoflavanoid isolated from SB, SMP, pure genistein and daidzein on lymphoma and leukaemia cells were compared using tetrazolium-based colorimetric (MTT) assay.
**Results:** The inhibitory effect of genistein on leukemia (*CEM*) cell line was stronger ($IC_{50}$ = 28.4 µM) than daidzein ($IC_{50}$ = >100 µM). For the *DG 75*, genistein had a better effect ($IC_{50}$ = 11.05 µM than daidzein ($IC_{50}$> 100 µM). The $IC_{50}$ for SMP were 193.92 µg/ml (DG-75) and 54.17 µg/ml (CEM). In terms of selectivity index (SI), genistein had the highest (>6.95) on *CEM* followed by genistein (>3.52) and then SB (>1.16).
**Conclusion:** SMP had a better anti-proliferative effect on both *CEM* and *DG-75* compared with SB.

*Keywords: Soybean; anti-proliferative; daidzein; genistein*.

## 1. INTRODUCTION

Soybean (SB) is an important source of protein and other nutrients in many developing countries due to its protein positive nutritional profile, being nearly equal to casein in biological value, low cost, and its availability [1,2]. It has been ranked as the world cheapest source of protein compared to other protein-rich foods such as meat, fish and egg and also an important source of human dietary protein with an average of 40% content, 30% carbohydrate and essential fatty acids and oil content of 20% and high content of essential mineral and vitamins [3-5]. The consumption of soybean has also been associated with the reduced risk of several chronic diseases such as cardiovascular diseases [6], osteoporosis, breast and prostate cancer [7,8]. The main component of soybean related to these effects are the isoflavones [9]. They usually exist in four forms; the aglycone forms (daidzein, glycitein and genistein), the β-glycoside forms (daidzin, glycitin and genistin), the 6-O-malonyl glucosides (malonyl daidzin, malonyl glycitin and malonyl genistin) and the 6-O-acetyl glucosides (acetyl daidzin, acetyl glycitin and acetyl genistin) [10]. The glycoside forms are the most predominant in soybean though these are hydrolyzed to aglycones component which is essential for absorption and this makes the aglycone forms the biologically active form of isoflavone [11]. The pharmacokinetics of absorption, distribution, metabolism (bioconversion in the gut and biotransformation in the liver) and elimination all contribute to the bioavailability and subsequent effectiveness of the isoflavones in SB and its processed derivatives like soy milk powder (SMP) [12,13]. The concentration of isoflavones in soybean ranges between 1.2 to 2.4 mg of total isoflavones per gram of sample distributed in different concentrations in the tissues of the seed, being higher in the embryo than in the endosperm [14,15]. Genistein, which is the predominant isoflavanoid has been found to inhibit cell growth of tumour cell lines from the various malignancies including breast, melanoma, prostrate, head and neck squamous cell carcinoma, leukaemia and lymphoma [16-20]. However, very little has been done on investigating the anti-proliferative effects of isoflavones isolated from soybean and soymilk powder on Burkitt's lymphoma (*DG 75*) and Leukaemia (*CEM*) cell lines which are two common childhood cancers in Africa [21]. The study therefore investigated the effect of these isolated isoflavanoids on the *DG 75* and *CEM* cell lines.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagent

Rosewell Park Memorial Institute (RPMI)-1640 culture medium, fetal Bovine serum (FBS), penicillin streptomycin L-glutamine (PSG), curcumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The T-Lymphoblast-like leukemia cell line (*CEM*), normal prostrate cell (*PNT2*) and Lymphoma cell line (*DG 75*) were donations from Dr. T. Uto (Nagasaki International University, Japan) and Keith Anderson (George Klein group, Department of Tumour biology, Karolinska Institute, Stockholm, Sweden). All reagents were of analytical grade and obtained from standard suppliers.

### 2.2 Plant Material

The certified raw soybean (*'Anidaso'* variety) was purchased from Ghana Grains Board, Kumasi.

This was given to the producer of "*Kwermec*" soya brand (A commercially available Soymilk powder brand available in shops in Ghana). This was to ensure the authenticity of the source of soybean as well as the uniformity of the product. Product safety and quality analysis such as Proximate, Mineral and Microbial analysis was then performed on the SMP before used for this work.

