Global Journal of Food Science and Nutrition

Research Article

doi: 10.39127/2475-2368/GJFSN:1000130
Ngoda PN, et al. Gl J Food Sci Nutri: GJFSN-130

Effect of Phytoestrogens as Endocrine-Disrupting Substances in Soybean-Based Gruels Using *In Vitro* MMV-Luc Cell Line

Peninah Njiraine Ngoda[1*], Christopher Elliot[2], Jackson Nkesiga[1], Lisa Connolly[2]

[1]Department of Dairy, Food Science and Technology, Egerton University, P.O Box 536 - 20115 Egerton, Njoro, Kenya
[2]Institute of Agri-Food and Land use, Queen's University Belfast, UK

***Corresponding author:** Peninah Njiraine Ngoda, Department of Dairy, Food Science and Technology, Egerton University, P.O Box 536 - 20115 Egerton, Njoro, Kenya. E-Mail: penjingoda@yahoo.co.uk

Citation: Ngoda PN, Elliot C, Nkesiga J, Connolly L (2023) Effect of Phytoestrogens as Endocrine-Disrupting Substances in Soybean-Based Gruels Using *In Vitro* MMV-Luc Cell Line. Gl J Foo Sci Nutri: GJFSN:130.

Received Date: February 27, 2023; **Accepted Date:** March 06, 2023; **Published Date:** March 10, 2023

Abstract

Soy-based weaning foods may be a source of endocrine-disruptive activity due to the abundant presence of phytoestrogens (PEs) in soy beans (Glycine Max. L.). PEs are weakly estrogenic compounds found in plants which mainly include lignans, isoflavones, prenylated phenols and coumarins. The bioactivity of PEs in soybean-based foods has not been fully explored. The aim of the study was to assess the endocrine-disrupting potential of different soy-based flours (at different heat and or storage states) which are commonly used in weaning gruels found in Kenya. The estrogenic bioactivity of the soy-based flour extracts was measured using previously developed reporter gene assays incorporating estrogen receptors. Agonist and antagonist bioactivity was compared to induction by the natural hormone 17β-estradiol (5 nM) hormone in the estrogen-responsive MMV-Luc cell line. Changes in the estrogenic activity of the flours after preparation as weaning gruels by heating at 100oC for half or full recommended time and storage at 20oC for 8 hrs were also assessed. The cooked flour blends were significantly estrogenic (their % maximum estrogenicity relative to the hormone was greater than 100%) as detected using the MMV-Luc cell line. Quantification of the estrogenicity of the flour extracts (both uncooked and those cooked and/or stored) on the MMV-Luc cell line revealed potent estrogenicity of (32.6 ± 6.6 to 617± 89.9 µg/kg of flour expressed as daidzein equivalents (DEQ). The effect of heat and storage on estrogenicity was flour blend specific. Upon being co-incubated with the hormone they had blend-specific effects on the estrogen hormone. Heat treatment and storage also had a flour blend-specific effect on the estrogenic activity in the MMV-Luc cell line. This could have important public health importance as infants and young children, have open developmental windows concerning sex organ development whose impact may be manifest in the future.

Keywords: *Phytoestrogens; Endocrine-disruptive; In Vitro MMV-Luc Cell Line; Transcriptive Activity; Soybeans-Based Gruels.*

Introduction

Soybean contains isoflavones, which are known to function in animals as phytoestrogens. Soybean isoflavones can have both beneficial and harmful effects on animals depending on the species, the age and sex of the animal, the dose, and the frequency of exposure [1]. Soybean bioactives such as isoflavones, phytosterols, and Bowman-Birk inhibitors have drawn more attention in recent years from a therapeutic perspective in inflammatory bowel diseases (IBD) because of their anti-inflammatory, anti-oxidative, and protective effects against intestinal permeability demonstrated in rodent models of IBD [2]. On the other hand, phytoestrogens are thought to be possible endocrine disruptors chemicals (EDCs), interfering with the proper operation of the reproductive and hormonal systems [3]. Endocrine-disrupting chemicals (EDCs) are substances found in the environment that interfere with hormone function. Some are naturally occurring, but the majority are synthetic materials that were dispersed without first being evaluated for their effects on human or animal health [4]. Endocrine-disrupting compounds (EDCs) are pervasive in both aquatic and terrestrial environments [5].

EDCs are a global challenge for the environment and human health. They are described as "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action." The number of chemicals with endocrine-acting characteristics is thought to be around 1000. EDCs include pesticides, fungicides, industrial chemicals, plasticizers, metals, pharmaceutical agents, phytoestrogens, personal care products, phenolic compounds found in some detergents and plastics, parabens as well as in food products and food packaging, and [6-8]. These substances are part of

a category of substances known as endocrine disruptors (EDCs). An EDC is a substance that can interfere with the production, secretion, transport, binding, action, or elimination of natural hormones in an organism or its offspring that are essential for the maintenance of homeostasis, reproduction, development, or behaviour of the organism [5]. EDCs known to mimic estrogen are widely recognized as estrogenic EDCs. Estrogen-receptor (ER) positive breast cancers are known to be at increased risk because of high levels of estrogen [6].

The environment contains EDCs that are naturally occurring. Endocrine-disrupting chemicals (EDCs) are a class of chemicals that can interact with numerous hormonal pathways in various ways. Exogenous compounds (EDCs) are substances or mixtures that can interact with the endocrine system and have negative effects on whole organisms, their offspring, or (sub) populations [9]. EDCs exposed in humans are primarily caused by ingestion, with some exposure also coming from inhalation and dermal uptake [8]. Our daily lives are filled with substances known as endocrine-disrupting molecules (EDCs). Growing epidemiological research demonstrates that EDCs may influence the onset or progression of breast cancer and consequently result in harmful health effects that last a lifetime, particularly when humans are exposed to a growing child [7].

There have been numerous studies on the endocrine-disrupting effects of PEs on animals and humans regarding isoflavones (0.01 µM -10 mM range) which include: the ability to cause goitre [10]; to defeminise the brain, affect the estrus cycle and affect ovarian function [11]; the lignan metabolite enterolactone affects estrogen signalling in both male and female rats and may impact gut microbiota, and circadian signalling [12]. It is well understood that gut microbiota converts plant lignans into more bioactive enterolignans, which are easily absorbed into the circulation and have a subsequent impact on health. The O-deglycosylation of plant lignans to the aglycone form of lignans and subsequent O-demethylation, dehydroxylation, and dehydrogenation of aglycones to produce enterolactone and/or enterodiol are key steps in this process [13].

In addition, rats exposed to genistein during pregnancy have a lower risk of developing non-alcoholic steatohepatitis, which is caused by a high-fat diet [14] and PEs have been shown to be anticarcinogenic in rats [15], among other public domain reports. According to Goldman *et al*. (1995) [16], infants and young children are a vulnerable subpopulation, particularly when it comes to formula and weaning foods made with soy [17,18]. EDCs may interfere with sex steroid hormone synthesis, action, and metabolism, which can lead to developmental and fertility issues, infertility, and hormone-sensitive cancers in both men and women [8]. Particular attention has been given to the negative effects of EDCs on the reproductive system over the past few decades. Energy homeostasis is disturbed as a result of the obesogenic effects of some EDCs. There have also been reports of interference with the hypothalamic-pituitary-thyroid and adrenal axes [8].

One of the main ways people are exposed to EDCs is through food. Other studies have successfully used complex chemical mixtures *in vitro* assays to measure exposure to estrogenic substances. One study concentrated on the estrogenic activity of water ([19]. The endocrine activity of fruits and vegetables using E-Screen has been investigated [20], while human serum has also been looked at [21]. A wide range of processed and packaged foods are being tested for their potential to expose people to estrogen, as a result of growing evidence showing how endocrine-disrupting chemicals (EDCs) affect humans [22].

Comparing studying the interactions of a single substance with a receptor to studying complex mixtures has the advantage of integrating mixture effects. By evaluating a complex mixture, people can look into potential endocrine disruptors that may not have previously been isolated from the mixture [23]. The development of breast cancer and mammary carcinogenesis depend heavily on estrogen receptor (ER) signalling. Progesterone receptor (PR), a molecule that is closely related to ER, is a crucial predictor of outcomes for endocrine therapies [24]. Further, endocrine-disrupting compounds (EDCs) are a new class of toxins that have negative effects on both human health and the environment. These endocrine disruptors are linked to diseases like cancer, cardiovascular risk, behavioural disorders, autoimmune defects, and reproductive diseases [25].

The prevalence of metabolic disorders has been found to be higher in areas where endocrine disruptors (EDCs) are present. A number of changes are brought about by exposure to EDCs, including microbial dysbiosis, the emergence of xenobiotic pathways, as well as related genes, enzymes, and metabolites involved in the metabolism of EDCs [26]. However, Scippo and Maghuin-Rogister (2007) [27] provided a thorough analysis of the presence of EDs in food and their potential health effects. They came up with the conclusion that while the investigation of mixture effects was a priority research area due to the additive effect of endocrine disruptors, individual low levels of EDs may be non-toxic. Another study by Ismail *et al*. (2021) [28] on the occurrence and distribution of endocrine-disrupting chemicals in mariculture fish and the human health implications found that there was no potential risk to the 36 consumers because the hazard index was below 1 (HI < 1).

The phytoestrogens may be used as a potent exogenous estrogen therapy alternative to premenopausal, postmenopausal, and menopausal women as well as to those women having low levels of endogenous estrogen due to the low estrogen phase of their monthly female reproductive cycles or due to intake of some medicines, like Tamoxifen [29]. Phytoestrogens are known as plant-derived substances that share structural similarities with endogenous

estrogens. Despite having the potential to be endocrine disruptors, studies have suggested that phytoestrogens may have some health benefits. As estrogens raise the incidence of breast, endometrial, and ovarian cancer, this is especially important for malignancies that are estrogen-dependent [30]. The current study revealed that climacteric syndrome symptoms could be reduced by consuming a cereal bar with phytoestrogens [31].

The natural hormone 17β-estradiol and PE genistein were tested singly. Results revealed that the combination of PEs and 17β-estradiol was additively agonistic such that the total estrogenicity was a sum of the estrogenicity due to PE and the estrogenicity due to the hormone. The study concluded that the PEs contribution to total estrogenicity was significant while for the synthetic compounds wasn't [32]. Many studies have shown that phytoestrogens have positive effects on lipid profiles, cognitive function, menopause, and oxidative stress, among other things [33]. A class of non-steroidal, polyphenolic plant-based compounds known as phytoestrogens are frequently used to treat menopause-related symptoms. They exhibit modest estrogen receptor (ER) affinity and preferentially bind to ER-B over ER-A, having both genomic and non-genomic effects [34].

One study sought to investigate the effect of the PE genistein at a level of 1 mg/kg b.w/day genistein and/or an antiandrogenic food contaminant (1 mg/kg b.w/day) vinclozolin, which is reported to affect male reproductive tract and fertility in adults was conducted by Lehraiki and colleagues [35]. This emphasizes how unpredictable results in complex mixtures might be compared to studies of single chemicals. Food extracts from soy-based gruel samples were used in this study. The net effect of pure PEs on ER reporter gene assay using MMV-Luc cell line has not been done by researchers. It has also not been done using reporter gene assays or other *in vitro* tools. The purpose of this study was to examine the bioactivity of estrogen in soy-based weaning gruel extracts using the MMV-Luc reporter gene cell lines, interaction with the natural hormone ligand 17β-estradiol (5 nM), and evaluation of uncooked, cooking time, and storage effects on the bioactivity.

Materials and Methods

Chemicals and Reagents

Dulbecco's Modified Eagles Medium (DMEM) (Cat. no. 61965-026), Penicillin 100 U/ml/ Streptomycin 100 μg/ml (Cat. no.15070-063), General Foetal bovine serum (FBS) (Cat. no. 10270-106), hormone deplete FBS (Cat. no. 12373-029) and trypsin (Cat. no.12604) were obtained from Invitrogen Ltd, Paisley, UK. Luciferase Assay System (Cat. No. E1501) consisting of Lyophilized luciferase Assay Substrate and Luciferase Assay Buffer, Cell Culture Lysis reagent 5X (Cat. no. E 194A, Promega, Southampton, UK); Trypan blue for automated counting (Cat. no. T 10282, Invitrogen Ltd, Paisley UK). Countess slides (Cat. no. 10283, Invitrogen, Paisley UK) Milli-Q water was of ultra- high purity (UHP, 18 MV/cm) from the Elgar water purifier (Marlow, Bucks, UK). The tissue flasks (25 and 75 cm^2) were ordered from BD Bioscience. The 50 ml, 20 ml, and 7 ml tubes were bought from SARSTEDT LTD. The 96 well plates were purchased from Greiner Bio-One (Frickenhausen, Germany). Glass bottles (Simax, Praha, Zech republic), Deacon 90 (East Sussex, UK), Virkon (Antec international, Sudbury, UK), DMSO (Dimethylsulfoxide) from Sigma-Aldricht, Poole Dorset, UK (Cat. no. D2650); Cryo-vial (Nalgene, Cat. no. 5000-1020, Roskilde, Denmark), Mr Frosty container (Cat. no.34863) and Iso- propanol (Cat. no.24137) both from Sigma Aldrich Poole Dorset, UK, PBS (phosphate buffer saline) (SAFC Biosciences Cat. No. 56064C, Lenexa, Kansas, USA); Acetic acid, Glacial, CH_3COOH, MW 60.05 g/mol, 99%, Sigma Aldrich; Hydrochloric acid, HCl, MW 36.46, sp gravity 1.18, Assay 35.4%, 11.4 M Vwr International ltd. Sodium Acetate Trihydrate, $CH_3COONa_3H_20$, MW136.08 g/mol, 99.5%, Sigma Aldrich, B-Glucorunidase Type H-5 from Helix Pomatia, 500 KU/20 ml lot number 049K3778, Sigma Aldrich, Diethyl ether,$(C_2H_5)_2O$, MW 74 g/mol.12, 99.5%, Sigma Aldrich, HPLC water, 18.01 g/mol, Sigma Aldrich; Methanol, CH_3OH, 32.04 g/mol, 99.7%, Sigma Aldrich; Cyclohexane, C_6H1_2, 84.16 g/mol, 99.7%, Sigma Aldrich; Ethyl acetate, $C_4H_8O_2$, 88.105 g/mol, 99.5%, Sigma Aldrich; Sigma C18 columns (2 g of stationary phase) and Silica (SiOH) columns (0.5 g of stationary phase) were from Phenomenex Ltd. 17-β estradiol (Cat. no. E2758) steroid hormones were from Sigma Chemicals, UK, Genistein 98% (Cat. no.G6649) Matairesinol 85 % (Cat. no. 40043) equol 99.0% (Cat. no. E45405), formononetin 99% (Cat. no. F47752), Apigenin 95% (Cat. no. A 3145), glycitein 98% (Cat. no. G2785), enterodiol, 99% (Cat. no. 45198), Daidzein 98% (Cat. no.D 7802), Daidzin 98% (Cat. no. 30408), Genistin (97%) (Cat. no. G0897), Acetone (Cat. no. 34850) and Methanol (Cat. no. M1175) were purchased from Sigma chemicals UK.

