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Estrogenic Potencies of Phytoestrogens Commonly Found in Soybean (*Glycine Max*) Assessed by use of MMV-Luc Cell Line Bioassay

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Abstract

Purpose: Phytoestrogens (PE) are weakly estrogenic compounds synthesized in plants, especially in climatic adversity. The endocrine disruptive activity of PEs and some of their human metabolites are of concern as they have been associated with positive and more so negative health effects.

Methodology: The transcriptional activities (TAs) of ten PEs (apigenin, daidzein, daidzin, equol, enterodiol, formononetin, genistein, genistin, glycitein, and matairesinol) were measured as induction of the expressed luciferase activity which is correlates with biological effects. These effects were compared to transcription caused by 17β-estradiol (5 nM) hormone (standards) *in vitro*. This was done by the use of estrogenic receptors in the MMV-Luc cell line using a reporter gene assay tool.

Findings: Standard curves for hormone standards were determined and the EC_{50} for 17β-estradiol was (4.5×10^{-11}M). The order of PEs potency for the ER (ERα and ERβ) in the MMV-Luc cell line, as determined from the EC_{50}s (M) of their dose-response curves were in the following order: equol (4.9×10^{-7}M) > formononetin (5.7×10^{-6}M) > apigenin(2.4×10^{-6}M) > genistein (2.1×10^{-6}M) > daidzein(3.3×10^{-5}M). Standard curves for the rest were not achieved in the MMV-Luc cell line at the tested concentrations. The % estrogenic potencies of these compounds varied from 9×10^{-3}M for equol to 1.4×10^{-4}M for daidzein, relative to 17β-estradiol whose percent potency was arbitrarily assigned a value of 100. This study successfully characterized the TA of ten PEs in MMV-Luc cell lines. By achieving and exceeding a full estrogenic effect in MMV-Luc cells, it was indicative that the phytoestrogen transcriptive activity was most likely through non-receptor mechanisms such as enzymatic pathways.

Recommendations: Although the potential for endocrine disruption by PE was noted *in vitro*, this study would recommend further *in vivo* tests. This is because toxicokinetics involving metabolism and bioavailability affects the potencies of the PEs. Further *in vitro* assays may be designed to investigate the additive effects of phytoestrogens at physiologically relevant concentrations.

Keywords: *Phytoestrogens, Gene Assays, Health Effects, Hormones, Transcriptive*

INTRODUCTION

Phytoestrogens are naturally occurring, polyphenolic, non-steroidal plant compounds that are structurally and functionally similar to estrogens, particularly 17β-estradiol and have estrogenic and/or antiestrogenic effects (Aronson, 2016; Memariani *et al*., 2020; Padmanabhan *et al*., 2021). Phytoestrogens are present naturally in plants such as legumes, soybeans, beans, nuts, cereals, flax seeds, sesame seeds, hops, and other plants that may exert estrogenic actions (Mostrom and Evans, 2018). Phytoestrogens (PEs) are plant metabolites which include isoflavones, flavanones, coumestans and lignans. PEs level varies within different crops depending on their cultivars, agricultural practices, attack by pests and climactic conditions (Ku *et al*. 2022; Latif *et al*., 2020; Resende *et al*., 2013; Omidvari *et al*., 2022a).

Soybeans are known to be a good source of PEs (Kładna *et al*., 2016). Structurally, all PEs are diphenolic compounds which have weak estrogenic properties due to their structural similarity to hormones (Yang *et al*., 2019). A PE can bind to the receptor and induce a response that is of comparable strength to that caused by natural ligands such as a hormone. This kind of PE would be classified as 'potent' and a strong agonist (Hall *et al*., 2019; Liu e*t al*., 2019a; Norris *et al*., 1999). Some will induce the same response but at increased concentration levels, the weak agonists. Other agonists will never induce as high a response as the hormone no matter what but increasing the dose will stop further increase of induction as there will be death through cytotoxicity and a decrease in the magnitude of observed induction (Kuang *et al*., 2021; Nguyen & Osipo, 2022; Mousavi & Adlercreutz, 1992, Wu *et al*, 2021). These are called partial agonists. Some PE will bind the receptor and block any further bioactivity by the receptor in an antagonistic manner (Hwang *et al*., 2020; Lehraiki *et al*., 2011; Suo *et al*., 2022; Tanwar *et al*., 2021).

As PEs can influence the endocrine system function then they can be classified as endocrine disrupters (EDs). An EDC has been defined as a chemical whose primary effect is on the endocrine system via effects on receptor-mediated hormone action, hormone synthesis or clearance (Pickering & Sumpter, 2003). PEs at various concentrations (0.5- 1000 mg/kg BW/day), administered orally or by injection, have been associated with certain adverse effects upon consumption such as cancer, infertility, and premature thelarche, mainly in animal models (Cai *et al*., 2021; Omidvari *et al*., 2022b; Palanza *et al*., 2016, Sweeney *et al*., 2016; Van Duursen *et al*., 2011; Wu *et al* 2020). Conversely, PEs have been acclaimed as health-promoting agents which may improve cardiovascular health, ameliorate menopausal symptoms, promote better bone density, prevent cancer, act as antioxidants, boost the immune system and even help strengthen body defences against viruses (Du *et al*., 2020; Kładna *et al*., 2016; Russo *et al*., 2016; Rowe & Baber, 2021; Sun *et al*., 2012).

There are various *in vitro* tests have been used to assess PE endocrine activity. These assays are based on different endpoints such as endogenous protein expression (pS2, PR, vitellogenin) (Cooper, 2019; Ni *et al.*, 2010, Wang *et al*., 2021), enzyme activity (Fujii *et al*., 2023; Thagavel *et al.*, 2019), receptor binding assays (Hegazy *et al.*, 2011; Matsumura *et al.*, 2005; Skledar *et al*., 2020), cell proliferation (Du *et al*., 2020; Nguyen & Osipo, 2022; Reiter *et al.*, 2011; Onoda *et al.*, 2011) and transcription activation assays (Sim *et al*., 2022; Willemsen *et al.*, 2004). Jobling (1998) and Connolly *et al*., (2009) chose to apply reporter gene assays as gene expression is sensitive, easy to work with and are available. A limitation of *in vitro* assays is that they do not provide information on the metabolism, bioavailability or elimination of compounds as *in vivo* assays do (Connolly *et al.*, 2009).