## 2.3 Extraction and Isolation

Isoflavones were isolated from soybean (SB) and soymilk powder (SMP) by the method described by Zhang et al. [22] with slight modification. Extraction of isoflavones from SB and SMP was performed using 70% ethanol. This was done by weighing 30 g of SB and SMP, separately and mixing them with 300 mL of 70% ethanol (1:10). The resulting mixture was heated to 50°C for 24 h using a water bath with a shaker (Clifton Shaking Bath, England). The extract was then separated from insoluble fractions by centrifugation at 2000 rpm (Mistral 3000 E, UK) for 15 min. The extract (filtrate) was hydrolyzed with 37% hydrochloric acid and the pH of resulting mixture adjusted to 1.5. The mixture was heated to 50°C for 12 h on the Clifton shaking water bath. The hydrolyzed product was then diluted with distilled water at the volume ratio of 1:5 (v/v) and stirred constantly at room temperature to precipitate isoflavone crystals which were separated by centrifuging at 2000 rpm for 1 hour (Mistral 3000 E, UK). The solids crystals deposited were then scoped and stored at 4°C for the HPLC and anti-proliferative assay.

## 2.4 HPLC Analysis

Isoflavone contents in the extract isolated from SB and SMP were analyzed using a reversed-phase C18 column (VP-ODS, 150×4.6 mm, 5 µm particle size) on the Shimadzu Prominence liquid chromatography: LC -20AB Pump, DGU-20A3 Degasser, CTO-19ASVP Oven column, SIL-ACHT Autosampler, and SPD-M20A PDA Detector. The sample injection volume of 20 µL was used. The mobile phase of 0.1% phosphoric acid (A) and methanol (B) was used (Table 1). A linear gradient elution from 10% to 90% B starting from 0 to 30 min at a flow rate of 1.0 ml/min was employed. The temperature of the column was maintained at 40°C, and the detection wavelength was set at 300 nm. The identification and quantification of the peaks were done by comparing the retention times and peak areas with the two standards; genistein and daidzein.

**Table 1. Gradient program for HPLC analysis**

| Time (Min) | % B | % A |
|---|---|---|
| 0.01 | 10 | 90 |
| 10 | 25 | 75 |
| 15 | 50 | 50 |
| 20 | 90 | 10 |
| 25 | 90 | 10 |
| 28 | 10 | 90 |
| 30 | 10 | 90 |

*% A- 0.1% Phosphoric acid, %B- 0.1% Methanol*

## 2.5 Cell Culture

Cells were cultured as described by Ham et al. [23] with slight modification. The CEM, DG 75 and PNT2 cells were cultured in RPMI 1640 medium. All culture media were supplemented with 1% PSG and 10% FBS. The cells were maintained in an incubator with 5% $CO_2$ concentration at 37°C and passaged on reaching about 90% confluence.

## 2.6 MTT Assay

The tetrazolium-based Colorimetric Assay (MTT) was adopted for the measurement of cell growth and viability test of daidzein, genistein, SB and SMP on the cancer and normal cell lines. This was done by adopting the method proposed by Ayisi et al. [24]. For the SB and SMP, dilution of the 50 mg/mL in 50% ethanol of stock extract was made and 20 µl of this sample was mixed with 80 µL of media to obtained 10 mg/mL concentration. Subsequently, 50 µL of the 10 mg/mL was added to 50 µl of media and 10% ethanol to obtain 5 mg/mL concentration. Further serial dilution was done to obtain concentrations of 2.5 mg/mL, 1.25 mg/mL and 0.625 mg/mL. The two isoflavanoid standards were also prepared by taking 10 µL of 10 mM of either genistein or daidzein and adding it to 90 µL of media and 10% DMSO to obtain 1 mM concentration. Further serial dilution was done to obtain concentrations of 0.5 mM, 0.25 mM, 0.125 mM and 0.0625 mM. Cells were seeded into 96-well plates at a density of 10,000 cells/well and pre-incubated in a humidified incubator at 37°C, 5% $CO_2$ for 24 h. Subsequently, 10 µL of each extract and standard dilutions were added to the cells in triplicate and the plates were incubate as stated above for 72 h. Curcumin was used as a positive control or standard in all assays. The cells were treated with 20 µL of 2.5 mg/mL MTT solution and re-incubated for further 4 h. Acidified isopropanol, 150 µL, was added to each well to

stop the reaction and then incubated in the dark at room temperature overnight. A colour control plate was also setup for each extract including the positive control, curcumin. The plates were incubated as describe above. Absorbance readings were done at 570 nm using spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria). The percentage cell viability was determined from the formula:

$$\% \text{ Cell Viability} = \frac{A_0 - A_1}{A_0} \times 100\%$$

where $A_0$ is Mean absorbance of control wells, $A_1$ is mean absorbance of test wells. The mean cell percentage viability obtained from triplicate determinations at each concentration was plotted on a dose response curve using Microsoft Excel. The values of inhibition concentrations at 50% ($IC_{50}$), that is, concentration of isoflavones in extracts (SB and SMP) or standard (daidzein and genistein) inducing 50% inhibition of cancer cells were determined from the dose response curves by nonlinear regression analysis.