Sample procurement

The flour blends were sampled from supermarkets and market in Nakuru, Kenya. Random representative samples (5) were taken of each brand from different shops and supermarkets around Nakuru County. For market blends, the vendors were chosen at random from the Municipal market and 5 representative samples from various points in the container were taken for remixing later. A verbal declaration of what was in the mixture and how to prepare it was given by the vendor and noted down. The samples were then shipped to Queen's University (IAFLU) and kept at 4^oC before heat treatment. After opening and cooking and or storing the samples were kept at -80^oC. The samples taken at the market (SP5, SP6 and SP7) contained more legumes than soya bean flour in comparison to those which were bought in the shops. Sample SP9, pure Soya, was purchased in the supermarkets. Traditionally a product such as SP9 is not used alone but is bought to be mixed later at home with other flours.

Production of flour and soy-based weaning gruels processing

The flour and gruels were prepared according to the method described by Bello *et al*. (2020) [36], Oyegoke *et al*. (2021) [37] and Marcel *et al*. (2021) [38]. The weaning flours in this study are made by blending flours from dried grains, dried tubers and dried legumes with soybean flour. Soya bean *Glycine max.* L. in combination with other flours from dried grains of sorghum (*Sorghum bicolour*), millet (*Eleusine coracana*), pearl millet (*Pennisetum glaucum* (L) R. Br.) or maize (*Zea mays*). Other foods mixed up with soy to make weaning foods include legumes; green grams (*Vigna radiata*), pigeon peas (*Cajanus cajan*), Kidney beans (*Phaseolus vulgaris*) peanuts (*Arachis Hypogaea*), sweet potato (*Ipomoea batatas*) flour, cassava (*Manihot esculanta*) flour, Sugar, cooking oil and milk.

Heat treatment or cooking method

The flour blends were heat treated (100°C) by boiling them in HPLC-grade water. The cooking times were chosen by referring to the recommendations on the label for cooking or via personal communication on point of purchase at the market. Precise amounts of flour were measured out (usually 50 grams) and the amount of water required was added and noted down (usually about 200 ml depending on the flour). Raw samples were taken soon after mixing the flour with HPLC water and cooled immediately from room temperature to 4°C. Another sample was at half the time recommended ("half-cooked") which meant the half-way time of recommended time for that flour (5-15 mins) and cooled immediately. Cooked meant boiled for the full recommended time (10- 30 minutes) depending on the sample. After the full heat treatment (cooking) the last sample was divided into two and one part cooled immediately while the other was left out for 8 hours before cooling at -20°C. The samples were then lyophilized in a freeze drier (Modulyod-230, Davidson and Hardy Ltd UK) for 72 hrs. They were then stored at -80°C in jars until needed.

Sample extraction

The extraction procedure was an adaptation of that developed by Antignac *et al.* (2009) [39]. One gram of soya-based weaning flour in its different cooked or storage states was dispersed in 20 ml acetate buffer 2 M (pH 5.2) followed by enzymatic hydrolysis by incubating (52°C/ 16 hrs) with 300 µl of purified *Helix pomatia* preparation.

Table 1. Ingredients of the flour blends as declared by vendors or on the labels (% of flour).

Blends Proportions	SP1	SP2	SP3	SP4	SP5 Market mix	SP6 Market mix	SP7 Market Mix	SP8	SP9	SP10
Soybean	25	25	20	20	10.52	21	21	25	100	25
Maize		65	25		42	42	42	25	-	25
Refined maize	25	-	-	30	-	-	-	-	-	-
Wheat	-	10	-	-	-	-	-	-	-	-
Finger millet	25	-	20	15	10.52	10.52	10.52	25		25
Amaranth Seeds	-	-	-	-	-	-	5.26	25		25
Bullash millet	-	-	15	10	-	5.26	-	-	-	-
Sorghum	25	-	15	10	10.52	10.52	10.52	-	-	-
Groundnuts	-	-	5	5	5.26	5.26	5.26	-	-	-
Green grams	-	-	-	10	5.26	-	-	-	-	-
Black beans	-	-	-	-	5.26	5.26	5.26			
Pigeon peas	-	-	-	-	10.52	5.26	-	-	-	-
Calcium	Yes	-	-	-	-	-	-	-	-	-
Vitamins	Yes	Yes	-	-	-	-	-	Yes	-	-
Sugar	-	Yes	-	-	-	-	-	-	-	-
Minerals	-	Yes	-	-	-	-	-	Yes	-	-
Glucose	-	-	Yes	-	-	-	-	-	-	-

The mixture was then centrifuged (4000 rpm/15 mins). The sample was liquid/liquid extracted twice with 10 ml diethyl ether resulting in two layers, after centrifugation (3500 rpm/10 mins) the organic layers were collected in a glass test tube. The aqueous layer (± 20 ml) was acid hydrolysed (3.2 ml hydrochloric acid (HCl) 35%) and the solution vortexed and incubated (80°C/2 hrs). Subsequently, liquid/liquid extraction of hydrolysate was carried out twice using 10 ml diethyl ether. Centrifugation (3500 rpm/10 mins) followed with the organic layer being added to the one collected before and evaporated to dryness under an N_2 stream at 45°C. The sample was reconstituted in water/ methanol (90/10) (v/v). Purification was carried out on a Reverse Phase SPE (C18) cartridge that was packed with 2g of stationary phase. The cartridge was conditioned with 10 ml methanol and an equal volume of water. The sample was loaded and the cartridge was washed with 5 ml water and 5 ml cyclohexane before elution with 7 ml methanol. The eluted samples were evaporated to dryness under an N_2 stream at 45°C and reconstituted in 0.1 ml ethanol and 0.1 ml ethyl acetate before adding cyclohexane (0.8 ml) and vortexing. The flow diagram of this process is shown in

Figure 1. To ensure there were no estrogenic hormones that may mask the PEs effect, a normal Phase SPE (SiOH) silica cartridge (0.5 g stationary phase) was used. The cartridge retains the hormones as the PEs pass through. That PEs is present in the flour samples was verified in the laboratory using UPLC/MS but the method was validated more by Antignac and colleagues (Antignac et al., 2009). The cartridge was conditioned by cyclohexane. The sample was then deposited and the cartridge was washed with 10 ml ethyl acetate and cyclohexane (30:70, v/v). The sample was eluted with 10 ml ethyl acetate/cyclohexane/ethanol 80:10:10, v/v/v. The eluates were evaporated to dryness in a nitrogen stream at 45°C (N_2, 45°C), then reconstituted in 50 µl methanol/DMSO (95:5, v/v) before putting in an HPLC vial that had a 150 µl micro insert. The extracts were then used immediately in the reporter gene assay procedure or stored in the dark at -18°C [39].

Reporter Gene Assay

RGA analysis, cells culture and luciferase assay were determined as described by Puranik *et al*. (2019), Ngoda *et al*. (2023) and Willemsen *et al*. (2004) [40-41].

Agonistic and antagonistic effect

The procedure used for evaluating the (ant) agonistic effect of PE extract was done as described by Puranik *et al*. (2019), and Willemsen *et al*. (2004) [40,42].

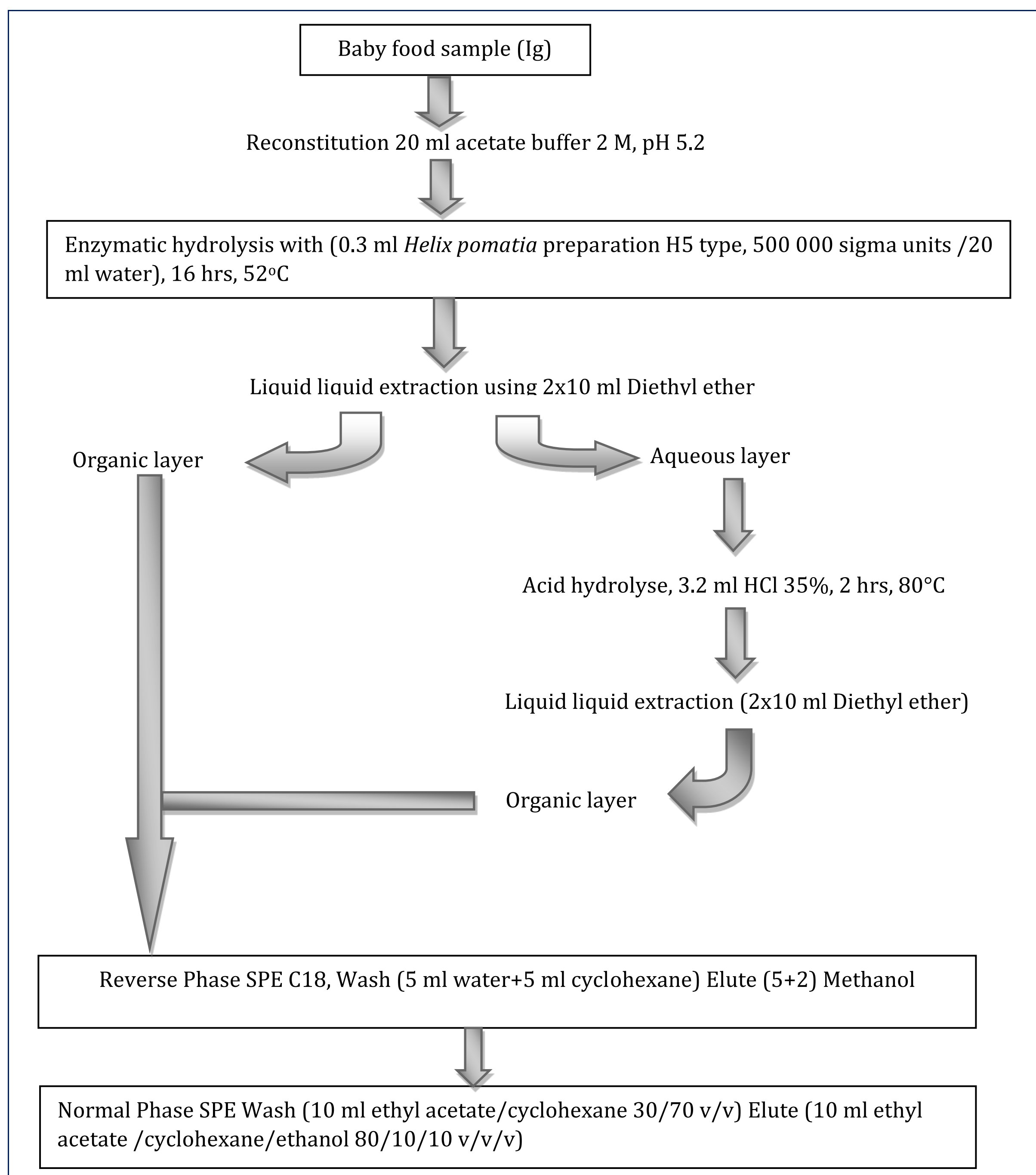

Figure 1. Flow Diagram Of Pe Extraction Procedure [39].

Cytotoxicity investigation using the MTT (3-(4, 5-Dimethyl-2 thiozolyl)-2, 5-diphenyl-2H-tetrazolium bromide) Assay
The extracts were assessed for cytotoxicity according to the method of Mosmann (1983), and Masuku *et al.* (2020) [43,44].

Extraction recoveries were calculated as per cent recoveries computed from the bioactivity of the extracted spiked daidzein compared to unextracted solvent standard daidzein at the same concentration.

$$\text{Recovery (\%)} = \left(\frac{\text{Average Calculated Spiked Concentration}}{\text{Average Expected Concentration}}\right) \times 100$$

Calculation of daidzein equivalents (DEQs) and Limit of quantitation (LOQ)
Calculation of DEQs was performed using non-linear interpolation from the daidzein-dose–response, best-fit curves. Study samples were assayed together on the same plate with a series of daidzein calibration standards. Positive quality controls, 17β-estradiol and negative control (0.05% MeOH in assay media) were on every plate as well. A negative control (solvent blank), pre-spike and post-spike of daidzein (4000 ng/ml) were incorporated in every extraction and on every plate for recovery determination. Bioactivity in fold induction over control (expressed as luciferase activity) was plotted on the Y-axis and the concentration on the x-axis. The y–intercept of the best-fit curve was used to calculate the daidzein equivalent concentration of the flour extract being analysed. The daidzein equivalents concentration was then corrected for recovery and any sample dilutions were factored in as well.

Limit of quantification (LOQ) was defined as the mean of all controls (Blanks) in the experiment plus three times the standard error of the mean (SEM). It was converted to estrogenic equivalents by multiplying by the daidzein potency relative to the standard 17β-estradiol.