On the other hand, EDCs may have structural similarities to hormones and can imitate indigenous hormones, have a similar mode of action, transport and storage in the body. Using

fluorescence microscopy Devin-Leclerc et al., (1998) found that the addition of agonists or antagonists led to ER-HSP90 dissociating and the chaperone relocating to the cytoplasm. Many EDCs can bind to the receptors and activate the receptor just like the hormone (estrogen mimics) resulting in transcription just as with hormones (Griekspoor *et al.,* 2007). Others cause estrogenicity by activating a different set of genes an example of which is where EDCs can also compete with the hormone for the binding site thus antagonising the hormone action and resulting in inhibition or reduction of the normal hormone action level.

In the case of the hormone estrogen the antagonists have been found to be either partial antagonists, where they act as antagonists in certain cells or certain promoter constructs and in estrogen receptors they are known as selective estrogen receptor modulators (SERMs). An EDC may interact in a manner that is opposite of what is expected in different cell contexts and which has been attributed to the availability of co-activators and co-repressors. Some antagonists consistently act as estrogen antagonists (full antagonists) no matter what type of cells are called selective estrogen receptor down regulators (SERDs). In part, they do that by targeting the receptor for degradation (Griekspoor *et al.,* 2007). In both cases, whether agonist or antagonist, binding changes the conformation of the ligand binding domains of the receptor which results in the effects that will be observed (Liu *et al*., 2019a; Ruff *et al.,* 2000).

Endocrine disrupters may also act in a dose-dependent manner to give different effects on the hormone receptor. Co-regulator proteins are important for transcription to occur. These coregulators are either coactivators or corepressors that either enhance or inhibit transactivation activity. They achieve these by the use of various enzymatic activities, such as those responsible for acetylation, methylation, ubiquitination and kinase activity. Ligand-dependent recruitment of co-regulators occurs through a hydrophobic cleft formed by helices 3, 4 and 12 in the AF-2 domain of the receptor (Gronemeyer *et al.,* 2004). In free receptors, this pocket is blocked by a short α-helix (H12) which is at the carboxy-terminal of the receptor. It prevents AF-2-mediated coregulator binding in the absence of a ligand. Upon hormone binding, this helix opens up to reveal a functional interface for coregulator recruitment through conserved LXXLL motifs in the cofactor. Antagonists induce a different structural change of H12 that blocks or modulates the recruitment of these essential coregulators (Griekspoor *et al.,* 2007).

However, not all coregulator binding occurs through the AF-2 region. Other structural changes within the receptor and dimerization are involved in coregulator recruitment. The AF-1 region of SHRs is important in the ligand-independent binding of coregulators. The exact coregulator requirements for transcription are dependent on cell type, and probably also on ligand and promoter context. This explains the variation in properties of some EDCs whereby, they can be partial antagonists in one tissue while having antagonistic properties or exhibit agonistic properties in others. Shaufele and colleagues showed that the receptor conformation is different for various ligands, which significantly influences the binding of specific coregulatory proteins (Schaufele *et al.,* 2000). Minute structural differences between the LBD of ERα and ERβ can result in profound differences in SRC-1 recruitment with the same ligand (Margeat *et al.,* 2003).

The current study's interest was in the receptor transcriptional activity of dietary PEs (apigenin, daidzein, daidzin, enterolactone, equol, formononetin, genistein, genistin, glycitein and matairesinol) in MMV-Luc cell lines. This method has the ability to determine the agonists and antagonists. The choice of the PEs was based on the fact that they are found in soya flour or cereal flour or are human metabolites of dietary PEs. The soya ones include genistin which constitutes 55-65% of isoflavones in soya, daidzin which constitutes 30-35% of isoflavones while glycitein, genistein, daidzein, and formononetin make up less than 10% of isoflavones

(Jefferson *et al*., 2012). Phytoestrogens consumption are rising quickly around the world, but their effects on human health are not yet fully explored. The objective of this study was to get the standard normalized curves of phytoestrogens and also to find out if the MMV-Luc cell line is sensitive to phytoestrogens.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's Modified Eagles Medium (DMEM) (Cat. No. 61965-026), Penicillin 100U/ml/ Streptomycin 100µg/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) was from (Promega, Southampton, UK); Trypan blue for automated counting (Cat. No. T 10282) from Invitrogen Ltd, Paisley UK). Milli-Q water was of ultra-high purity (UHP, 18MV/cm) from the Elgar water purifier (Marlow, Bucks, UK), DMSO (Dimethylsulfoxide) from Sigma-Aldricht, Poole Dorset, UK (Cat. No. D2650); PBS (phosphate buffer saline) (SAFC Biosciences Cat. No. 56064C, Lenexa, Kansas, USA); HPLC water, 18.01g/mol, SigmaAldrich; Methanol,CH_3OH, 32.04g/mol, 99.7%, SigmaAldrich; estradiol (Cat. No. E2758) steroid hormone was 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) and Methanol (Cat. No. M1175) were purchased from Sigma chemicals UK.

Reporter Gene Assay

Estrogen-responsive (MMV-Luc) cell lines were as previously produced (Willemsen *et al.*, 2004). Prior to running the assay, cells were cultured for at least two passages in hormone-free assay media (DMEM, 10% hormone-depleted serum to remove endogenous hormones). The estrogen assay was performed using phenol red-free DMEM due to the estrogenicity of phenol red. Cells were seeded at a concentration of 4×10^5 cells/ml, into white-walled 96 well plates with clear flat bottoms (Greiner Bio-One, Frickenhausen, Germany**)** at 100µl/well and incubated for 24 hrs. The following day, standards were prepared 1:100 (v/v) by adding 10 µl of the relevant steroid hormone to 1 ml of assay media giving a final methanol concentration of 0.5%. The following standards hormone (17β-estradiol) and PE concentrations used are shown in table 1. Antagonist tests were carried out by incubating the PEs and (their metabolites) at the shown concentrations table 1 with 5 nM of 17β-estradiol (MMV-Luc). The cells were incubated for 24 hrs for the MMV-Luc cell line.

Table 1: Concentrations used for reporter gene assays

Compound	Concentrations used in	Compound	Concentrations used in
17β-estradiol	0.05pM-10 nM	Equol	0.25 nM – 20 µM
Apigenin	0.25 nM – 80 µM	Formononetin	0.5 nM – 115 µM
Daidzein	0.25 nM – 50 µM	Genistein	0.5 nM – 150 µM
Daidzin	0.1 nM – 20 µM	Genistin	0.1 nM-20 µM
Enterodiol	0.5 nM – 10 µM	Glycitein	1.0 nM – 100 µM
Matairesinol	0.5 nM – 80 µM		

The supernatant was discarded and the cells were washed twice with phosphate-buffered saline (PBS) prior to lyses with 20 µl cell culture lysis buffer (Promega, Southampton, UK). Finally, 100 µl luciferase (Promega, Southampton, UK) was injected into each well and luciferase activity was measured using the Mithras Multimode Reader (Berthold, Other, Germany). All experiments were performed thrice (n=3) for all cell lines and the transcriptional activity of the various compounds was measured and compared with the negative control (0.5% methanol in media). Each experimental point was performed in triplicate (three wells on the plate). The % Relative potencies for the PEs were calculated as:

$$\text{Relative potencies (\%)} = \frac{\text{EC}_{50} \text{ for the hormone}}{\text{EC}_{50} \text{ for the PE}} \times 100$$

The dose-response curves were plotted by means of triplicate readings for a minimum of three experiments.