The selectivity index (SI) defined as the ratio of the $IC_{50}$ obtained from the experiment on normal cell vs. cancer cells was determined using the formula below. Samples with an SI greater than 2 were considered to have a high selectivity towards cancerous cells [25].

$$SI = \frac{IC_{50} \text{ of the extract on normal human cells}}{IC_{50} \text{ of the extract on cancer cells}}$$

### 2.7 Statistical Analysis

All the tests were done in triplicates and results expressed as means of the three values obtained. The percentage cell viability graph was drawn using Microsoft Office Excel 2010.

## 3. RESULTS

### 3.1 Isoflavanoid Content of Soybean (SB) and Soymilk Powder (SMP)

Genistein and daidzein in SB and SMP were identified by HPLC analysis. Chromatograms of pure genistein and daidzein are shown in Fig. 1 (a, b) with retention times 20 mins and 22 mins, respectively. Fig. 1 (c) represents the chromatograms of the isoflavanoid isolated from SB and SMP, respectively. Peaks corresponding to the retention times for genistein and daidzein were identified and the concentrations of these compounds were calculated based on the areas under the curves (Table 2). It was observed that the concentration of both daidzein and genistein were higher in SMP compared to SB. The highest content of daidzein was found in SMP (6.247 µg/ml per 100 mg) while the lowest was in SB (0.392 µg/ml per 100 mg).

**Table 2. Concentrations of Genistein, Daidzein in SB and SMP**

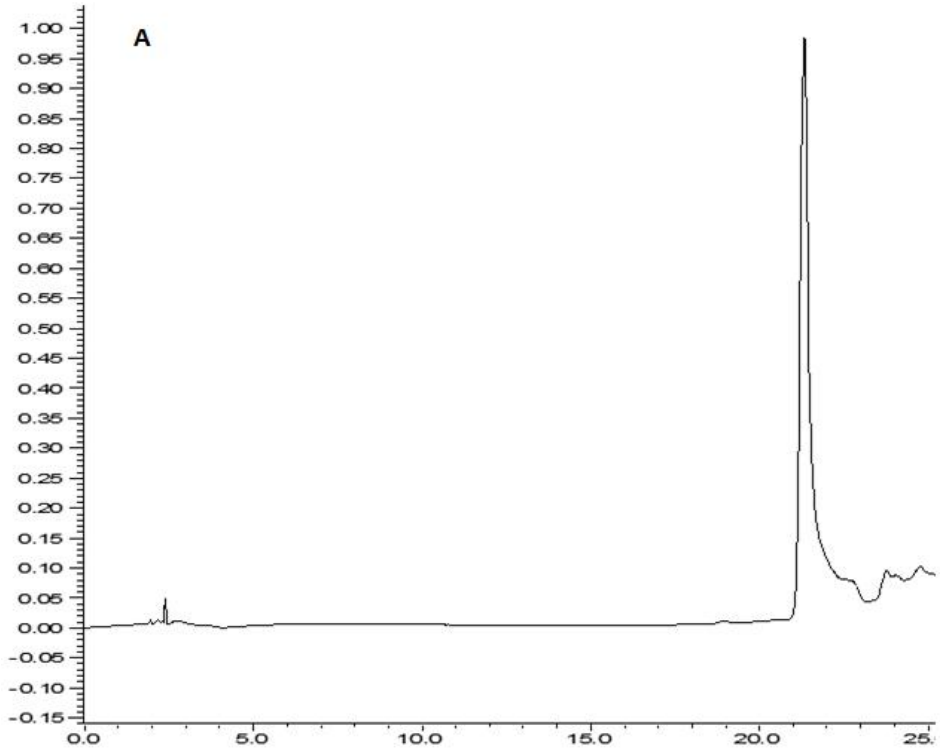
| Samples | Conc. (mg/mL) per 100 mg |
|---|---|
| **Genistein** | |
| G-SB | 0.000586 |
| G-SMP | 0.005517 |
| **Daidzein** | |
| D-SB | 0.000392 |
| D-SMP | 0.006247 |