UPLC-MS Analyses
Ultra-performance liquid chromatography/Mass spectrometer (UPLC-MS) was used to determine if the extracts had PEs. Nine of the 10 PEs were detected with the exemption of equol. However, quantitation was not done due to technological problems. UPLC-MS analyses were done using a Waters Acquity UPLC module interfaced with a Waters Premiere XE triple mass spectrometer (Manchester, UK). A C18 column (2.1 x 50 mm, 1.7 µm particle size (Waters, Manchester, UK) was used. The solvents used to elute the analytes were (A) HPLC water with 0.5% acetic acid (B) Methanol. The automated programming ensured the mobile phase composition was (A/B; v/v), 70:30 at 0 minutes to 0:100 which was held from minute 13 to 15 and then held at 70:30 from 20 to 30 mins for re-equilibration. The mobile phase flow rate was 0.3ml/min and the injected volume was 10 µl. Nitrogen was the nebulisation and cone gas whose flow rate was 1100 L/hr and 100 L/hr, respectively. The source and desolvation temperatures were at 130 and 400°C, respectively. The Mass Spectrometer system was operated in negative electrospray ionisation mode. A diagnostic signal was monitored for each PE using the capillary and cone potential in addition to the collision energy and acquisition parameters

Data Analysis
Data were subjected to analysis of variance (ANOVA) using GraphPad Prism version 5.00 for Windows (GraphPad Software Inc., San Diego, California, USA). All data were expressed as mean ± SD. Means were separated using Bonferroni post-tests at a 5% significant level. Means of at least three independent experiments (n =3) with each experimental point performed in triplicate. For reporter gene assays, normalised calibration curves were fitted with a sigmoidal dose-response curve equation, and values were reported as % maximal luciferase activity ± SEM.

Results

Dose-response curves are reported to show that the cell line could respond to the positive control 17β-estradiol. Daidzein dose response curve in MMV-Luc cell line.

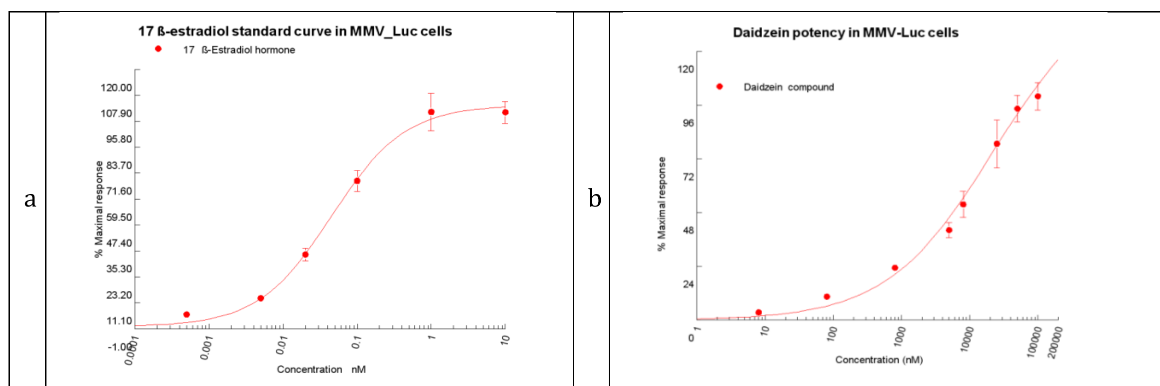

Figure 2. Dose-response standard curves of (a) 17β-estradiol and (b) Daidzein in MMV cell line that was treated with increasing concentration of the hormone or chemical daidzein and luciferase activity measured after 24 hrs. Values are % maximal luciferase activity ± SEM (normalised to 100%). Means of at least three independent experiments (n =3) with each experimental point performed in triplicate.

Maximum ant (agonism) achieved by the cooked flour blends without and in the presence of 17β-estradiol (E2) in MMV-Luc cells using reporter gene assay was as follows

Table 2. Maximum % estrogenic when MMV-Luc cells are exposed to flour extracts relative to hormone standards (17β-estradiol (5 nM) for an incubation time of 24 hrs.

Type of cooked flour blend (Sample)	Average Maximum % agonist TA in the absence of E2 (5 nM)	Average Maximum % ant(agonist) TA in the presence of E2 (5 nM)
SP1	***	*** (Agonist)
SP2	***	*** (Agonist)
SP3	***	*** (Agonist)
SP4	***	*** (Agonist)
SP5	***	*** (Agonist)
SP6	***	*** (Agonist)
SP7	***	**(Antagonistic)
SP8	***	*** (Agonist)
SP9	***	*** (Agonist)
SP10	***	*** (Agonist)

Key (determining number of stars)

*No effect - Slight agonist (< 25% induction Maximum of hormone), **Medium agonist (25-75 % induction Maximum of hormone); ***Strong agonist (> 75% induction Maximum of hormone) (Adapted from Willemsen *et al.,* 2004) [42].

Key (determining combinatorial effect with hormone (The word in brackets after stars)

Agonist (effect > than that for hormone alone, Antagonistic (the effect was less than that of the respective hormones)

Calculation of daidzein equivalents (DEQs) and Limit of quantitation (LOQ)

The LOQ for the weaning flours was 4.61 μg DEQ/kg for the MMV-Luc cell line which is equivalent when converted to estrogenic equivalents (EEQ) to 392 pg EEQ/Kg for the MMV-Luc cell line. This was lower compared to the LOQ of 0.91 ng EEQ /kg for food (meat, chocolates, bread) and 1.37 ng EEQ/kg for infant formula [45]. The slight difference could have arisen from the differences in the foods that were used in the two studies and the use of yeast estrogen assay while the MMV-Luc cell line derived from mammalian origin was used and hence more sensitive.

Recovery of daidzein as detected in MMV-Luc cell line

Daidzein percent recovery which was an average of 4 means, was 62.67% (**Table 4**).

Table 3. Percent daidzein compound recovery values.

Compound	Average calculated spiked concentration (ng/ml)	Average expected concentration (ng/ml)	% Recovery
Daidzein	2328	4000	58.25
	2529.2	4000	63.23
	2418.4	4000	60.46
	2750	4000	68.75
	2506.4	**4000**	**62.67**

Values are mean of triplicate readings of 3 experiments and each experimental point taken in triplicate

Quantification of flour's estrogenic activity in DEQs in MMV-Luc cells

Quantification of flour blend extract's estrogenic and androgenic bioactivity was determined in MMV-Luc cells in DEQs by measuring the expressed luciferase enzyme. It was preferable to use DEQs as opposed to hormone equivalents. This was due to the fact the extraction method used involved the use of silica columns whose purpose was to remove all hormones that would have masked the bioactivity of PEs. It would have been impossible to determine the recovery of the method as no spiked hormone would have been recovered. Daidzein equivalents or other PE equivalents would have been appropriate as it would enable comparisons of the values obtained by the two cell lines to be done.

For quantification, a daidzein calibration curve was used in every plate and the recovery efficiency of extracted daidzein was determined. Serial dilutions of PE extracts were made until concentrations that were not cytotoxic to the cells were obtained and the bioactivity of the flour blend was estimated. The final estimate was calculated by taking into account the dilution factor and recoveries that were observed. All experiments were performed in triplicate using triplicate samples in three independent experiments.

Effect of heat and storage on the bioactivity of SP1-SP4 Flour Blends in DEQ

There was a significant difference ($p<0.05$) among all tested samples (SP1, SP2, SP3, SP4, SP5, SP8, SP9 and SP10) except SP6 and SP7 (Table 4). Therefore, the null hypothesis was rejected and concluded that the mean bioactivity was not statistically identical when the flour blends SP1, SP2, SP3, SP4, SP5, SP8, SP9 and SP10 are Raw, Half-Cooked, Cooked and Stored (8 hrs). The increase in bioactivity for flour blend SP1 flour blend occurred only when the flour blend was cooked. Hence for this type of flour blend, half the recommended heat treatment (boiling) does not change the bioactivity significantly in the raw flour blend, and only boiling for the fully recommended time can lead to an increase in bioactivity. When the cooked flour blend is stored the gain in bioactivity obtained by cooking is lost, and the level of bioactivity becomes identical to the one in the raw flour blend. Therefore, for this type of flour blend, the storage has an ameliorating effect on the bioactivity. It would be interesting to see if all conditions on gruel safety and organoleptic characteristics can be met with the reduced heat treatment when one is trying to avoid bioactivity in the flour. Storing would also be recommended for this type of flour. This type of flour had very little bioactivity and the differences may not really affect that much. The graphical representation of the SP1 flour blend bioactivity is in Figure 3 a and the tabulated results are in Table 4.

For the SP2 flour blend in Figure 3 b, there was an increase in bioactivity when the raw flour blend was half cooked. However, there was no significant increase in bioactivity when the half cooked flour blend was fully cooked. On the other hand, storing the cooked flour blend resulted in an increase in bioactivity. Therefore, for this type of flour blend, the heat and storage of the cooked flour has an increasing effect on the bioactivity. For this flour blend there was a tendency for it to burn probably because of the wheat component. If one aims at decreasing the effects of increasing bioactivity then consuming the gruel immediately and avoiding storage would be a solution for SP2. The increase in bioactivity for the SP3 flour blend in Figure 3 c, occurs only when the flour blend is cooked for recommended time. Since boiling for half the recommended time does not significantly increase the bioactivity in the raw flour blend then it would be interesting to find out if the microbial quality, biochemical and acceptability considerations can be met at this level of heating and recommend that time of heating. Avoiding a full cook also would save on fuel. When the cooked flour blend is stored the gain in bioactivity obtained by cooking is lost, and the level of bioactivity becomes identical to the one in the raw flour blend. Therefore, for this type of flour blend, the storage has a reducing effect on the bioactivity. So encouragement on storing the gruel for eight hours before drinking can be given to those who want to avoid any increase in bioactivity. The SP3 flour blend however, had minimal estrogenicity just like SP1 and was mainly cereal based. Flour blend SP4 in **Figure 3 d**, the level of heat during the treatment has a significant effect in the bioactivity. The higher the heat level, the higher the bioactivity.

Table 4. Bioactivity of flour blends (μg/Kg of flour) in different flour blends in different states of heat and storing as characterised in MMV-Luc cell line.

Blends	Daidzein equivalents in Raw flour	DEQ when flour is boiled for half the recommended time	DEQ when flour is boiled for the fully recommended time	DEQ when flour is boiled for the fully recommended time and stored for 8 hrs at 20°C
SP1	$(32.65\pm 6.63)^{a}$	$(37.60 \pm 1.92)^{a}$	$(49.87 \pm 6.54)^{b}$	$(37.95 \pm 3.78)^{a}$
SP2	$(305.99 \pm 15.64)^{a}$	$(388.25 \pm 50.55)^{b}$	$(524.0 \pm 221.3)^{bc}$	$(617.53 \pm 89.98)^{cd}$
SP3	$(49.79 \pm 6.48)^{a}$	$(51.80 \pm 9.64)^{a}$	$(75.44 \pm 8.60)^{b}$	$(63.77 \pm 11.34)^{a}$
SP4	$(275.87 \pm 35.86)^{a}$	$(272.59 \pm 17.30)^{ac}$	$(380.05 \pm 97.4)^{b}$	$(282.17 \pm 24.52)^{b}$
SP5	$(271.38 \pm 22.93)^{a}$	$(295.10 \pm 26.16)^{b}$	$(260.49\pm 28.24)^{ac}$	$(302.14 \pm 22.50)^{bd}$
SP6	$(271.02 \pm 31.60)^{a}$	$(281.71 \pm 42.24)^{a}$	$(281.30 \pm 28.29)^{a}$	$(244.09 \pm 23.82)^{a}$
SP7	$(242.53 \pm 34.05)^{a}$	$(237.29 \pm 31.01)^{a}$	$(255.86 \pm 28.29)^{a}$	$(232.53\pm 14.57)^{a}$
SP8	$(308.76\pm 31.42)^{a}$	$(329.53 \pm 42.84)^{ac}$	$(453.71\pm29.73)^{b}$	$(377.38 \pm 29.02)^{d}$
SP9	$(2336.60 \pm 446.40)^{a}$	$(1876.38 \pm 170.27)^{b}$	$(3216.17\pm 311.41)^{c}$	$(3244.14 \pm 334.67)^{cd}$
SP10	$(337.00 \pm 27.95)^{a}$	$(283.10 \pm 69.42)^{ab}$	$(266.14 \pm 36.75)^{b}$	$(259.87 \pm 47.14)^{b}$

Values are (Means ± SD). Means in the same row with different alphabet (in superscript) are significantly different at $p<0.05$. The recommended time was 10 minutes for SP1, SP2 and SP10, 12.5 minutes for SP3 and SP4, 17.5 minutes for SP8 and lastly 30 minutes for SP5, SP6, SP7 and SP9.

On the other hand, the storage caused a loss in the gain in bioactivity during the full cook. For this type of flour investigation as to the safety and acceptability at half cook can be followed up to avoid increase in bioactivity.