Cytotoxicity Assay

PE standard or hormone concentration as that used for reporter gene assays was tested for cytotoxicity. After the requisite cell exposure to hormone or PE for 24hrs in the estrogen cell line respectively, the medium was aspirated from the wells and discarded. In this assay MTT solution (5 mg in 20 ml of PBS) was diluted in media (1:2.5) and 50 µl of this dilution was added to each of the seeded wells of a 96-well plate (BD Science). It was then wrapped in tin foil and incubated for four hours at 37^{o}C/8% CO_2 after which excess MTT was removed and 200 µl DMSO was added. It was then shaken for 10 minutes and read in the plate reader at a wavelength of 570 nm. For cytotoxicity analysis, the toxic effect was derived from the absorbance values using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{(\text{B} - \text{A})}{\text{B}} \times 100$$

Where B is the mean absorbance of negative control and A is the mean absorbance of the sample

Data Analysis

Data were analyzed using Slidewrite Plus V6 software. The RGA data was fitted using the sigmoidal dose-response curve equation, where X isthe logarithm of concentration, Y the response, and bottom and top are fixed to 0% and 100% respectively of the maximum response:

$$\text{Y} = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{(\log \text{EC}_{50} - \text{X})}}$$

Fold induction was calculated as the ratio of a response when compared to the negative control (n-fold). The concentration that produced a 50% increase in maximal response (EC_{50}) was used as a measurement of the assay's sensitivity (Willemsen *et al.*, 2004, Cai *et al*., 2012).Values are Mean fold induction ± SD of three independent experiments (n = 3) with each experimental point performed in triplicate.

RESULTS

Non-normalized Dose-Response Curves in MMV-Luc Cells

The un-normalized dose-response curves shown in figure 1 depict the five phytoestrogens were weak agonists compared to the 17β-estradiol. This is because increasingly high concentrations of PEs (~100 nM) were required for any agonist activity to be induced by the phytoestrogens.

Consequently, higher individual PE concentration (> 500 nM) was required to achieve a full estrogenic effect. From the same figure 1 the dotted line through 17β-estradiol maximum fold induction level, intercepts the other curves at various concentrations which shows that equol required a concentration of 521 nM, genistein 1.212 µM, apigenin 2.239 µM, formononetin 2.818 µM while daidzein required 7.079 µM. That is compared to 1 nM of 17β-estradiol hormones. The PEs, however, achieved superagonist status (transcription activity was higher than that of 17β-estradiol (5 nM). Apigenin achieved an average maximum fold induction of 15, daidzein 12, formononetin 10, equol 9, and genistein 17 at the tested concentrations. This was against a maximum average fold induction of about 5for 17β-estradiol at its' best performance in the MMV-Luc cell line.

Four other PEs matairesinol, enterodiol, genistin, and daidzin standard curves were not achieved in MMV-Luc cell lines at the tested concentration. They exhibited maximum medium agonistic activity which varied from 27- 50% of17β-estradiol (5 nM) induction whose induction had arbitrarily been assigned 100%. These can be classified as partial agonists (Mostrom and Evans, 2018; Woods, 2003). The four had no trend to enable a proper curve to be fitted. The tenth PE glycitein was anti-estrogenic in the MMV-Luc cell line at the tested concentrations and had very slightly agonistic activity at a maximum 20% induction of 17β-estradiol (5nM) induction whose induction had arbitrarily been assigned 100%**.** The limitation of these non-normalized curves is that the slope of the curve and height is not taken into account for a more standardized comparison and hence the use of normalised curves in the next section.

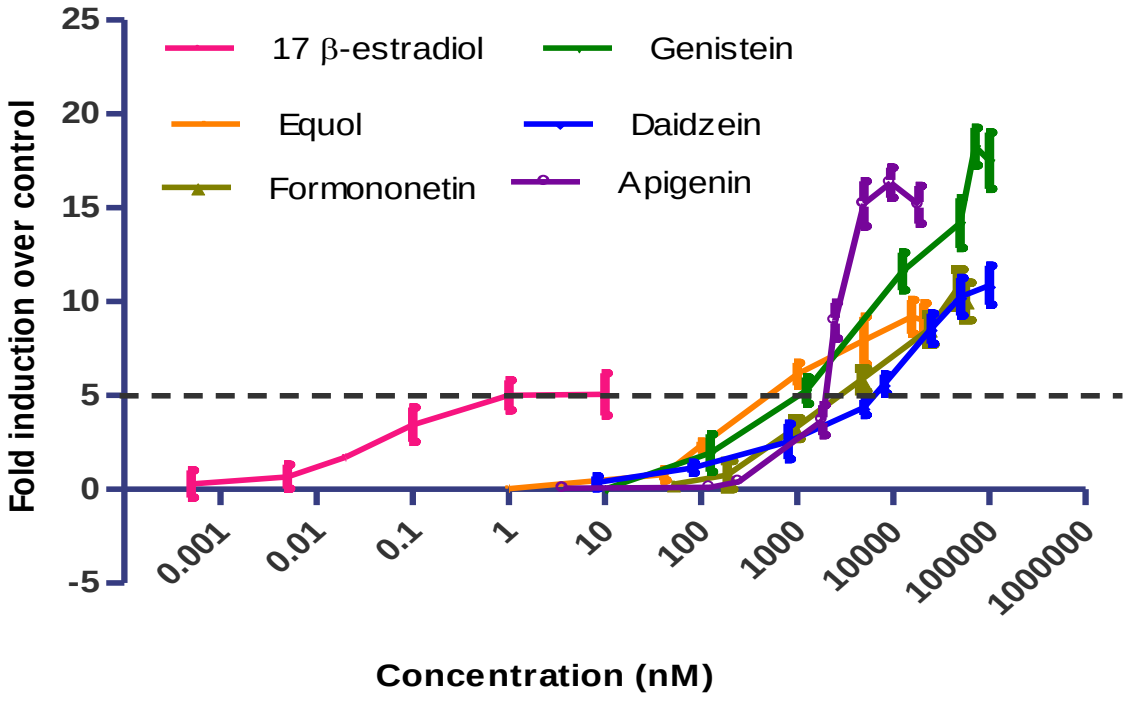


Figure 1: Dose-response curves of 17β-estradiol, apigenin, daidzein, equol, formononetin and genistein

Values are Mean fold induction ± SD in relation to untreated cells. The MMV cell line was treated with increasing concentrations of the hormone or compound (means are of three independent experiments (n = 3) with each experimental point performed in triplicate). The luciferase activity was measured after 24hrs. The dotted line depicts a full estrogenic effect.