### 3.2 Anti-proliferative effect of isoflavonone extracts

The effects of test substances on *DG-75* cells are as shown in Fig. 2. Genistein inhibited the growth of DG-75 cells with $IC_{50}$ value of 11.05 µM (2.98 µg/ml) whereas daidzein shows less inhibitory effect $IC_{50}$ > 100 µM (25.4 µg/ml). The effect of SB on the lymphoma cell line was not drastic compared to SMP which was able to clear about 50% of the viable cells at 193.92 µg/ml. However, the $IC_{50}$ for SB was greater than 1000 µg/ml as indicated in Fig. 2(D). The anti-proliferative effect of a standard cancer drug curcumin was also tested on the lymphoma cell lines. Curcumin inhibited the growth of DG 75, giving an $IC_{50}$ value of 10.57 µM (0.00389 µg/ml) as indicated in Fig. 2(E).

The anti-proliferative effects of genistein, daidzein, SMP, SB and curcumin on CEM cells are as shown in Fig. 3. Genistein exhibited a strong inhibitory effect with $IC_{50}$ value of 28.4 µM (7.66 µg/ml) while daidzein showed less inhibitory effect $IC_{50}$ > 100 µM (25.4 µg/ml). The effect of SMP on CEM was observed to be more profound with $IC_{50}$ = 54.17 µg/ml compared to that of the SB ($IC_{50}$= 858.88 µg/ml).

The effects of daidzein, genistein, curcumin and isoflavanoids from SB and SMP on PNT2 cells have been shown in Fig. 4. Daidzein and genistein had minimal effect on the normal cell lines at an $IC_{50}$ >100 µM (25.4 µg/ml) and $IC_{50}$ >100 µM (27.6 µg/ml) respectively Figs. 4 (A and B). SB showed no inhibitory effect on PNT2 cells ($IC_{50}$ >1000 µg/ml) whilst SMP inhibited the growth of normal cells ($IC_{50}$ = 375.61 µg/ml). Curcumin inhibited the growth of the PNT2 cells ($IC_{50}$ =0.00594 mg/ml).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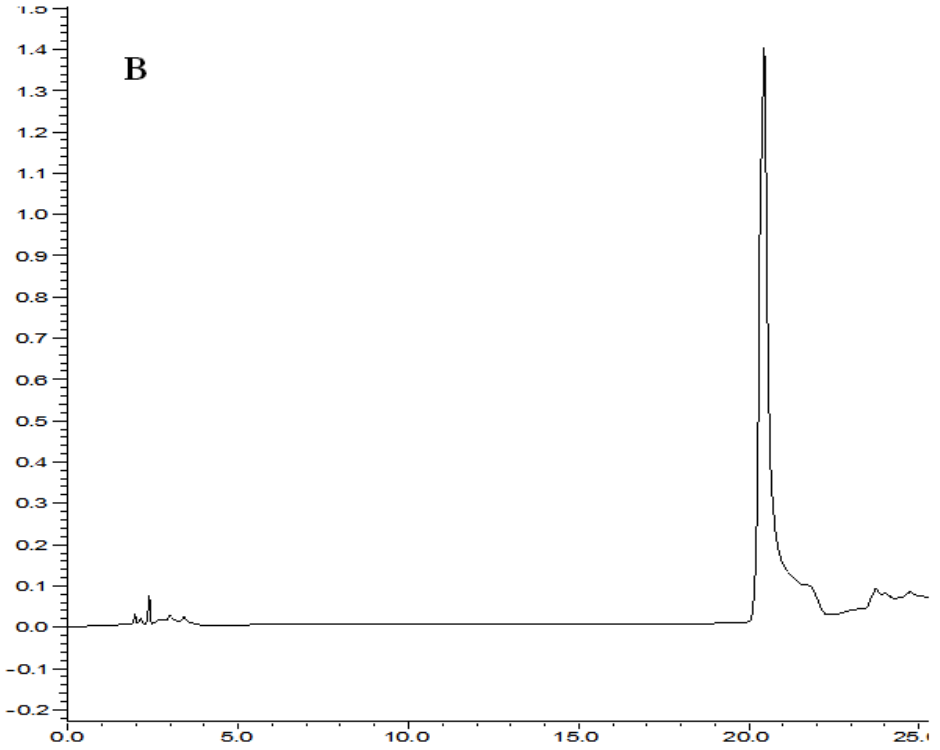
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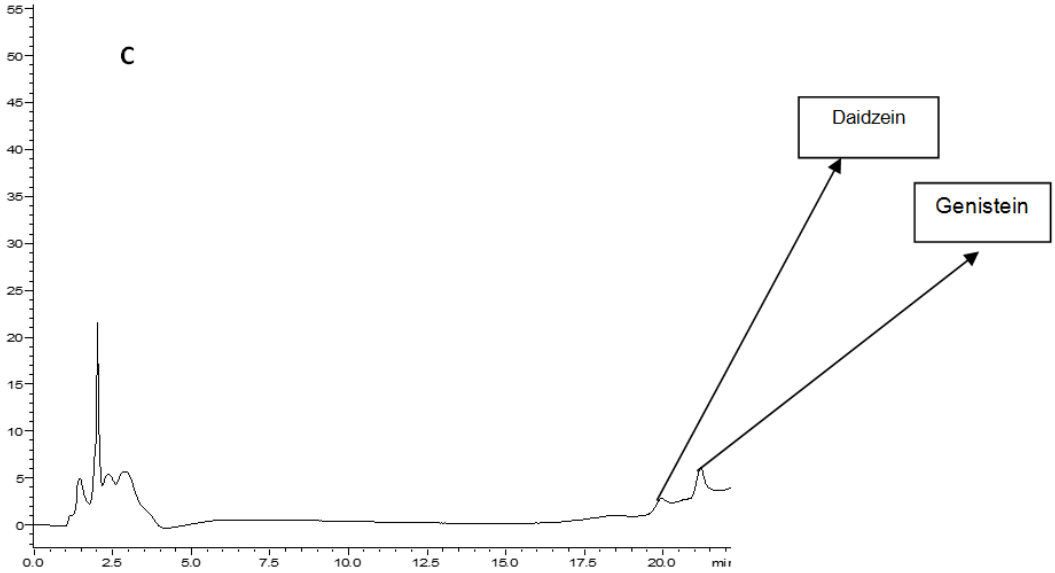