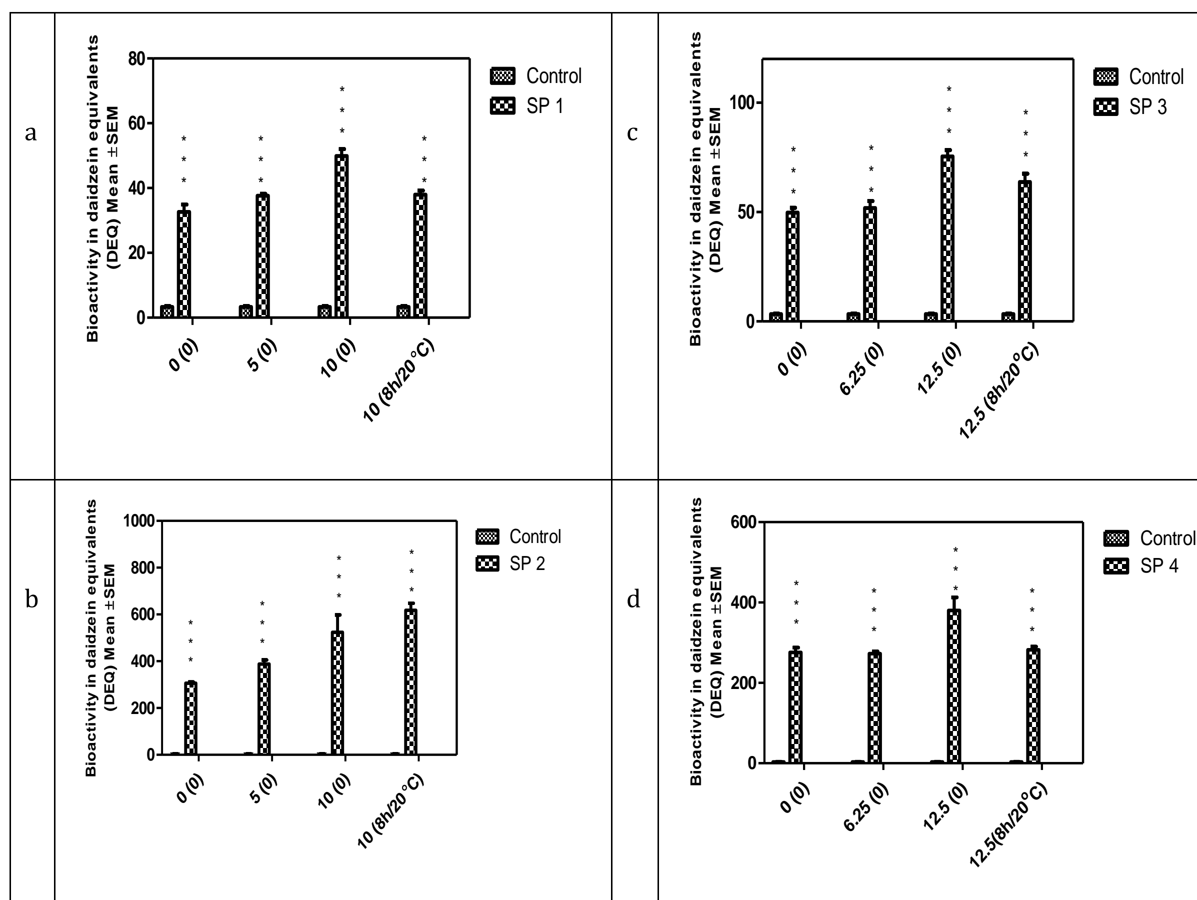

Figure 3. Effect of heat and storage on the bioactivity of flour blend extracts (a), (b), (c), (d), representing SP1-SP4, respectively, in MMV-Luc cells after 24 hrs exposure. Values are Means ± SD μg/kg of flour in DEQ (n = 3) (with each experimental point performed in triplicate) against time in minutes (storage/temperature of storage) *** means significantly different from control at p<0.001.

Effect of heat and storage on the bioactivity of SP5-SP10 Flour Blends in DEQ

For SP5 flour blend in Figure 4 e, the full cook heating had a decreasing effect on the bioactivity. However, the storage of the cooked flour blend has an increasing effect on the bioactivity. For those who want to avoid bioactivity gruel cooked for the recommended time and not stored would be the solution. For SP8 in Figure 4 h, the boiling at the recommended time leads to a significant increase in bioactivity while storing the cooked flour blend leads to a significant decrease in the bioactivity. Hence, for the SP8 flour blend, it would be interesting to see if the recommended time can be shortened to reduce the increase in bioactivity without jeopardizing safety and acceptability. Storage for eight hours decreased bioactivity significantly and hence can be a solution in reducing bioactivity for this kind of flour.

For SP9 flour blend in Figure 4 i, a significant level of heat leads to an increase in bioactivity while storing the cooked flour blend keeps the bioactivity unchanged. Hence, for SP9 flour blend, a high level of heat has an increasing effect on the bioactivity whereas the storage has no impact on the bioactivity. When SP10 flour blend (Figure 4 j) was fully cooked, there was a decrease in bioactivity while storing the cooked flour blend keeps the bioactivity level unchanged. Therefore, for this type of flour blend boiling at the recommended time has a reducing effect on the bioactivity whereas the storage of the cooked flour blend has no effect on the bioactivity. For this type of flour, it would be recommended to boil it for the full time and it wouldn't matter whether storage was done or the gruel was taken immediately. However, there was no significant difference ($p>0.05$) for samples SP6 and SP7 which implies that the null hypothesis is held. Hence, the mean bioactivity was statistically identical when SP6 and SP7 blends are raw, half-cooked, cooked or cooked and stored (Table 4). Therefore, for these type of flour blends, the heat and the storage of the cooked flour blend have no effect on the bioactivity (Figure 4 f, g).

Summary of the effect of heat on different cooked states and storage in MMV-Luc cell line

When the samples were half cooked 70% of them had their bioactivity unchanged from the raw flour, 20% had their bioactivity significantly increased while the other 10% had their bioactivity decreased. Upon fully cooking 60% had

their bioactivity increasing, 20% had their bioactivity decreased and 20% had their bioactivity remain the same. When the fully cooked samples were stored for 8 hrs at 20°C 50% of them registered no change in bioactivity, 30% decreased bioactivity and 20% had their bioactivity increasing (Figure 5).

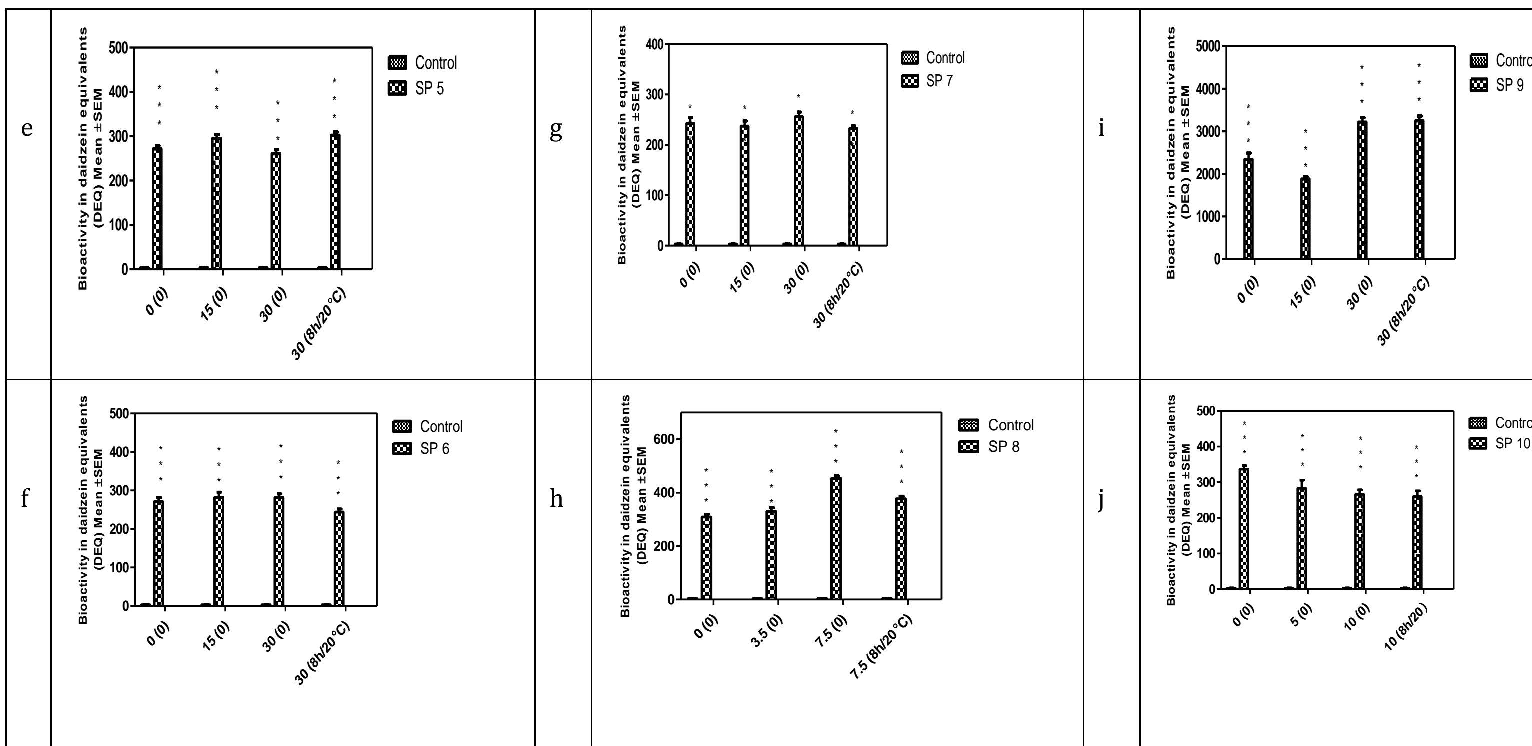

Figure 4. Effect of heat and storage on the bioactivity of flour blends extracts (e), (f), (g), (h), (i) and (j) representing SP5, SP6, SP7, SP8, SP9 and SP10, respectively, in MMV-Luc cells after 24 hrs exposure. Values are Means ± SD μg/kg of flour in DEQ (n = 3) (with each experimental point performed in triplicate) against time in minutes (storage/temperature of storage) *,*** means significantly different from control at p<0.05, p<0.001, respectively.

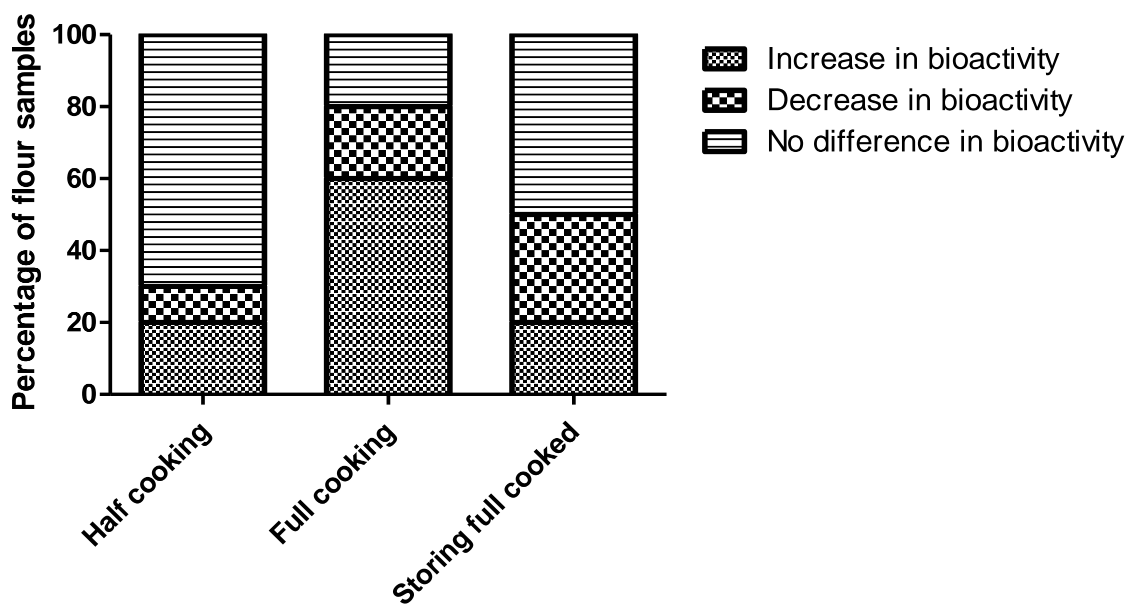

Figure 5. Effect of heat and storage on the samples' bioactivity is summarised as percentages of the total having either increase, decrease or no difference in bioactivity, in all the flour extracts tested on the MMV-Luc cell line (Figure 3-4).

Discussion

Effect Of Heating and Storing on Phytoestrogens

Effects of processing, heat treatments, and storage of soy/soy-products or PEs have been investigated by various researchers but estrogenic bioactivity of PEs in soybean and soybean-based food has not been fully explored. The seven classes that make up phytoestrogens are isoflavones, flavones, flavanones, chalcones, coumestans, lignans, and stilbenes. Isoflavones, lignans, and coumestans are the principal ones [33]. The extraction method is temperature sensitive, according to studies that examined isoflavone extraction strategies in various food matrices [45]; the same finding was reached when processing PE compounds at various temperatures [47,48]. According to Alide *et al*. (2020) [49] observations, cooking time had no significant impact on the phytochemicals and antioxidant activity, while cooking temperature had a significant impact on the total phenolic content and total flavonoid content. Engül *et al*. (2014) [50] discovered that different methods of cooking (boiling, steaming, stir-frying, and microwave cooking) had an impact on the overall polyphenol content and antioxidant activities of vegetables. It demonstrates that the effects of all cooking methods on the antioxidant and overall polyphenol content were more inconsistent. In general, cooked vegetables had lower antioxidant activity and total polyphenolic content than raw samples [50].