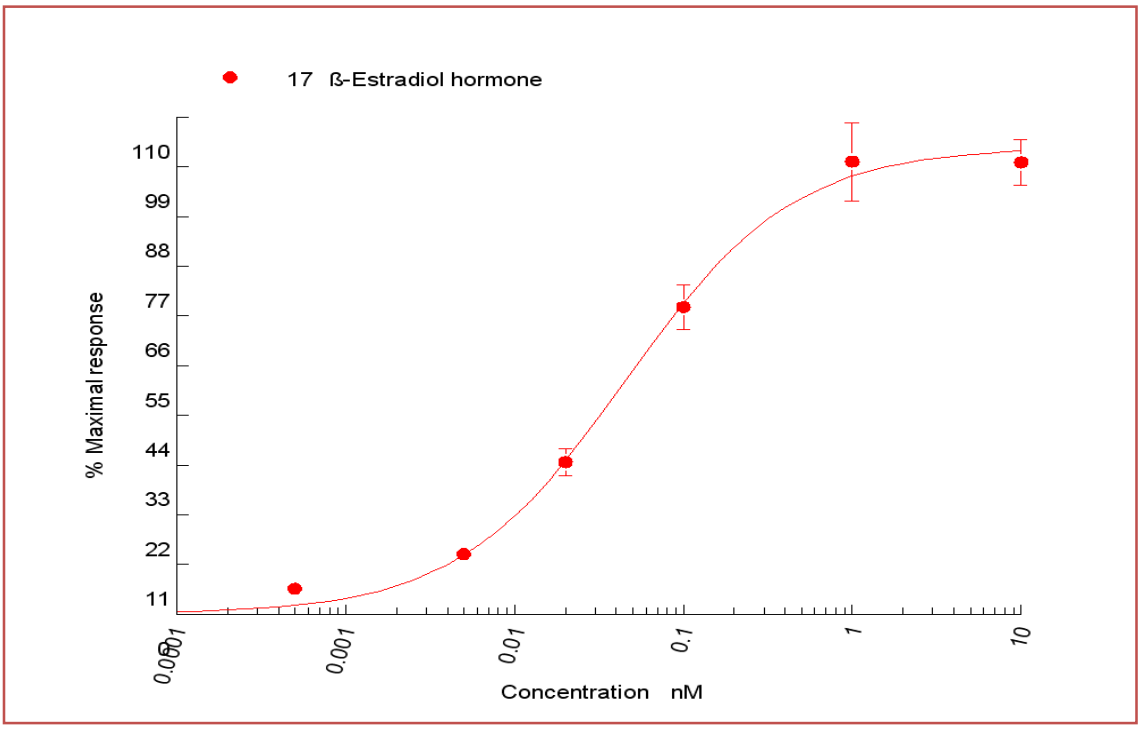
Normalised Dose-Response Curves in MMV-Luc Cells

Five dose-response curves for isoflavones daidzein and its metabolite equol, formononetin, genistein and a flavone, apigenin was determined. Fourof the PEs (daidzein, formononetin, genistein, and apigenin) and the daidzein metabolite equol were weak agonists and induced estrogenicity comparable to 17β-estradiol at higher concentrations figures 2,3,4,5).The EC_{50} which is the concentration that gives half-maximal luciferase expression was calculated and reported for the curves table 2 potencies of the PEs relative to estrogenic hormone standard, 17β-estradiol and androgenic hormone standard testosterone have been reported in the same table. In the current study, the rank order of EC_{50} (M) for the PEs was equol (4.9×10^{-7}) > formononetin (5.7×10^{-6}) > apigenin (2.4×10^{-6}) > genistein (2.1×10^{-6}) > daidzein (3.3×10^{-5}). Dose-response curves were not established with daidzin, genistin, enterodiol, glyciten and matairesinol at the tested concentrations.

Table 2: The EC50 of select PE and % potency relative to 17β-estradiol

Compound	EC_{50} M	% Relative potency
17 β-Estradiol	4.5×10^{-11}	100
Equol	4.9×10^{-7}	0.009
Formononetin	5.7×10^{-6}	0.0008
Genistein	2.1×10^{-6}	0.0021
Apigenin	2.4×10^{-6}	0.0018
Daidzein	3.3×10^{-5}	0.00014