**Fig. 1. HPLC chromatogram of genistein (A), daidzein (B), SMP/SB (C)**

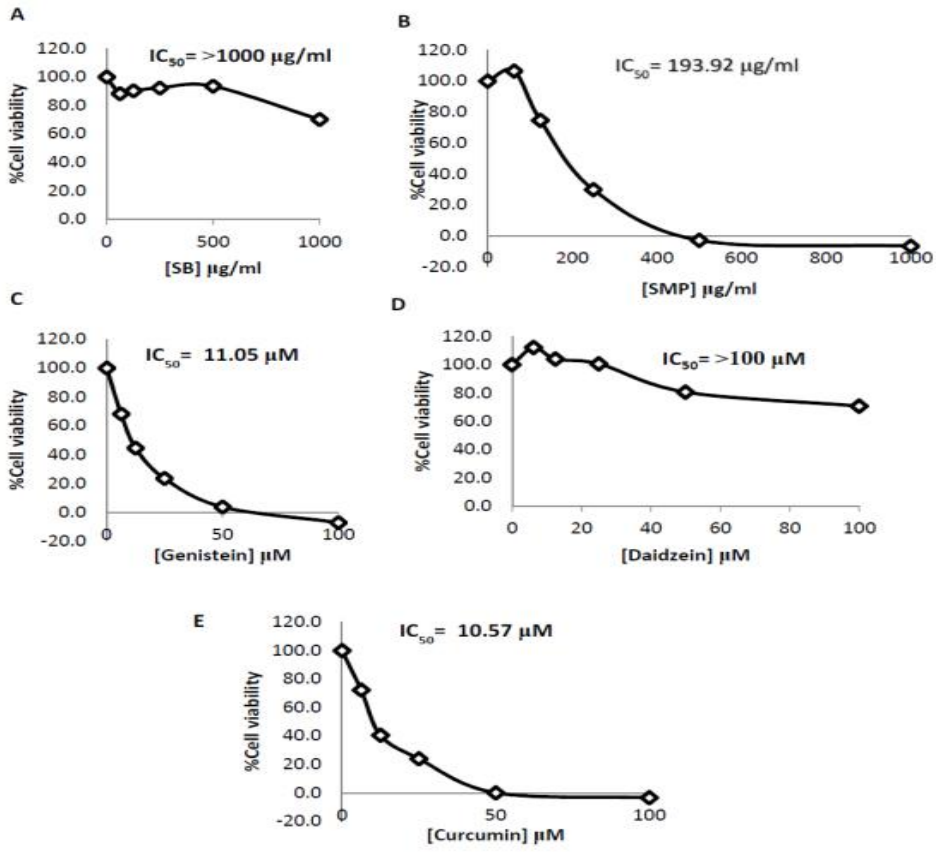


**Fig. 2. Percentage cell viability of SB (A), SMP (B), Genistein (C), Daidzein (D), and Curcumin (E) on lymphoma cell line (DG-75)**