Maximum agonism and antagonism achieved of the cooked flour extracts in MMV-Luc cells the presence and absence of natural ligand estrogen hormone

Plants such as legumes, soybeans, beans, nuts, cereals, flax seeds, sesame seeds, hops, and other plants that may have estrogenic effects naturally contain phytoestrogens [41]. Since soybean use in human and animal food has become more internationalized, exposure to phytoestrogen has increased [51]. Consumption of soya-based flours in making weaning gruels is common in Kenya comprising 50% of the gruel-taking children in Nakuru in the current study. Infants and young children are vulnerable to EDC insults due to their critical windows of development being open. The concern about estrogenic is mainly to the neonate (0-28 days after birth) as organ development is still being effected 3-4 weeks post-natal [52]. Organs such as the mammary glands are organised at this time for later development at puberty [53]. Endocrine-disrupting chemicals (EDCs) are harmful because they mimic or block the binding of natural hormones to receptors, preventing the expected hormonal signalling. Receptors are consequently triggered without being needed [25]. The rising prevalence of metabolic diseases has been linked to endocrine disruptors. EDCs have been theorized to change the adipose tissue, pancreas, liver, gastrointestinal system, muscle, and brain homeostatic and hedonic pathways, which may enhance vulnerability to various conditions. The effects of EDCs on the gut microbiota have been shown in a small number of studies to raise the risk of metabolic diseases like obesity and diabetes [26].

According to different studies, consuming foods or beverages containing a lot of soy throughout adolescence can affect how the reproductive system develops, alter how menstruation occurs as an adult [54], and even predict menarche [55]. In addition, urogenital epithelium and uterine cells in infant females who have been fed soy formula since birth showed signs of an estrogen response, and the cells of the vagina had a greater maturation index when compared to infants fed cow-milk formula or breast milk. Also, females who were fed soy formula saw a slower decline in uterine volumes during the first few days of life [56]. Changes in the composition of the microbiota and changes in the production of microbial metabolites could have a significant effect on host metabolism and the emergence of diseases because the products and byproducts released following the microbial metabolism of EDCs can also be absorbed by the host. An additional strategy for the treatment and prevention of metabolic disorders could be the restoration of gut microbiota abnormalities brought on by EDC [26].

Animal and *in vitro* studies have demonstrated that either directly interacting with hormone receptors or through epigenetic and cell-cycle regulatory mechanisms, endocrine disruptive substances have an impact on the hormone-dependent pathways necessary for male and female gonadal development. The majority of research in human populations suggests a link between exposure to EDCs and diseases of the male and/or female reproductive systems, including infertility, endometriosis, breast cancer, testicular cancer, and poor sperm quality and/or function [57]. EDCs also raise the incidence of hormone-sensitive malignancies. They also induce diabetes, obesity, metabolic problems, and thyroid homeostasis. One of the main sources of numerous EDCs is sewage effluents, which eventually get to huge water bodies and may pollute the drinking water supply [58]. It seems inevitable that people will be exposed to these EDCs through food, and this could have serious long-term negative effects on people's health and wellbeing, especially in pregnant women, developing fetuses, and young children, who are frequently regarded as highly susceptible populations to EDC exposures. Many EDCs may affect the onset or course of breast cancer, according to a growing corpus of epidemiological research [7].

Estrogenic and antestrogenic activity MMV-Luc Cell line

Soybean-based food is employed as a protein source and also a high amount of phytoestrogens [58]. A range of phytoestrogens, including daidzein, zenistein, and biochalin A, are found in foods made from soy [3]. Since phytoestrogens and 17-β-estradiol share a structural resemblance, this is the primary route by which they may exercise their potential impacts on the body [33]. The biology of various cell types can be changed by estrogens and estrogen-like substances. Whereas G protein-coupled estrogen receptor 1 (GPER-1) is a non-classical estrogen receptor that is mostly found in the plasma membrane, estrogen receptors alpha (ERα) and beta (ERβ) are members of the so-known classical family of estrogen receptors [57]. The gruel extracts exhibited strong agonistic effects (the estrogenicity was at least > 75% of the hormone set at 100% using the key developed by Willemsen *et al.,* 2004) [42]. All

the cooked samples of the cooked samples with the exception of one (SP7) antagonistically acted agonistically with 17β-estradiol meaning the hormone expression was not antagonised in the presence of cooked PE extracts. This was not easily explained although the study noted that it was the only type of flour that had amaranthus seeds as a component that was unique to it compared to the other flour blends. The estrogenicity of the food sample extracts is in agreement with other studies which reported estrogenic activity in many food samples [45]. The hormone action not being inhibited by PEs is in agreement with a study where the natural hormone 17β-estradiol and a combination of PEs and 17β-estradiol were additively agonistic such that the total estrogenicity was a sum of the PE and the hormone [32]. This is of significance as that means that the PEs in the flour blend samples can act as endocrine disruptors on their own and may enhance hormone action. Our environment is full of estrogen-like compounds, including phytoestrogens and xenoestrogens. Estrogen-dependent changes in cell biology and tissue homeostasis have drawn interest in the study of human health and disease [59].

Phytoestrogens' ability to mimic estrogen hormones in baby food/soya milk has had controversial actual and potential health implications as observed in human and animal studies. This is exemplified in the case where continuous pre and post-natal exposure to high levels of soy phytoestrogens caused alterations in the onset of the vaginal opening, weight and size of the offspring and DNA methylation [60]. This can have evolutionary consequences. Studies involving PEs' possible linkages with the function and characteristics of selected reproductive tissues have been carried out. Several studies have documented significant effects on infants who eat foods containing soy. As estrogen receptors are found in all tissues and organs, including the central nervous system (CNS), they can influence cell survival, differentiation, and proliferation [59].

For example, in the Bernbaum *et al.,* 2008 [61], study, PEs were found to have a preserving effect on infantile breast tissue leading to a slower decrease in the size of the tissue after birth. Infant breast tissue is prominent at birth, probably due to maternal estrogens, but decreases with time. The said study showed that all babies had maximal breast tissue at birth, which waned as children grew older, but for soya-fed babies there seemed to be re-estrogenisation at 6 months. On the contrary, a study by Gilchrist *et al.,* 2010 [62] showed that there was no evidence of differences in breast-bud size, uterus, prostrate and testicular volumes in four-month-old soya-fed infants and infants not fed soya. Rowe and Baber (2021) [34] conducted a systematic review and meta-analysis of studies examining the effects of phytoestrogens on post-reproductive health and discovered that while many studies examining dietary and supplemental phytoestrogens have been carried out, the evidence of clinical efficacy is inconsistent and unreliable. With the consumption of phytoestrogen, there does seem to be a reduction in the vasomotor symptoms of menopause, although it is probably small and slow to start. Phytoestrogens also appear to improve markers of cardiovascular risk and bone mineral density. Breast, endometrial, or colorectal cancer appear to be unaffected by phytoestrogens, and phytoestrogen intake may even be beneficial (Rowe & Baber 2021) [34].

Another study that had some babies in their samples found no hormonal and metabolic disorders after feeding soy protein formula to children (7-96 moths) for six months. None of the enrolled girls showed signs of precocious puberty and none of the boys presented gynecomastia; bone age was within the normal range. The serum level of pertinent enzymes and hormones was normal as were the urinary biomarkers [63]. On the other hand observed the highest accumulation of equol and daidzein in the kidney, followed by liver, reproductive tract, thyroid and muscle from ewes that long-term exposure to red clover silage (daily intake of 157.6 mg/kg b.w. of phytoestrogens). Furthermore, ewes supplemented with subterranean clover (formononetin biochanin A, genistein, and daidzein) had pathophysiological and morphological modifications in their reproductive system, pituitary, adrenal, and thyroid glands. Moreover, genistein stimulates the expression of genes linked to the growth of breast cancer cells in women with estrogen-dependent breast cancer [63]. Genistein and daidzein may also be growth factors for human estrogen-dependent tumour cells, both *in vitro*. Genistein interacts with thyroid hormone receptors at nutritional doses (1 mM *in vitro*) [64] and inhibits thyroid peroxidase [65]. Men who received dietary isoflavone supplements for 10 weeks (100 mg/day) in the form of soy-containing meals such as soy milk drinks or puddings, soy flour, or soybeans showed enhanced cognitive performance. Following daily treatment with oral isoflavone (116 mg) in the form of capsules (68 mg daidzein, 12 mg genistein, and 36 mg glycitein) for six weeks, it was shown that men's spatial memory was greatly improved [66].

Products made from soybeans, which have high isoflavone concentrations, are the most typical sources of phytoestrogen. This substance, which shares structural similarities with estrogen, has the ability to either agonist or antagonist the estrogen receptor. Studies on animals show that phytoestrogen has major effects on sexual development, including delayed pubertal timing, reduced ovarian and estrous cycle function, and altered hypothalamic and pituitary activities [67]. Phytoestrogens have a variety of impacts on sexual function, according to a systematic review and meta-analysis that sought to examine how well they treat sexual disorders and the severity of dyspareunia. According to findings in the literature, maritime pine bark, T. foenum-graecum L., and F. vulgare could be used as treatments for sexual dysfunctions while soy, red clover, genistein, and flaxseed did not appear to have any promising results [11,68].

In addition, numerous research involving animals have shown that administering xenoestrogens, such as genistein, in the early postnatal period may affect the estrogen receptors in the brain [69]. Early exposure to phytoestrogens may have long-lasting effects on the development of the reproductive and other systems, as well as the differentiation of brain structures, neuronal networks, endocrine secretion, and behaviours [33]. Genistein could cause long-term neurobehavioral and endocrine changes [70]. However, according to Chang *et al*. (2021), genistein administration markedly enhanced the sucrose preference ratio, locomotor activity, and monoamines while lowering serum cortisol levels. In the brain tissue of control rats, the mRNA expression of brain-derived neurotrophic factor (BDNF) was markedly decreased by 0.73%. Nevertheless, genistein supplementation markedly elevated BDNF, and mRNA expression by 107% and 229.6%, respectively, in groups 10 mg and 100 mg. These findings show that genistein supplementation may be helpful in treating depression. Genistein reduced chronic inflammatory response in the vasculature prevented the inflammatory injury of vascular endothelial cells (VECs), and significantly alleviated inflammation, possibly through downregulating miR-21 [72].

Wei-Yun & Cailin (2021) [73] investigated the impact of genistein on hyperuricemia and renal protection in hyperuricemic mice and discovered that genistein at the dosage of 10 and 20 mg/kg b.w improved renal function, renal histological characteristics, and antioxidant activities. In hyperuricemic mice, genistein also reduced renal fibrosis by inhibiting the JAK2/STAT3 and Wnt/β-catenin signalling pathways. The research revealed that genistein may be utilized as a natural supplement to treat uric acid (UA)-related illnesses and has significant potential to prevent hyperuricemia and its associated disorders [73]. Šošić-Jurjević and colleagues found that isoflavones improved T3 availability in the liver of middle-aged (MA) males rats, despite reducing serum T4, by using thirteen-month-old Wistar rats and injecting subcutaneously with 35 mg/kg b.w./day of genistein, daidzein, or vehicle (controls) for four weeks. The liver's neutral route for converting cholesterol into bile acids may be activated as a result of an increase in T3 in the liver. Purified isoflavones do not provide a hypocholesterolemic impact but appear to be able to affect several targets involved in the control of cholesterol metabolism and oxysterol synthesis in a sex-specific manner [74]. Again, no clinically significant decreases in cerebrospinal fluid (CSF) heparan sulfate were linked with high doses of genistein aglycone (160 mg/kg/day), and no signs of therapeutic effectiveness were found. Nevertheless, urine glycosaminoglycan levels dropped statistically [75].

However, extrapolation of this data to potential effects on babies is sometimes difficult due to the difference in exposure routes, injection in rats while exposure in infants would be via feeding (orally) babies. Circulating levels of genistein in the neonate regardless of the dose have been found to predict future negative impacts on the female rat reproductive system; the maximum administered dose is 50 mg/kg b.w [11]. Another recent study found that soya and milk protein supplements (normal amount of feed - not specifically quantified) modulate mammary gland development in pre-pubertal mice [74]. Estrogens can have neuroprotective effects by functioning as anti-oxidants, fostering DNA repair, increasing the expression of growth factors, and regulating cerebral blood flow. Moreover, estrogen-dependent signalling pathways are involved in controlling the equilibrium between differentiation and proliferation of neural stem/progenitor cells (NSPCs), which affects neurogenic processes [58].

Quantification and effect of heat and storage on flour blend estrogenic activity in daidzein equivalents (DEQ /kg flour blend) as measured from expressed luciferase activity in MMV-Luc cell line

The high DEQs in some cooked sample extracts (SP2, SP3, and SP10) in the MMV-Luc cell line may have been due to the chemical modification of PEs. Isoflavones exist in various structural forms for example genistein in soy can exist as 6"-*O*-malonyl-β-glucoside, as - β glucoside, or as 6"-*O*-acetyl l-β-glucoside. Cooking soya foods alters glucoside conjugates depending on the type of cooking. The 6"-*O*-acetyl l-β–glucoside is most affected and easily converted to β glucoside in moist cooking such as gruel making [75]. If the gruel was burned as was the tendency with some of our samples SP3 and SP2 then more aglycones and a decrease in total isoflavones would have resulted [75]. The by-products of repeatedly heated cooking oils (RCO) contain polycyclic aromatic hydrocarbons (PAHs) and aldehydes, which are known to have cancer-causing, mutagenic, and tumour-causing qualities. It has been discovered through numerous experimental studies that a number of PAHs contain estrogenic or anti-estrogenic characteristics that initiate and activate steroidogenic pathways. Beno[a]pyrene, a compound with anti-estrogenic actions that are present in greater amounts in RCO, has anti-estrogenic qualities [76].

Of note, however, is that the different blends were affected differently by cooking and storage. The chemical composition and flour particle behaviour of each type of flour may have been different as different foods vary in isoflavone composition. The length of time the flours had been held at the granaries before milling before getting to the shops or market was not known. Storage is a factor in isoflavone levels in soy beans [77].