a



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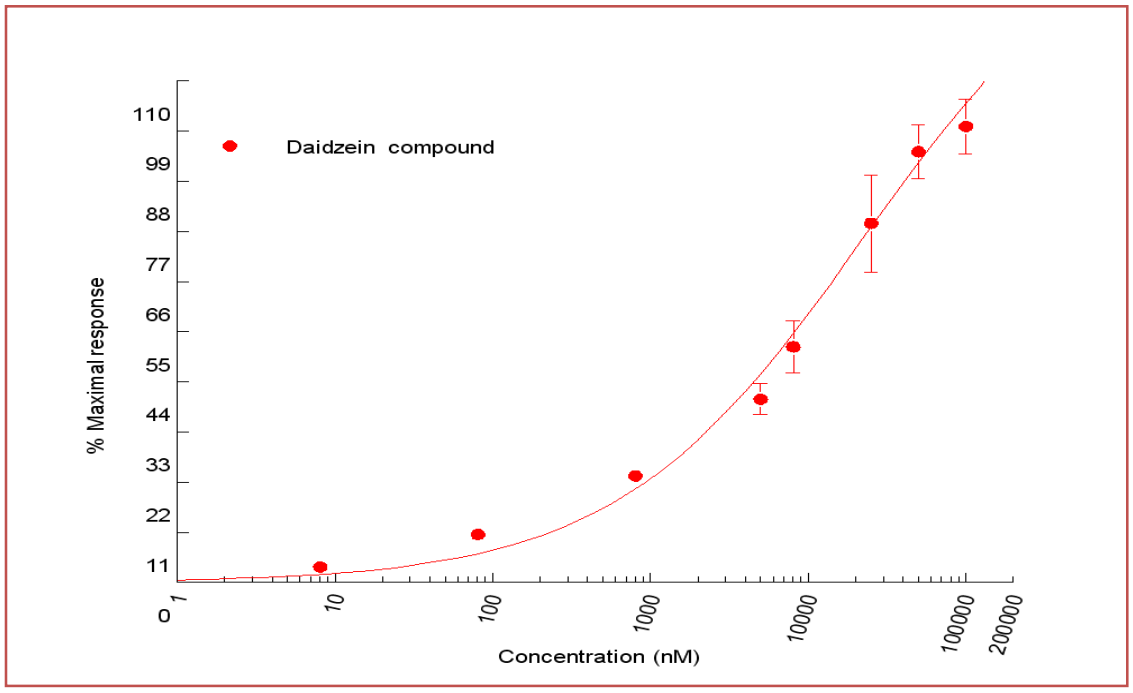
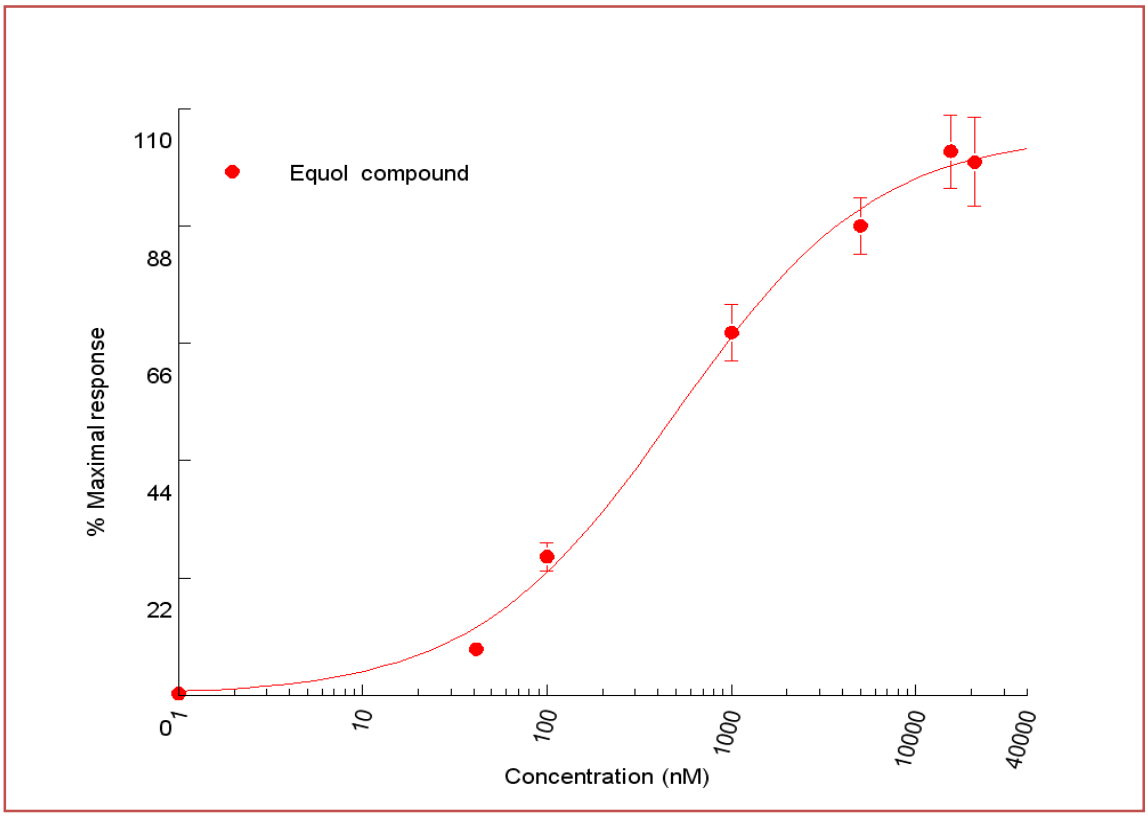


Figure 2: Dose-response and standard curves of (a) 17β-estradiol and (b) daidzein

The MMV cell line was treated with increasing concentration of the hormone or compound and luciferase activity was used after 24hrs.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.

a



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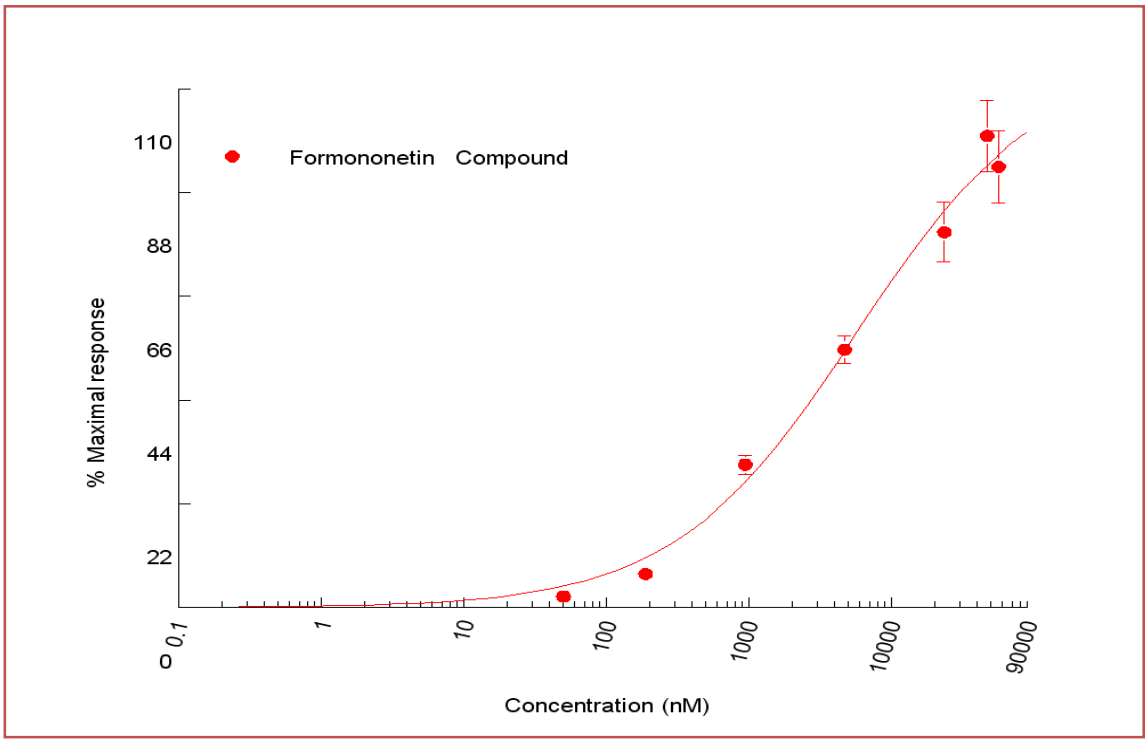
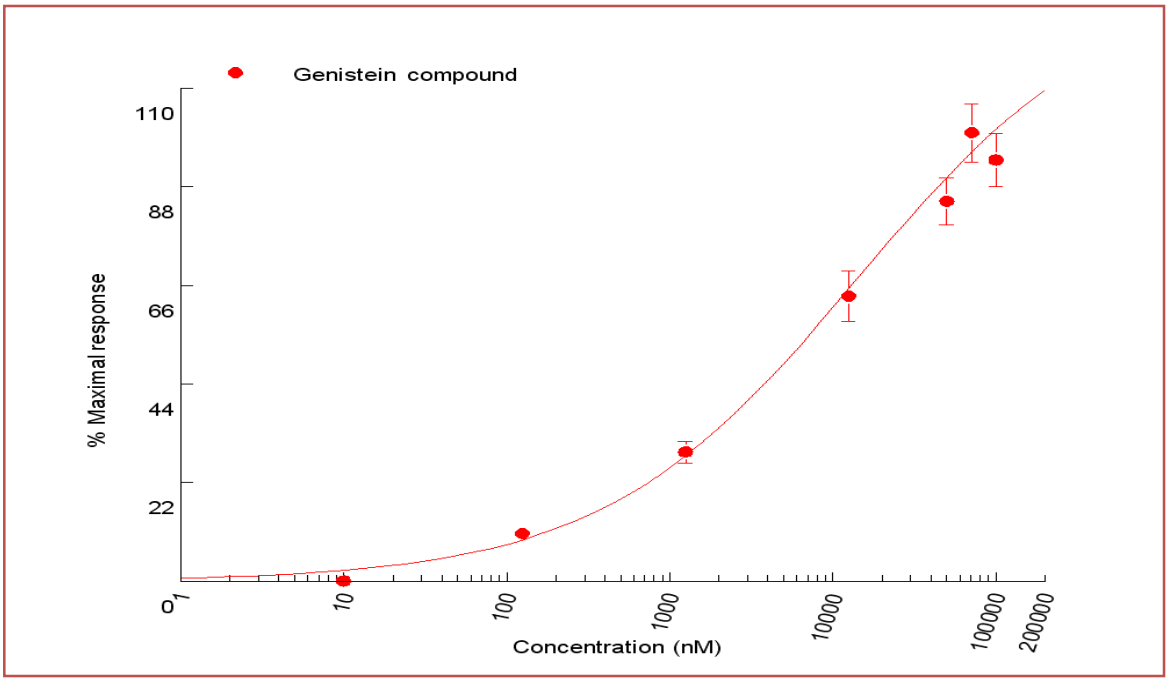