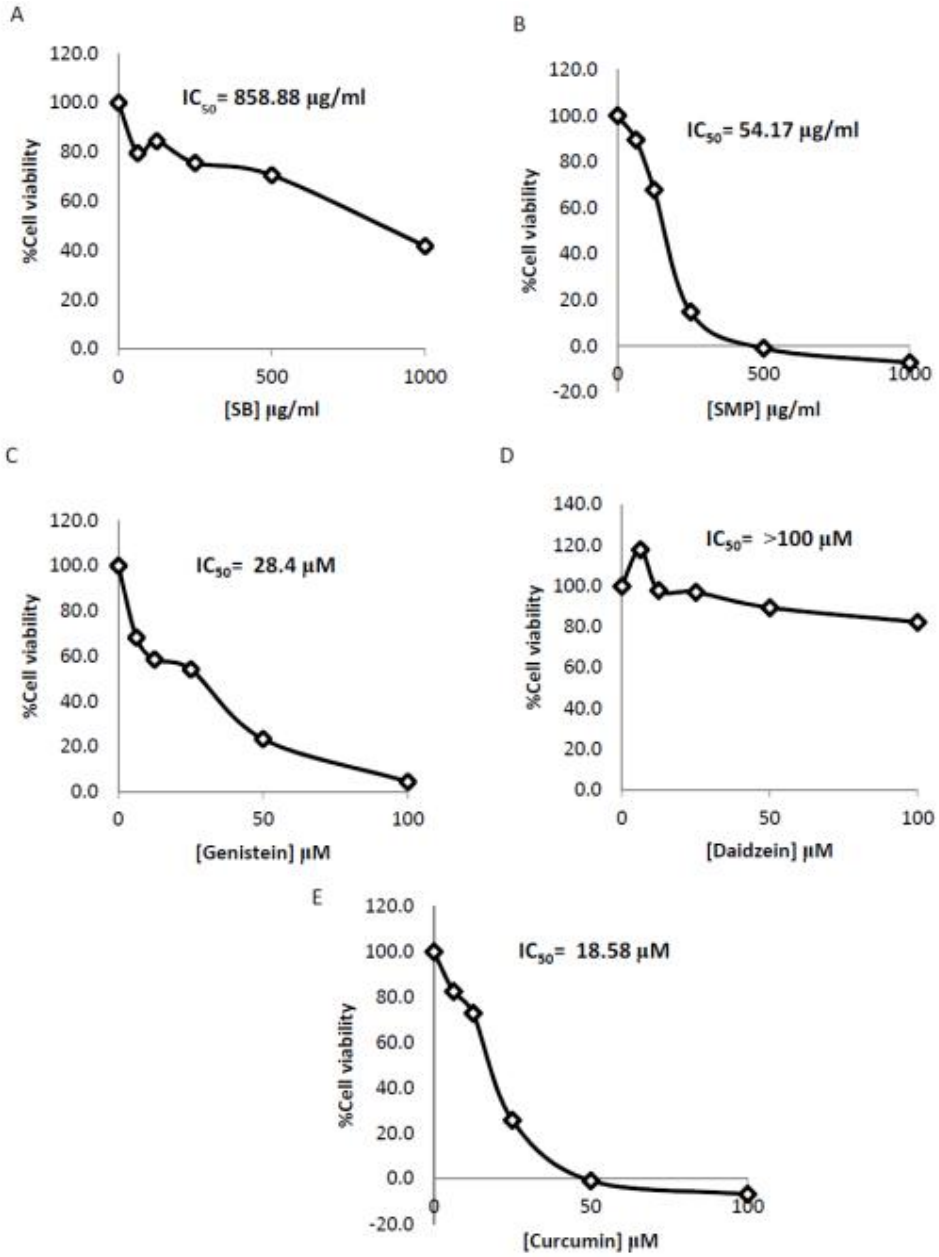


**Fig. 3. Percentage cell viability of genistein (A), daidzein (B), SB (C), SMP (D) and curcumin (E) on CEM cells**

Table 3 shows the selectivity indices of test substances towards the cell lines. SMP had a selectivity of 6.95 and 1.95 towards CEM and DG-75, respectively. These selectivity indices are better than that of SB.