Estrogenicity of the flours ranged from 32.6 ± 6.61 μg/Kg to 617 ± 89.9 μg /Kg of the flour blend, expressed as daidzein equivalents (DEQ). This translates, in estrogen equivalents (EEQ) for the flours as 1.81± 0.487 ng/Kg – 43.90 ± 6.16 ng/Kg of the flour blend. The soya-cooked flour blend extract on its own had an estrogenic activity of a maximum of 3506.6 ± 518 μg/Kg of cooked flour blend (DEQ). This in estrogen equivalents is equal to 258.08 ± 24.60 ng/kg flour

blend. This was significant as to achieve a full estrogenic effect one needed DEQ of 24.81± 2.78 µg/Kg of flour blend that translates to values of 1.824 ± 0.204 ng/Kg estrogenic equivalents (EEQ) that were identical to 1.44 ng/ml (50 nM) of 17β-estradiol. All the flour blend samples had a DEQ of more than 24.81 µg/Kg. The Procedural blanks had low estrogenicity (3.29 ±0.430 µg/Kg of flour blend in DEQ), which translated to 300 ± 32 pg/Kg (EEQ). These values (1.81± 0.487 ng/Kg – 43.90 ± 6.16 ng/Kg EEQ) are higher than those reported by Behr *et al.,* 2011 for infant formulas which ranged from 14-22 ng/Kg EEQ as analysed with the yeast estrogen assay. The difference could have been due to their extraction method, the type of food extract that did not include soya and the use of different cell lines. While net bioactivity was considered for the total mixture of flour blend PEs, their study involved a totality of the infant food extract that included the proteins and possible steroid hormones which were removed in our extraction procedure by incorporating the acetate buffer to precipitate proteins and using of silica column to remove any hormones. When Morgan and colleagues examined the estrogenic activity of soybean parts, they found that it was particularly high in the tissues (roots, leaves, and shoots) that phytoestrogens use to engage in outreach activities like allelopathy, mutualist recruitment, defence, and synthesis for systemic distribution. In organs (flowers, seeds, and pods) that use phytoestrogens primarily for local regulation, there was less estrogenic action [78].

Behr *et al.*(2011) [45], reported the total dietary estrogen equivalents for infant food per day (net bioactivity) was 1.46 ng EEQ/day. This was from babies that were fed exclusively on soya-based milk at an assumed volume of 750 ml per day. In our case, the soya-based infant gruels were only part of a varied diet and the estrogenicity load was not concluded on the babies based on the total diet but can be reported on the estrogenicity load of the flour extracts in the weaning gruels sample (Behr *et al.,* 2011) [45]. In the Behr study, significant estrogenicity was detected in 60% of the infant formulas, by use of the YES (yeast estrogen screen) tool. In our study, significant estrogenicity was detected in 100% of the flour blend extracts ($p<0.0001$) which could be attributable to the presence of soya in our food samples. The sensitivity for the identification of phytoestrogens was enhanced in a yeast estrogen screening (YES) assay. The findings demonstrated the effectiveness of the newly designed YES techniques for simple high-throughput screening or detection of estrogen-like chemicals [82].

The flour blend mass consumed per day by the babies in Kenya was calculated and ranged from 0.5 to 70 grams with an average of 34.21 g/day. Although this may seem little, the fact that it is only part of the estrogenic load cannot be underestimated. Other sources of estrogenicity will come from other components of the gruel such as cow's milk, butter or other foods. The polycarbonate from baby bottles and other plastic utensils' may add considerable estrogenic load [83]. Breast milk may also add to the estrogenic load as it is known to have all kinds of chemicals from pesticides, herbicides and other estrogenic contaminants [84,86]. Androgenicity detected in daidzein equivalent when flour extracts are combined with hormone (testosterone) ranged from 367.5 ± 50 µg/kg of flour blend to below detection for one of the tested samples; sample SP 53. The soya flour blend had the highest androgenicity at 3506.76 ± 518 µg/Kg of the flour blend. Androgenicity of procedural blanks was 125.19 ±12.32 µg/Kg and hence the handling and the procedures did not add significant luciferase gene activity. The androgen cell line was however less sensitive than the estrogen cell line.

Our study would have to be collaborated with *in vivo* studies in animal models before concluding that the flour blends have estrogenic effects and proscribing the flour. However, going by other policies around the world about soya in baby food/milk the debate is contentious. Committees set up to look into the soya milk used as a weaning food in the US have reported no good reasons for using soya milk-based weaning foods over cow's milk and have cautioned on the potential adverse health effects which may result from using soya [85,86]. The American National Toxicology Program (USA-NTP) found that Sprague-Dawley rats treated to genistein (8–40 mg/kg b.w /day) had significantly more mammary and pituitary adenomas and carcinomas in their progeny [87]. Moreover, genistein stimulates the expression of genes linked to the growth of breast cancer cells in women with estrogen-dependent breast cancer [63]. Genistein and daidzein may also be growth factors for human estrogen-dependent tumour cells, both *in vitro* [89] and in animal models of xenograft nude mice [88]. Similarly, soy isoflavones demonstrated alteration in the mammary density in Western postmenopausal women [51].

In a meta-analysis to quantitatively assess the high soy isoflavone intake linked to an increased risk of uterine fibroids in premenopausal women, it was shown that high soy isoflavone intake or soy-based meals between infancy and adulthood were linked to an elevated risk of uterine fibroids [90]. In addition, consuming a lot of soy milk, soy-based foods, or giving genistein to infants can disrupt the dopaminergic system [91], nitrergic [90,93] and vasopressinergic systems [90], in the urogenital epithelium and the uterus, uterine volume [56], menstruation in the adulthood [54] and experimental research on mice have shown altered gene expression and microRNA profiles in the hippocampus and hypothalamus, indicating changes to neurobehavioral and neuroendocrine processes [71]. Phytoestrogens, such as genistein, may be helpful as part of a treatment plan for lung fibrosis caused by hydrochloric acid and chronic lung dysfunction since they are crucial in the etiology of pulmonary fibrosis [94].

The term "phytoestrogens" refers to a class of so-called functional dietary ingredients that are non-steroidal, secondary metabotypes that imitate or modulate the activity of endogenous estrogen and are produced by the

metabolism of plant-derived precursors. These molecules participate in a number of physiological and pathological processes involving metabolism, immunological response, skin, cardiovascular, neurological, and bone remodelling [33].

Along with ER activities, phytoestrogens have direct effects on androgen receptors in the brain that may effectively regulate neural circuit processes. Male mice given low phytoestrogen diets showed decreased long-term potentiation (LTP) in the ventral hippocampus, profound reductions in second messengers associated with plasticity in the hippocampus synapses, decreased inter-male aggression, altered territorial indication behaviour, and a general disruption of social behaviour [95,96]. Moreover, acute equol perfusion was able to reverse this LTP deficit, indicating a potential influence of phytoestrogen on hippocampal plasticity and memory [95]. Phytoestrogens have a number of advantages for the body, including antioxidants, neuroprotection, immune system improvement, and cardiovascular protection. After consumption, phytoestrogens have complex biological effects because of how the gut bacteria interact with them and how their metabolism differs, which affects how much of them are bioavailable [33]. Due to their antioxidant effects, phytoestrogen chemicals are already prescribed by doctors all over the world as supplements or food ingredients that promote health. According to numerous studies, eating foods high in phytoestrogens lowers your chance of developing menopause symptoms, cardiovascular disease, and a number of cancers, including prostate and uterine cancer [28]. Notwithstanding these health benefits, the current investigation by Lee *et al*. (2022) [30] found that phytoestrogens were positively related to breast and endometrial cancer, however, the relationships varied by phytoestrogen type. Due to the potential for acting as endocrine disruptors and posing a risk of negative health impacts, phytoestrogens' influence may also call into question their use [55,97]. It is crucial to proceed cautiously while outlining any risks or advantages for this reason. Because of this, there is endless controversy regarding the potential health advantages and hazards of phytoestrogens.

Conclusions and Recommendations

The reporter gene assay was applied to characterise the soya-based flour blend extracts in the estrogen cell lines. In MMV-Luc cells 9/10 of the cooked flour extracts were estrogenic and 1/10 was anti-estrogenic. Reporter gene assays enabled us to discriminate between antagonists and agonists. The reporter gene assays were able to discriminate the changes in bioactivity due to the co-incubation of PEs and hormone (17β-estradiol (5 nM) in the MMV-Luc cell line. These findings are of importance as the flour blends were capable of giving a full estrogenic effect in the MMV-Luc cell line. PEs have been associated with precocious puberty, ovarian differentiation alterations, cancer, and transgenerational effects on the urogenital tract amongst other effects. In males, PEs have been associated with increased reproductive disorders which include testicular cancers, reduced semen, prostate cancers, low testosterone levels, gynecomastia, reduced fecundity and fertility rates. These reproductive disorders have been interlinked to endocrine disruption in the neonatal, perinatal and postanal stages as tested mainly in animals. It would be wise to be cautious when using soya-based weaning foods.

There was a significant effect of heat treatment and storage for most of the flour samples. The effect was flour blend specific but in general, half-cooking did not affect the estrogenic activity of 70% of the flour samples, 20% had their bioactivity significantly increased while the other 10% had their bioactivity significantly decreased. Upon fully cooking 60% had their bioactivity increasing, 20% had their bioactivity decreased and 20% had their bioactivity remain the same. When the fully cooked samples were stored for 8 hrs at 20°C; 50% of them registered no change in bioactivity, 30% decreased bioactivity and 20% had their bioactivity increasing significantly. The effect of storing the gruel for 8h does not increase the bioactivity (80% of tested samples) but individual formulations would have to be tested for the effect is not uniform and the cause for the variations would require further physicochemical analyses. Different flour blends vary in isoflavone composition which may be altered by cooking. The altered composition of isoflavone conjugates will alter the rate of absorption and probably the metabolism outcome. This underlines the need for more care to be taken when making conclusions on data involving heat treatment of isoflavones. The estimated soya flour blend composition did not have a significant correlation with the bioactivities as the blends were too complex and in most cases the quantities declared were unverifiable.

On the other hand, an animal feeding study can be conducted to evaluate the toxicological end points of the flour mixes. Further studies involving the suitability of organoleptic, chemical and microbial characteristics of the gruel when cooked at half the recommended time as estrogenic activity is significantly lower than at the fully recommended time for 60% of the samples. In addition, chemical analyses may be carried out to identify the estrogenic and antiestrogenic compounds in the extracts using UPLC/MS, GC or HPLC and the fractions applied to cell lines. Finally, food modelling using flours from known varieties grown in research stations where climatic conditions, farming practices and storage time can be controlled prior to the application of the *in vitro* tests to the complex flour blend extracts.

Acknowledgements

I would like to thank the Commonwealth scholarship Commission in the UK, for funding the research.