Figure 3: Dose-response standard curves of (a) equol and (b) formononetin

The MMV cell line was treated with increasing concentration of the compound and luciferase activity was measured after 24 hrs. Values are % maximal luciferase activity ± SEM (normalised to 100%). Means of at least three experiments (n =3) with each experimental point performed in triplicate.

Normalised Dose-Response Curves for Genistein and Apigenin in the MMV-Luc Cell Lines

a



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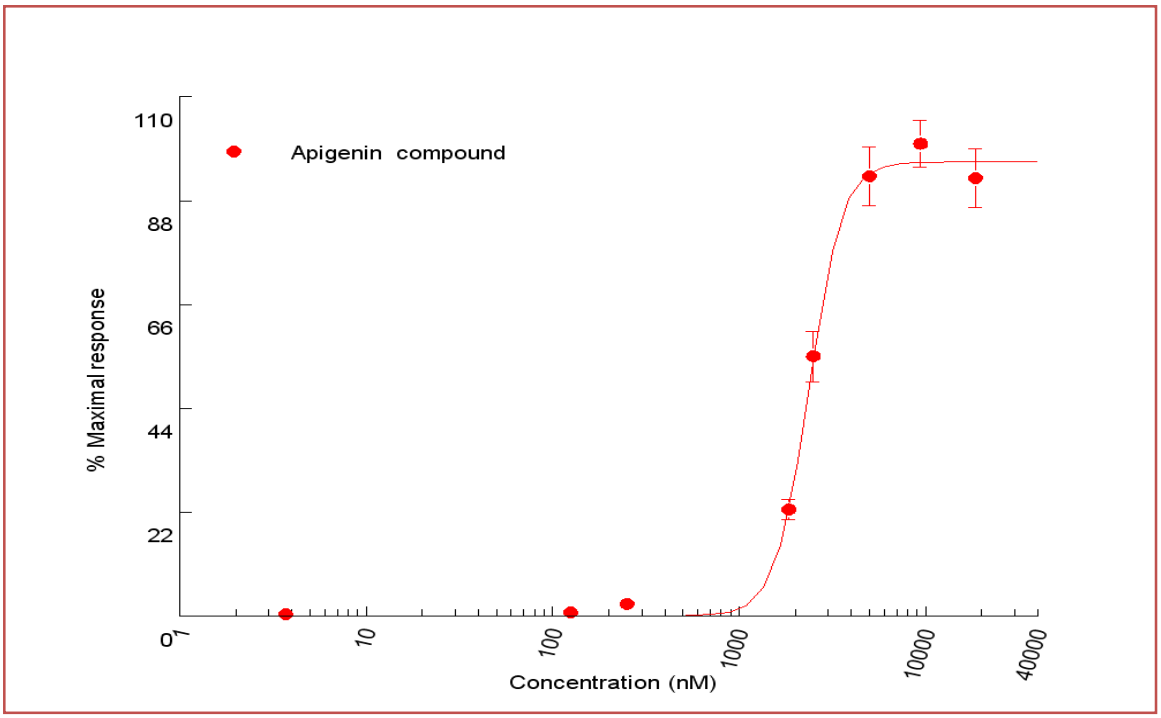

Figure 4: Dose-response standard curves of (a) genistein and (b) apigenin

The MMV cell line was treated with increasing concentration of the compound and luciferase activity was measured after 24 hrs. Values are % maximal luciferase activity ± SEM (normalised to 100%). Means are of at least three experiments n =3 with each experimental point performed in triplicate.