## 4. DISCUSSION

In this study anti-proliferative effects of two isoflavanoid standards i.e. daidzein and genistein were compared with the isoflavanoids isolated from soybean and soymilk powder. Using HPLC assay, the two chromatogram for the isoflavanoid standard showed distinct peaks and that confirmed the purity of the standards (Fig. 1). The elution time of the two standards corresponds with two peaks found in the chromatogram in Fig. 1(c). Soybean, which has been implicated as a rich source of isoflavones, is believed to contain about 1.2-2.4 mg of total

isoflavanoids per gram of a sample as observed by Rostagno et al. [14]. In quantifying the concentration of daidzein and genistein in the two samples, it was clear that SMP had higher concentration of both genistein and daidzein compared to their concentration in SB. This could probably be as a result of the processing of the SB into its milk powder (SMP), which is a concentrate of the former. Genistein has been identified as the major isoflavone constituent of soybean that is found naturally as the glycoside genistin though this is hydrolyzed to genistein by natural microflora in the intestine [26] and this is consistent with our findings where the concentration of genistein was higher (0.586 µg/ml) compared to 0. 392 µg/ml for daidzein.

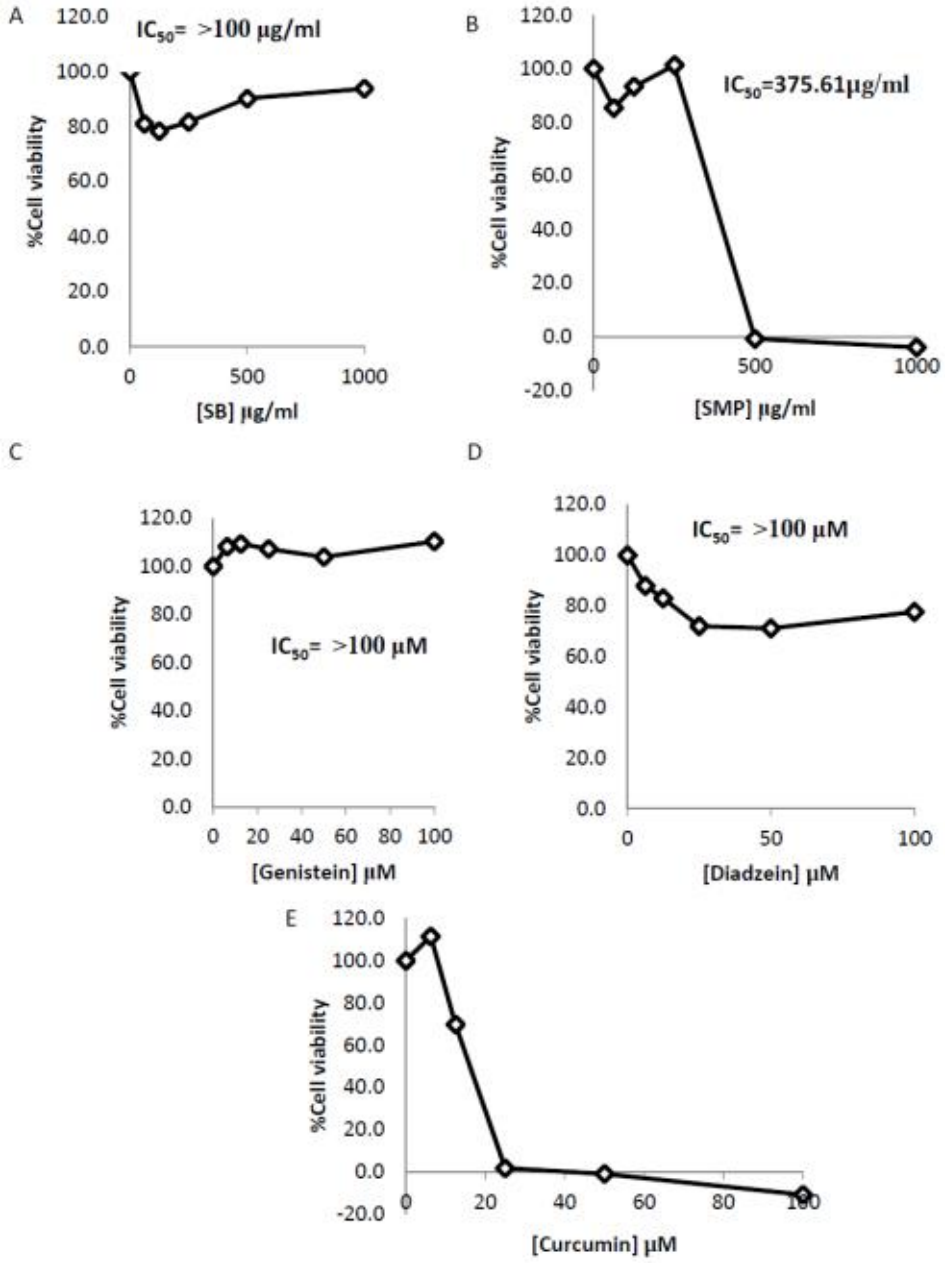


**Fig. 4. Percentage cell viability of SB (A), SMP (B), genistein (C), daidzein (D), and Curcumin (E) on *PNT2* Cell line**

**Table 3. Selectivity Indices of genistein, daidzein, SMP, SB and Curcumin**

| Sample | DG 75 | CEM |
|---|---|---|
| Genistein | >9.03 | >3.52 |
| Daidzein | 1 | 1 |
| SB | 1 | >1.16 |
| SMP | >1.94 | >6.95 |
| Curcumin | 1.52 | 0.87 |

Numerous mechanisms for isoflavones have been suggested for their inhibition of cancer cells. These include classical competitive activity to estrogen which has been considered in estrogen-related cancer prevention. Also, binding of isoflavones to endoplasmic reticulum which results in the inhibition of cell cycle in the prevention of cancer has been suggested [8]. In this study, it has been shown that genistein had a stronger inhibitory effects on the growth of lymphoma cell line DG-75 ($IC_{50}$= 296.6 mg/ml) compared to daidzein which gave an $IC_{50}$ value of 2542.3 mg/ml (Figs. 4c, d). Similarly the inhibitory effect of genistein on leukaemia cell line (CEM) was stronger ($IC_{50}$ = 767.5 mg/ml) than that of daidzein ($IC_{50}$= 2542.3 mg/ml). This observation is consistent with what was reported by Matsukawa et al. [27] that daidzein induced cell cycle arrest