Conflicts of Interest

The authors declare no conflicts of interest

Reference

1. Grgic, D., Varga, E., Novak, B., Müller, A. and Marko, D. (2021). Isoflavones in Animals: Metabolism and Effects in Livestock and Occurrence in Feed. Toxins, 13(12), 1-33. https://doi.org/10.3390/toxins13120836.
2. Basson, A.R., Ahmed, S., Almutairi, R., Seo, B. and Cominelli, F. (2021). Regulation of Intestinal Inflammation by Soybean and Soy-Derived Compounds. *Foods*, 10(4), 1-30. https://doi.org/10.3390/foods10040774
3. Kwack, S.J., Kim, K.B., Kim, H.S., Yoon, K.S. and Lee, B.M. (2009). Risk assessment of soybean-based phytoestrogens. *Journal of Toxicology and Environmental Health, Part A*; 72(21-22), 1254-61. DOI: 10.1080/15287390903212212
4. Darbre, D.P. (2019). The history of endocrine-disrupting chemicals. *ScienceDirect,* 2451-9650. https://doi.org/10.1016/j.coemr.2019.06.007
5. Marlatt, L.V., Bayen, S., Castaneda-Cort`es, D., Delb`es, G., Grigorova, P., Langlois, S.V., Martyniuk, J.C., Metcalfe, D.C., Parent, L., Rwigemera, A., Thomson, P. and Van Der Kraak, G. (2022). Impacts of endocrine disrupting chemicals on reproduction in wildlife and humans. *Environmental Research*, 208, 1-20. https://doi.org/10.1016/j.envres.2021.112584
6. Plessis, D.M., Fourie, C., Stone, W. and Engelbrecht, A. (2023). The impact of endocrine disrupting compounds and carcinogens in wastewater: Implications for breast cancer. *Biochimie*, 2023. https://doi.org/10.1016/j.biochi.2023.02.006.
7. Wan, Y.L.M., Co, A. V. & Hani El-Nezami (2021): Endocrine disrupting chemicals and breast cancer: a systematic review of epidemiological studies. *Critical Reviews in Food Science and Nutrition*, 1-29. https://doi.org/10.1080/10408398.2021.1903382
8. Yilmaz, B., Terekeci, H., Sandal, S. & Kelestimur, F. (2019). Endocrine disrupting chemicals: exposure, effects on human health, mechanism of action, models for testing and strategies for prevention. *Reviews in Endocrine and Metabolic Disorders,* 1-21. https://doi.org/10.1007/s11154-019-09521-z
9. Cediel-Ulloa, A., Lupu, L.D., Johansson, Y., Hinojosa, M., Özel, F. & Rüegg, J. (2022) Impact of endocrine disrupting chemicals on neurodevelopment: the need for better testing strategies for endocrine disruption-induced developmental neurotoxicity. *Expert Review of Endocrinology & Metabolism*, 17:2, 131-141, https://doi.org/10.1080/17446651.2022.2044788
10. Doerge, D. R., & Chang, H. C. (2002). Inactivation of thyroid peroxidase by soy isoflavones, *in vitro* and *in vivo*. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 777, 269–279. https://doi.org/10.1016/S1570-0232(02)00214-3
11. Kouki, T., Kishitake, M., Okamoto, M., Oosuka, I., Takebe, M. and Yamanouchi, K. (2003). Effects of neonatal treatment with phytoestrogens, genistein and daidzein, on sex difference in female rat brain function: estrous cycle and lordosis. *Hormones and behavior, 44*(2), 140-145. https://doi.org/10.1016/S0018-506X(03)00122-3
12. Damdimopoulou, P., Nurmi, T., Salminen, A., Damdimopoulos, A.E., Kotka, M., Van Der Saag, P., Strauss, L., Poutanen, M., Pongratz, I. and Makela, S. (2011). A single dose of enterolactone activates estrogen signaling and regulates expression of circadian clock genes in mice. *The Journal of nutrition, 141*(9), 1583-1589. DOI: 10.3945/jn.111.140277
13. Li, Y., Wang, F., Jun Li, J., Ivey, L.K., Wilkinson, E.J., Wang, D.D., Li, R., Liu, G., Eliassen, A.H., Chan, T.A., Clish, B.C., Huttenhower, C., Hu, B.K., Sun, Q. and Rimm, B. E. (2022). Dietary lignans, plasma enterolactone levels, and metabolic risk in men: exploring the role of the gut microbiome. *BMC Microbiology*, *22*(82), 1-14. https://doi.org/10.1186/s12866-022-02495-0
14. Huang, C., Qiao, X. and Dong, B. (2011). Neonatal exposure to genistein ameliorates high-fat diet-induced non-alcoholic steatohepatitis in rats. *British Journal of Nutrition, 106*(1), 105-113. DOI: 10.1017/S0007114510005799
15. Mabrok, H.B., Klopfleisch, R., Ghanem, K.Z., Clavel, T., Blaut, M. and Loh, G. (2012). Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model of breast cancer. *Carcinogenesis, 33*(1), 203-8. DOI: 10.1093/carcin/bgr256
16. Goldman, L.R. (1995). Case studies of environmental risks to children. *The Future of children / Center for the Future of Children, the David and Lucile Packard Foundation, 5*(2), 27-33. DOI:10.2307/1602355
17. Jefferson, W.N. and Williams, C.J. (2011). Circulating levels of genistein in the neonate, apart from dose and route, predict future adverse female reproductive outcomes. *Reproductive toxicology (Elmsford, N.Y.), 31*(3), 272-279. DOI: 10.1016/j.reprotox.2010.10.001
18. Jefferson, W.N., Padilla-Banks, E. and Newbold, R.R. (2007). Disruption of the developing female reproductive system by phytoestrogens: genistein as an example. *Molecular nutrition & food research, 51*(7), 832-844. DOI: 10.1002/mnfr.200600258
19. Fernandez, M.P., Buchanan, I.D. and Ikonomou, M.G. (2008). Seasonal variability of the reduction in estrogenic activity at a municipal WWTP. *Water research, 42*(12), 3075-3081. https://doi.org/10.1016/j.watres.2008.02.022
20. Schiliro, T., Gorrasi, I., Longo, A., Coluccia, S. and Gilli, G. (2011). Endocrine disrupting activity in fruits and vegetables evaluated with the E-screen assay in relation to pesticide residues. *The Journal of steroid biochemistry and molecular biology, 127*(1-2), 139-146. DOI: 10.1016/j.jsbmb.2011.03.002
21. Soto, A.M., Michaelson, C.L., Prechtl, N.V., Weill, B.C., Sonnenschein, C., Olea-Serrano, F. and Olea, N. (1998). Assays to measure estrogen and androgen agonists and antagonists. *Advances in Experimental Medicine and Biology,* 444, 9-23. DOI: 10.1007/978-1-4899-0089-0_3
22. Omoruyi I.M. and Pohjanvirta, R. (2017). Estrogenic Activities of Food Supplements and Beers as Assessed by a Yeast Bioreporter Assay. *Journal of Dietary Supplements,* *15*(5), 665-672. DOI: 10.1080/19390211.2017.1380104

23. Wagner, M. and Oehlmann, J. (2009). Endocrine disruptors in bottled mineral water: total estrogenic burden and migration from plastic bottles. *Environmental science and pollution research international, 16*(3), 278-286. https://doi.org/10.1007/s11356-009-0107-7
24. Fei, F., Siegal, P.G. and Wei, s. (2021). Characterization of estrogen receptor-low-positive breast cancer. *Breast Cancer Research and Treatment*, 1-11. https://doi.org/10.1007/s10549-021-06148-0
25. Ismanto, A., Hadibarata, T., Kristanti, A.R., Maslukah, L., Safinatunnajah, N. and Kusumastuti, W. (2022). Endocrine disrupting chemicals (EDCs) in environmental matrices: Occurrence, fate, health impact, physio-chemical and bioremediation technology. *Environmental Pollution*, 302. https://doi.org/10.1016/j.envpol.2022.119061
26. Gálvez-Ontiveros Y, Páez S, Monteagudo C, Rivas A. (2020). Endocrine Disruptors in Food: Impact on Gut Microbiota and Metabolic Diseases. *Nutrients*, *12*(4), 1-40. DOI: 10.3390/nu12041158
27. Scippo, M. and Maghuin-Rogister, G. (2007). Endocrine disruptors in food: potential impact on human health. *Annales de Medecine Veterinaire,* 151(1), 44-54.
28. Ismail, H.A.N., Aris, Z.A., Wee, Y.S., Nasir, M.H., Razak,R.M., Kamarulzaman, H.N. and Omar, T.F.T. (2021). Occurrence and distribution of endocrine-disrupting chemicals in mariculture fish and the human health implications. *Food Chemistry*, 345, https://doi.org/10.1016/j.foodchem.2020.128806.
29. Himanshu, V.B., Tandon A, Goyal, M., Mishra, R. and Deshpande, M. (2021). Neuroprotective effects of phytoestrogens: A potential alternative to estrogen therapy in alzheimer's disease patients. *Journal of geriatric mental health*, 8, 83-92. DOI: 10.4103/jgmh.jgmh_33_21
30. Lee, W.A., Poynor, V. and McEligot, J.A. (2022). Urinary Phytoestrogen Levels Are Associated with Female Hormonal Cancers: An Analysis of NHANES Data From 1999 to 2010. *Nutrition and Cancer*, *74*(8), 2748-2756, https://doi.org/10.1080/01635581.2021.2020304
31. Frigo, M., de Barros, E., dos Santos, B.C.P., Peres, L.G., Weber, J., Zanelatto, C. & Koehnlein, A.E. (2022) Effects of a Cereal Bar with a Combination of Phytoestrogens on the Climacteric Symptoms: A Placebo-Controlled, Randomized Trial. *Journal of the American Nutrition Association, 41*(3), 325-332. https://doi.org/10.1080/07315724.2021.1884143
32. Van Meeuwen, J.A., van den Berg, M., Sanderson, J.T., Verhoef, A. and Piersma, A.H. (2007). Estrogenic effects of mixtures of phyto- and synthetic chemicals on uterine growth of prepubertal rats. *Toxicology Letters*; 170(2), 165-176. https://doi.org/10.1016/j.toxlet.2007.03.003.
33. Petrine, C.J. and Del Bianco-Borges, B. (2020). The influence of phytoestrogens on different physiological and pathological processes: An overview. *Phytotherapy Research*. 2020; 1–18. https://doi.org/10.1002/ptr.6816
34. Rowe, J.I. & Baber, J.R. (2021). The effects of phytoestrogens on postmenopausal health. *Climacteric*, *24*(1), 57-63, https://doi.org/10.1080/13697137.2020.1863356
35. Lehraiki, A., Messiaen, S., Berges, R., Canivenc-Lavier, M.C., Auger, J., Habert, R. and Levacher, C. (2011). Antagonistic effects of gestational dietary exposure to low-dose vinclozolin and genistein on rat fetal germ cell development. *Reproductive toxicology (Elmsford, N.Y.),* 31(4), 424-430. https://doi.org/10.1016/j.reprotox.2010.12.005
36. Bello A.A., Gernah D.I., Ariahu C.C. and Ikya J.K, (2020). Physico-Chemical and Sensory Properties of Complementary Foods from Blends of Malted and Non-Malted Sorghum, Soybean and Moringa Oleifera Seed Flours. *American Journal of Food Science and Technology*, 8(1), 1-13. doi: 10.12691/ajfst-8-1-1.
37. Oyegoke, T.G., Adedayo, E.O., Fasuyi, F.O. and D. A. Oyegoke, D.A. (2021). Proximate Analysis and Sensory Evaluation of Complimentary Food Formulated from Yellow Maize, Soybean, Millet and Carrot Composite Flours. *Achievers Journal of Scientific Research*; 3(1), 144-151.
38. Marcel, M.R., Chacha, J.S. and Ofoedu, C.E. (2021). Nutritional evaluation of complementary porridge formulated from orange-fleshed sweet potato, amaranth grain, pumpkin seed, and soybean flours. *Food Science & Nutrition*; 10, 536–553. DOI: 10.1002/fsn3.2675.
39. Antignac, J., Gaudin-Hirret, I., Naegeli, H., Cariou, R., Elliott, C. and Le Bizec, B. (2009). Multi-functional sample preparation procedure for measuring phytoestrogens in milk, cereals, and baby-food by liquid-chromatography tandem mass spectrometry with subsequent determination of their estrogenic activity using transcriptomic assay. *Analytica Chimica Acta, 637*(1-2), 55-63. DOI: 10.1016/j.aca.2008.11.050
40. Puranik, V.N., Srivastava, P., Bhatt, G., Mary, J.S.J.D., Limaye, M.A. and Sivaraman, J. (2019). Determination and analysis of agonist and antagonist potential of naturally occurring flavonoids for estrogen receptor (ERα) by various parameters and molecular modelling approach. *Scientific Reports*, *9*(1), 1-11. https://doi.org/10.1038/s41598-019-43768-5 1
41. Ngoda, P.N., Elliot, C. and Connolly, L. (2023). Estrogenic Potencies of Phytoestrogens Commonly Found in Soybean (*Glycine Max*) Assessed by use of MMV-Luc Cell Line Bioassay. *American Journal of Food Sciences and Nutrition*; 5(1), 1 – 22. DOI: https://doi.org/*10.47672/ajfsn.1354*
42. Willemsen, P., Scippo, M.L., Kausel, G., Figueroa, J., Maghuin-Rogister, G., Martial, J.A. and Muller, M. (2004). Use of reporter cell lines for detection of endocrine-disrupter activity. *Analytical and bioanalytical chemistry*, *378*(3), 655-663. DOI: 10.1007/s00216-003-2217-2
43. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods,* 65(1-2), pp. 55-63.

44. Masuku, N.P, Unuofin, J.O. and Lebelo, S.L. (2020). Phytochemical content, antioxidant activities and androgenic properties of four South African medicinal plants. *Journal of Herbmed Pharmacology*; *9*(3), 245-256. Doi: 10.34172/jhp.2020.32
45. Behr, M., Oehlmann, J. and Wagner, M. (2011). Estrogens in the daily diet: in vitro analysis indicates that estrogenic activity is omnipresent in foodstuff and infant formula. *Food and Chemical Toxicology*; 49(10), 2681-8. DOI: 10.1016/j.fct.2011.07.039
46. Biesaga, M. (2011). Influence of extraction methods on stability of flavonoids. *Journal of Chromatography A,* 1218(18), 2505-2512. https://doi.org/10.1016/j.chroma.2011.02.059
47. Stintzing, F.C., Hoffmann, M. and Carle, R. (2006). Thermal degradation kinetics of isoflavone aglycones from soy and red clover. *Molecular nutrition & food research,* 50(4-5), 373-377. DOI: 10.1002/mnfr.200500187
48. Yue, X., Abdallah, A.M. and Xu, Z. (2010). Thermal dynamic properties of isoflavones during dry heating. *International Journal of Food Science and Technology*, 45(9), 1878-1882. https://doi.org/10.1111/j.1365-2621.2010.02346.x
49. Alide, T., Wangila, P. & Kiprop, A. (2020). Effect of cooking temperature and time on total phenolic content, total flavonoid content and total in vitro antioxidant activity of garlic. *BMC Res Notes*, 13(1), 1-7. https://doi.org/10.1186/s13104-020-05404-8
50. Şengül, M., Yildiz, H. & Kavaz, A. (2014) The Effect of Cooking on Total Polyphenolic Content and Antioxidant Activity of Selected Vegetables. *International Journal of Food Properties*, 17:3, 481-490. https://doi.org/10.1080/10942912.2011.619292
51. Isidoro, B., Lope, V., Whelan, D., Pedraz, C., Sánchez-Contador, C., Santamariña, C., … Pollán, M. (2016). Use of hormone therapy and isoflavones and mammographic density in Spain. *Menopause*, 23(5), 556–564. https://doi.org/10.1097/GME.0000000000000569
52. Dencker, L. and Eriksson, P. (1998). Susceptibility in utero and upon neonatal exposure. *Food additives and contaminants*, 15 Suppl, 37-43. DOI: 10.1080/02652039809374613
53. Russo, P.A. and Seidman, E. (1990). An unusual histiocytoid proliferation in infancy. *Human pathology,* 21(5), 564-567. https://doi.org/10.1016/0046-8177(90)90016-X
54. Upson, K., Harmon, Q. E., Laughlin-Tommaso, S. K., Umbach, D. M., & Baird, D. D. (2016). Soy-based infant formula feeding and heavy menstrual bleeding among young African American women. *Epidemiology*, 27(5), 716–725. https://doi.org/10.1097/EDE.0000000000000508
55. Csaba, G. (2018). Effect of endocrine disruptor phytoestrogens on the immune system: Present and future. *Acta Microbiologica et Immunologica Hungarica*, 65(1), 1–14. https://doi.org/10.1556/030.65.2018.018
56. Adgent, M. A., Umbach, D. M., Zemel, B. S., Kelly, A., Schall, J. I., Ford, E. G., … Stallings, V. A. (2018). A longitudinal study of estrogenresponsive tissues and hormone concentrations in infants fed soy formula. *Journal of Clinical Endocrinology and Metabolism*, 103(5), 1899–1909. https://doi.org/10.1210/jc.2017-02249
57. Kumar M, Sarma DK, Shubham S, Kumawat M, Verma V, Prakash A and Tiwari R (2020) Environmental Endocrine-Disrupting Chemical Exposure: Role in Non-Communicable Diseases. *Frontiers in Public Health*; 8, 1-28. doi: 10.3389/fpubh.2020.553850
58. Meusel, E., Menanteau-Ledouble, S., Naylor, M., Kaiser, H. and El-Matbouli, M. (2022). Gonad development in farmed male and female South African abalone, Haliotis midae, fed artificial and natural diets under a range of husbandry conditions. *Aquaculture International*; 30, 1279–1293. https://doi.org/10.1007/s10499-022-00850-6
59. Bustamante-Barrientos, F.A., Méndez-Ruette, M., Ortloff, A., Luz-Crawford, P., Rivera, F.J., Figueroa, C.D., Molina, L. and Bátiz, L.F. (2021). The Impact of Estrogen and Estrogen-Like Molecules in Neurogenesis and Neurodegeneration: Beneficial or Harmful? *Frontiers in Cellular Neuroscience*, 15, 1-19. DOI: 10.3389/fncel.2021.636176
60. Guerrero-Bosagna, C.M., Sabat, P., Valdovinos, F.S., Valladares, L.E. and Clark, S.J. (2008). Epigenetic and phenotypic changes result from a continuous pre and post-natal dietary exposure to phytoestrogens in an experimental population of mice. *BMC physiology*, 8(17), 1-19. https://doi.org/10.1186/1472-6793-8-17
61. Bernbaum, J.C., Umbach, D.M., Ragan, N.B., Ballard, J.L., Archer, J.I., Schmidt-Davis, H. and Rogan, W.J. (2008). Pilot studies of estrogen-related physical findings in infants. *Environmental health perspectives*, 116(3), 416-420. DOI: 10.1289/ehp.10409
62. Gilchrist, J.M., Moore, M.B., Andres, A., Estroff, J.A. and Badger, T.M. (2010). Ultrasonographic patterns of reproductive organs in infants fed soy formula: comparisons to infants fed breast milk and milk formula. *The Journal of pediatrics*, 156(2), 215-220. https://doi.org/10.1016/j.jpeds.2009.08.043
63. Giampietro, P.G., Bruno, G., Furcolo, G., Casati, A., Brunetti, E., Spadoni, G.L. and Galli, E. (2004). Soy protein formulas in children: no hormonal effects in long-term feeding. *Journal of pediatric endocrinology & metabolism: JPEM*, 17(2), 191-196. DOI: 10.1515/jpem.2004.17.2.191
64. Shike, M., Doane, A. S., Russo, L., Cabal, R., Reis-Filo, J., Gerald, W., Cody, H., Khanin, R., Bromberg, J. and Norton, L. (2014). The effects of soy supplementation on gene expression in breast cancer: A randomized placebo-controlled study. *Journal of the National Cancer Institute*, 106(9), 1–12. https://doi.org/10.1093/jnci/dju189
65. Hofmann, P. J., Schomburg, L., & Köhrle, J. (2009). Interference of endocrine disrupters with thyroid hormone receptor-dependent transactivation. *Toxicological Sciences*, 110(1), 125–137. https://doi.org/10.1093/toxsci/kfp086
66. Doerge, D.R. and Sheehan, D.M. (2002). Goitrogenic and estrogenic activity of soy isoflavones. *Environmental health perspectives, 110*(3), 349-353. DOI: 10.1289/ehp.02110s3349