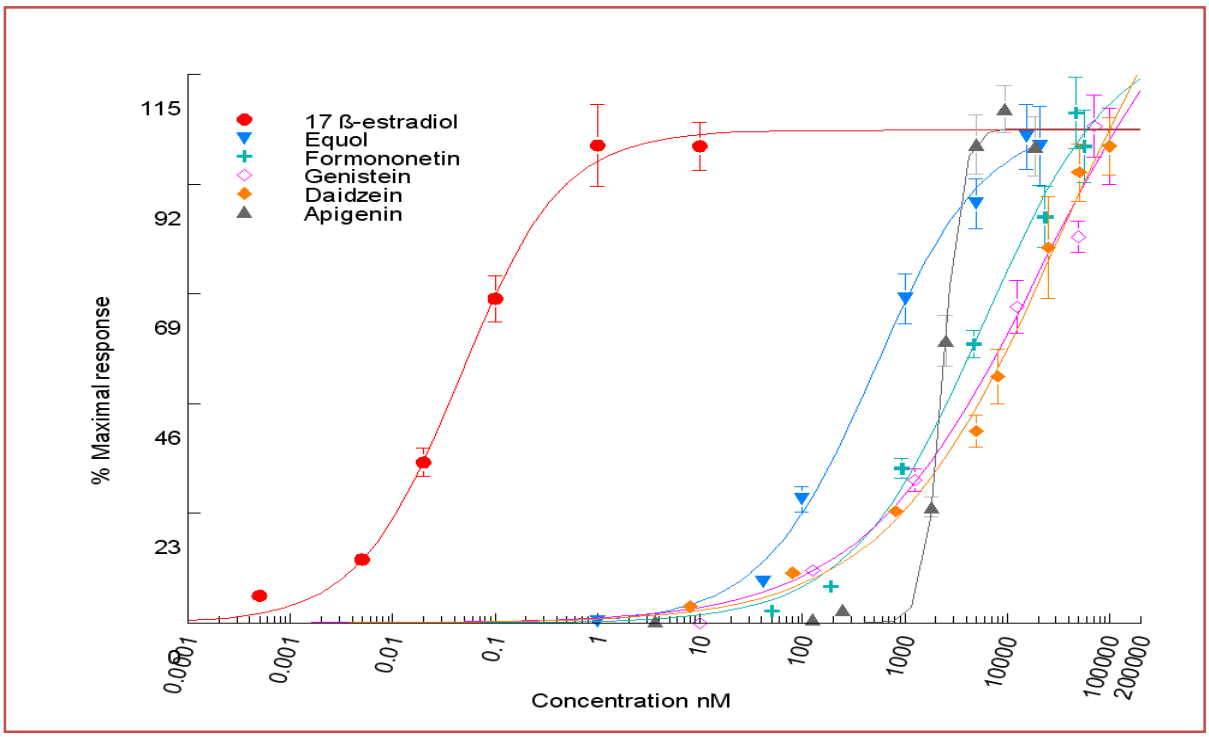

Figure 5: Dose response standard curves in the MMV-Luc cell line of the 17β-estradiol and different phytoestrogens

The MMV cell line was treated with increasing concentration of the hormone or compound and luciferase activity measured after 24 hrs. Values are % maximal luciferase activity ± SEM (normalised to 100%). Means are of at least three experiments (n=3) with each experimental point performed in triplicate.

DISCUSSION

Fold Inductions of Phytoestrogens in MMV-Luc Cell Line

Fold Inductions in MMV-Luc Cell Line (Non-Normalised Curves/Data)

Fold inductions (expressed as luciferase activity over control) in figure 1, revealed that five phytoestrogens were weak agonists compared to the 17β-estradiol as increasingly high concentrations were required for any agonist activity to be induced by the phytoestrogens. While 1nM of 17β-estradiol was enough to achieve a full estrogenic effect in the MMV-Luc cell line, higher individual PE concentrations were required to achieve a full estrogenic effect. From the graph showing a sample of non-normalised curves, equol required a concentration of 521 nM, genistein 1.212 µM, apigenin 2.239 µM, formononetin 2.818 µM while daidzein required 7.079 µM to achieve full estrogenicity identical to that of 17β-estradiol. The PEs, however, achieved superagonist status (transcription activity was higher than that of 17β-estradiol (5 nM) after that.

Apigenin achieved an average maximum fold induction of 15, daidzein 12, formononetin 10, equol 9 and genistein 17 at the tested concentrations. This was against a maximum average fold induction of about 5 for 17β-estradiol at its' best performance in the MMV-Luc cell line. In our study equol exhibited a full agonist effect at an increased concentration which is in contradiction with observations elsewhere where the MCF-7 cell line was used (Harris *et al.*, 2005). In that study, equol was a partial agonist and never expressed superinduction. There was concurrence with our study and Harris and colleagues pertaining to daidzein achieving a higher fold induction compared to the metabolite equol (double fold induction in the Harris and colleagues study and one and a half for our study). Equol is important as it has been associated with many positive health effects. Not all people are equol producers, only 30-50% of the people can metabolise daidzein into equol (Lampe *et al*., 1998).

Fujitani *et al*. (2021), reported that various studies show that 20-30% of westerners can produce equol while Asians range from 40 to 60%. Equol is considered chemoprotective in specific endocrine-related pathologies, such as breast cancer, prostate cancer, cardiovascular diseases, and menopausal symptoms (Hod *et al*., 2021).These positive effects include a lower risk of breast cancers (Rietjens*et al*., 2017) at a concentration of 800 nM an amount that is larger than the EC_{50} for equol in this study 470nM (Table 2). Other positive health effects include protection against cardiovascular disease, cognitive decline, and climacteric symptoms in post-menopausal women (Fujitani *et al*., 2021; Hayashi*et al*., 2021).

It is possible to get maximal inductions with phytoestrogens that are higher than that of 17β-estradiol (Takeuchi *et al.*, 2009; Wang *et al*., 2021) which some studies have attributed to the post-transcription effect on enzyme stability through phosphorylation, acetylation and ubiquitination, that include second messenger pathways. This can thus be classified as an artefact of the assay that may not have any biological significance (Sotoca *et al.*, 2010). An example of a post-transcription effect is that where blocking ubiquitination or proteasomal degradation of ERα results in overstimulation of ER-responsive gene transcription in MCF cells (Boscaro *et al*., 2020; Fan *et al*., 2004; Hwang *et al*., 2020; Yang *et al*., 2019).

Ligands or combinations of ligands that cause conformational changes that may lead to those types of blockages may result in post-translational effects. Changes in the chemical structure *in vitro* as with the case of genistein into orobol seen earlier as well as in other examples where direct interaction of genistein with the luciferase will increase the enzyme's half-life and hence cause higher induction (Jin *et al*., 2020; Thompson 1997; Wang *et al*., 2021). Luciferase stabilizing compounds are luciferase inhibitors (Auld *et al.,* 2008). Accumulation of stabilized luciferase reporter enzyme has been found to enhance the observed bioluminescence activity (Auld *et al.,* 2008; Baktiarova *et al.,* 2006; Mueller *et al.,* 2004; Pohjanvirta & Nasri, 2022).