at G1, but genistein almost completely arrested the cell cycle progression at G2/M. Akiyama et al. [28] also studied the inhibitory action of genistein on protein tyrosine kinase and found out that genistein selectively showed strong inhibition on epidermal growth factor receptor, but daidzein did not show this inhibitory action. This goes to confirm that the inhibitory effect of genistein on cell growth is stronger than daidzein.

The growth inhibitory effect of the crude isoflavones isolated from SB and SMP were also explored. From Figs. 2 and 3, SMP had a greater inhibitory effect on both lymphoma and leukaemia cell lines compared to SB. This could probably be due to the higher concentration of isoflavone content of SMP compared SB as indicated in Table 1. Thus, the higher the isoflavone content, the greater its growth inhibitory effect.

Curcumin is a known potent anti-cancer agent and exerts its effect on various stages of cancer development, i.e. oncogene activation [29], cancer cell proliferation [30], apoptosis evasion [31], anoikis resistance [32] and metastasis [33]. This was therefore used as a standard drug for the anticancer assay to compare the anti-proliferative effect of the two isoflavones standards, SMP and SB. The $IC_{50}$ for curcumin was therefore less than all other standards and samples used for this analysis.

With respect to the SI for the lymphoma cell line (*DG 75*), genistein had the highest SI followed by SMP and curcumin. A drug/extract with SI>2 is adjudged to have a better selectivity index for a particular cancerous cell [25]. Therefore genistein and to some extent SMP had a best selectivity towards DG 75. The SI of the various extracts and standards were also evaluated for the leukaemia cell line (CEM). SMP had the best of the selectivity index followed by Genistein and then SB. This also clearly indicates that genistein and SMP were better anti-proliferative agents on the leukaemia cell line. SMP is therefore a potent candidate as a therapy for BL and Prostrate cancer.

## 5. CONCLUSION

In conclusion, isoflavones were isolated from SB and SMP with SMP having the highest concentration of Genistein (0.000586 mg/ml) and daidzein (0.00624 mg/ml). SMP had a better anti-proliferative on *DG-75*, (SI=1.94) and *CEM*, (SI=6.95). It is recommended that future studies looks at the combining effect of genistein and daidzein or any addition and their synergistic or antagonistic effect on lymphoma and prostate cancer cell lines.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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