67. Domańska, A., Orzechowski, A., Litwiniuk, A., Kalisz, M., Bik, W. and Baranowska-Bik, A. (2021). The beneficial role of natural endocrine disruptors: Phytoestrogens in Alzheimer's disease. *Oxidative Medicine and Cellular Longevity*; 2021:1-17. https://doi.org/10.1155/2021/3961445.
68. Kim, S.H. and Park, M.J. (2012). Effects of phytoestrogen on sexual development. *Korean Journal of Pediatrics*; 55(8), 265-71. DOI: 10.3345/kjp.2012.55.8.265
69. Najafi, N.M. & Ghazanfarpour, M. (2018). Effect of phytoestrogens on sexual function in menopausal women: a systematic review and meta-analysis. *Climacteric*, *21*(5), 437-445. https://doi.org/10.1080/13697137.2018.1472566
70. Marraudino, M., Farinetti, A., Arevalo, M. A., Gotti, S., Panzica, G. C., & Garcia-Segura, L. M. (2019). Sexually dimorphic effect of Genistein on hypothalamic neuronal differentiation in vitro. *International Journal of Molecular Sciences*, 20(10), 1–9. https://doi.org/10.3390/ijms20102465
71. Butler, M. C., Long, C. N., Kinkade, J. A., Green, M. T., Martin, R. E., Marshall, B. L., Willemse, T.E., Schenk, A.K., Mao, J. and Rosenfeld, C. S. (2020). Endocrine disruption of gene expression and microRNA profiles in hippocampus and hypothalamus of California mice: Association of gene expression changes with behavioural outcomes. *Journal of Neuroendocrinology*, 32, 1–22. https://doi.org/10.1111/jne.12847
72. Xie, X., Cong, L., Liu, S., Xiang, L., & Fu, X. (2021). Genistein alleviates chronic vascular inflammatory response via the miR-21/NF-κB p65 axis in lipopolysaccharide-treated mice. *Molecular Medicine Reports*, 23, 1-11. https://doi.org/10.3892/mmr.2021.11831
73. Wei-Yun, B. & Cailin, Z. (2021). Genistein ameliorates hyperuricemia-associated nephropathy in hyperuricemic mice. *Food and Agricultural Immunology*, 32(1), 778-797. https://doi.org/10.1080/09540105.2021.1996540
74. Šošić-Jurjević, B., Lütjohann, D., Renko, K., Filipović, B., Radulović, N., Ajdžanović, V., Trifunović, S., Nestorović, N., Živanović, J., Stojanoski, M.M., Köhrle, J. and Milošević, V. (2019). The isoflavones genistein and daidzein increase hepatic concentration of thyroid hormones and affect cholesterol metabolism in middle-aged male rats. *The Journal of Steroid Biochemistry and Molecular Biology*, 190, 1-10. https://doi.org/10.1016/j.jsbmb.2019.03.009
75. Ghosh, A., Rust, S., Langford-Smith, K., Weisberg, D., Canal, M., Breen, C., Hepburn, M., Tylee, K., Vaz, F.M., Vail, A., Wijburg, F., O'Leary, C., Parker, H., Wraith, J.E., Bigger, B.W. and Jones, S.A. (2021). High dose genistein in Sanfilippo syndrome: A randomised controlled trial. *Journal of Inherited Metabolic Disease*, 44(5):1248-1262. DOI: 10.1002/jimd.12407
76. Alston-Mills, B., Lepri, J.J. and Martin, C.A. (2011). Modulation of mammary gland development in pre-pubertal mice as affected by soya and milk protein supplements. *The British journal of nutrition*, 106(4), 502-509. doi:10.1017/S0007114511000389
77. Coward, L., Smith, M., Kirk, M. and Barnes, S. (1998). Chemical modification of isoflavones in soyfoods during cooking and processing. *American Journal of Clinical Nutrition*, 68(6), 1486S-1491S. https://doi.org/10.1093/ajcn/68.6.1486S
78. Ganesan, K. and Baojun Xu, B. (2020). Deep frying cooking oils promote the high risk of metastases in the breast-A critical review. *Food and Chemical Toxicology*, 144, 1-12. https://doi.org/10.1016/j.fct.2020.111648.
79. Lee, J.H. and Cho, K.M. (2012). Changes occurring in compositional components of black soybeans maintained at room temperature for different storage periods. *Food Chemistry*, 131(1), 161-169. https://doi.org/10.1016/j.foodchem.2011.08.052
80. Morgan, H.E., Dillaway, D. & Edwards, T.M. (2014) Estrogenicity of soybeans (Glycine max) varies by plant organ and developmental stage. *Endocrine Disruptors*, 2(1). e28490. https://doi.org/10.4161/endo.28490
81. Le, H.H., Carlson, E.M., Chua, J.P. and Belcher, S.M. (2008). Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. *Toxicology letters*, 176(2), 149-156. https://doi.org/10.1016/j.toxlet.2007.11.001
82. Sophon, N., Phiwpesh, C., Darith, S., Cherdshewasart, W. and Boonchird, C. (2021). Improved yeast-screening assay for phytoestrogens. *Bioscience, Biotechnology, and Biochemistry*, 85(6), 1422-1432. DOI: 10.1093/bbb/zbab051
83. Harris, C.A., Woolridge, M.W. and Hay, A.W. (2002). Organochlorine pesticide residues in breastmilk: difficulties in sample collection and in assessing intakes. *Food additives and contaminants*, 19(6), 547-554. DOI: 10.1080/02652030110113771
84. Dordevic, M., Sazdanovic, P., Dordevic, G. and Jovanovic, B. (2010). Morbidity in newborns exposed to organophosphorus pesticides. *Medicinski pregled*, 63(5-6), 414-417. DOI: 10.2298/mpns1006414d
85. Agostoni, C., Axelsson, I., Goulet, O., Koletzko, B., Michaelsen, K.F., Puntis, J., Rieu, D., Rigo, J., Shamir, R., Szajewska, H., Turck, D. And Espghan Committee on Nutrition. (2006). Soy protein infant formulae and follow-on formulae: a commentary by the ESPGHAN Committee on Nutrition. *Journal of pediatric gastroenterology and nutrition*, 42(4), 352-61. DOI: 10.1097/01.mpg.0000189358.38427.cd
86. Bhatia, J., Greer, F. and American Academy of Pediatrics Committee on Nutrition. (2008). Use of soy protein-based formulas in infant feeding. *Pediatrics*, 121(5), 1062-1068. https://doi.org/10.1542/peds.2008-0564
87. National Toxicology Program. (2008). Toxicology and carcinogenesis studies of genistein (Cas no. 446-72-0) in Sprague-Dawley rats (feed study). *National Toxicology Program Technical Report Series*, 545, 1–240
88. Du, M., Yang X., Hartman J. A., Cooke P. S., Doerge D. R., Ju Y. H., Helferich W. G. (2012). Low-dose dietary genistein negates the therapeutic effect of tamoxifen in athymic nude mice. *Carcinogenesis*, 33,(4), 895–901. http://dx.doi.org/10.1093/carcin/bgs017.

89. Carreau, C., Flouriot, G., Bennetau-Pelissero, C., & Potier, M. (2009). Respective contribution exerted by AF-1 and AF-2 transactivation functions in estrogen receptor α induced transcriptional activity by isoflavones and equol: Consequence on breast cancer cell proliferation. *Molecular Nutrition and Food Research*, 53(5), 652–658. https://doi.org/10.1002/mnfr.200800061
90. Qin, H., Lin, Z., Vásquez, E., Luan, X., Guo, F., & Xu, L. (2019). High soy isoflavone or soy-based foods intake during infancy and in adulthood is associated with an increased risk of uterine fibroids in premenopausal women: A meta-analysis. *Nutrition Research*, 71, 30–42. https://doi.org/10.1016/j.nutres.2019.06.002
91. Ponti, G., Farinetti, A., Marraudino, M., Panzica, G. C., & Gotti, S. (2019). Postnatal genistein administration selectively abolishes sexual dimorphism in specific hypothalamic dopaminergic system in mice. *Brain Research*, 1724, 1–10. https://doi.org/10.1016/j.brainres.2019.146434
92. Ponti, G., Rodriguez-Gomez, A., Farinetti, A., Marraudino, M., Filice, F., Foglio, B., Sciacca, G., Panzica, G.C. and Gotti, S. (2017). Early postnatal genistein administration permanently affects nitrergic and vasopressinergic systems in a sex specific way. *Neuroscience*, 346, 203–215. https://doi.org/10.1016/j.neuroscience.2017.01.024
93. Rodriguez-Gomez, A., Filice, F., Gotti, S., & Panzica, G. C. (2014). Perinatal exposure to genistein affects the normal development of anxiety and aggressive behaviors and nitric oxide system in CD1 male mice. *Physiology & Behavior*, 133, 107–114. https://doi.org/10.1016/j.physbeh.2014.05.020
94. Solopov, P., Biancatelli, R.M.L.C., Dimitropoulou, C. and Catravas, J.D. (2021). Dietary Phytoestrogens Ameliorate Hydrochloric Acid-Induced Chronic Lung Injury and Pulmonary Fibrosis in Mice. *Nutrients*; 13(10), 1-13. https://doi.org/10.3390/nu13103599
95. Çalıs¸kan, G., Raza, S. A., Demiray, Y. E., Kul, E., Sandhu, K. V., & Stork, O. (2019). Depletion of dietary phytoestrogens reduces hippocampal plasticity and contextual fear memory stability in adult male mouse. *Nutritional Neuroscience*, 8305, 1–12. https://doi.org/10.1080/1028415X.2019.1698826
96. Sandhu, K. V., Demiray, Y. E., Yanagawa, Y., & Stork, O. (2020). Dietary phytoestrogens modulate aggression and activity in social behaviour circuits of male mice. *Hormones and Behavior*, 119, 1–9.https://doi.org/10.1016/j.yhbeh.2019.104637.
97. Rietjens, I. M. C. M., Louisse, J., & Beekmann, K. (2017). The potential health effects of dietary phytoestrogens. *British Journal of Pharmacology*, 174(11), 1263–1280. https://doi.org/10.1111/bph.13622.
98. Setchell, K.D., Zimmer-Nechemias, L., Cai, J. and Heubi, J.E. (1998). Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. *The American Journal of Clinical Nutrition,* 68(6 Suppl), 1453S-1461S. DOI: 10.1093/ajcn/68.6.1453S.

Copyright: © 2023 *Ngoda PN. This Open Access Article is licensed under a Creative Commons Attribution 4.0 International (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.*