Genistein, formononetin, daidzein, equol and apigenin exhibited superagonist status at higher concentrations in the MMV-Luc cell line. This meant that the total estrogenicity was much higher than for the natural hormone. This could have been due to non-ER/ERE mediated mechanisms. The non-genomic effects mediated by cytoplasmic or membrane ERs involves nitric oxide and calcium flux release via rapid cellular responses. They may also be due to the activation of different signalling pathways such as mitogen-activated protein kinase (MAPK), AMP-activated protein kinase (AMPK) and phosphoinositide 3-kinase (PI3K) as observed in various cells (Razandi *et al*., 2004; Richards *et al*., 2001; Pohjanvirta & Nasri, 2022; Wang *et al*., 2021).

Studies by Amer*et al*. (2010) and Ma *et al*. (2010) in the same year reported genistein, daidzein and equol to be highly estrogenic in the ER_{β} in the rat brain cells in a concentration-dependent manner which is in agreement with our study that the PEs are estrogenic. In a meta-analysis of different studies conducted by Montani et al. (2008) variation of results in superinduction effects of the same chemical under different studies and a multitude of cell lines had conclusions that have been supported by others (Li, 2015) Montani *et al*., 2008). Superinduction was found to be related to the type of serum used to supplement the basal media, how the effect is measured (whether cells are endogenous or transfected and if one is using firefly luciferase or *Renilla reniformis* luciferase), and the number of estrogen response elements (ERE) and nature of the promoter. It was not correlated with a concentration in media, exposure period or cell model which other studies concur (Auld *et al.,* 2008; Pohjanvirta & Nasri, 2022; Sotoca *et al.,* 2010; Wang *et al*., 2021).

The cell model not affecting superinduction is not surprising because the type of cell will have different characteristics such as what he has listed above and hence require different serum and basal media. However, it is possible to have different cell lines coming to the same conclusion regarding the superinduction of a chemical as demonstrated by the current study and Sotoca *et al*. (2010) that found genistein to be superinduced by the presence of 17β-estradiol. Four of the phytoestrogens (matairesinol, enterodiol, daidzin and genistin) were of partial agonist proportions in the MMV-Luc cell line at the tested concentrations. They did not achieve a full estrogenic effect on their own. However when in the presence of 5nM 17β-estradiol differential fold inductions were observed at different concentrations visually depict this although values there are given as percentages of hormone). These observations include their agonist activity as with enterodiol, daidzin and genistin. Matairesinol was an agonist and antagonist at higher concentrations (70 µM). Glycitein was consistently antagonistic at the tested concentrations. However, in the presence of 5 nM 17β-estradiol, it enhanced the hormone activity.

EC_{50}s and Relative Potencies of PEs in the MMV-Luc Cell Line

The EC_{50}values obtained in the current study for 17β-estradiol and the PEs as in table 2 shows that the PEs are less potent relative to 17β-estradiol. This is in partial agreement with various studies as explained below (Matsumura *et al.,* 2005; Harris *et al.,* 2005; Kalita and Milligan, 2010; Frizzell *et al.,* 2011). In the current study, the rank order of EC_{50} (M) for the PEs was

equol (4.9 x 10^{-7}) > formononetin (5.7 x 10^{-6}) > apigenin (2.4 x 10^{-6}) > genistein (2.1 x 10^{-6}) > daidzein (3.3 x 10^{-5}). This conflicted with another study where the relative potency of PEs (relative to 17β-estradiol whose IC_{50} (M) was 1 x 10^{-11} as determined by $IC_{50}$by use of transcription of estrogen-responsive ERE-CAT reporter gene. The cell line Matsumura and colleagues used was MCF-7.Three PEs were ranked in the order; genistein (4 x 10^{-8}M) > equol (1 x 10^{-7}M) > daidzein (3 x 10^{-7}M) (Matsumura *et al.,* 2005). Genistein (GEN), also known as 4', 5, 7-trihydroxyisoflavone, is the most extensively researched isoflavone phytoestrogen substance and is widely present in soy foods. Considering how closely it resembles endogenous17β-estradiol (E2). GEN binds to estrogen receptors (ERs) both *in vivo* and *in vitro* (Liu *et al*., 2019b). In this study equol was more potent compared to genistein. Genistein has been observed to be bio-transformed in certain cases *in vitro* assays into orobol (metabolite) which is its primary oxidative metabolite (Breinholt *et al.,* 2003; Kulling, 2001) and this can lead to inconsistencies in the observed potency if the biotransformation to orobol has occurred. Pairing this kind of study with physic-chemical analysis such as UPLC or HPLC would help confirm chemical changes.

Induction activity results from ER activities of whole cells, such as MCF-7 suffer from crosstalk with other endogenous receptors such as the progesterone receptors (Willemsen *et al.,* 2004). Kalita & Milligan. (2010) also compared the estrogenicity of 17β-estradiol in comparison to PEs by using Ishikawa (human endometrial adenocarcinoma) cells and recombinant yeast cells. The EC_{50} (M) values obtained for 17β-estradiol and the PEs in Ishikawa cells were as follows: 17β-estradiol 3.3 x 10^{-10}, genistein 2.89 x 10^{-7}, genistin 5.57 x 10^{-7}, daidzein 4.37 x 10^{-7}, daidzin 4.46 x 10^{-7}, equol 1.83 x 10^{-7}, apigenin 2.26 x 10^{-6}. In the recombinant yeast cell assay, the following EC_{50}s (M) were obtained; 17β-estradiol 2.2 x 10^{-10}, genistein 2.14 x 10^{-7}, genistin 2.37 x 10^{-6}, daidzein 1.45 x 10^{-6}, daidzin > 10^{-5}, equol 1.38 x 10^{-7}, apigenin >10^{-5}.

From the two studies by Kalita and Milligan (2010), the EC_{50} (M) values for 17β-estradiol were comparable to the one in this study, the genistein EC_{50} (M) value for the recombinant yeast assay was closer than that analysed by the use of Ishikawa cells. The EC_{50} values for genistin were not achieved in this study case at the tested concentration. Daidzein EC_{50} (M) values were closer to those obtained using the yeast recombinant cell line but about 100-fold different from the Ishikawa cells. For equol our EC_{50} was comparable and close to both the Ishikawa assay and yeast recombinant assay. Harris *et al*. (2005) used MCF -7 cells and found differential EC_{50} s dependent on the ER isoform (ERα and ERβ). From numerous studies reported, ERα and ERβ have been verified as important transcriptional factors, which regulate the expression of specific genes in different tissues or organs in a ligand-dependent manner. Some of their biofunctions and roles of signalling in different physiological processes have also been demonstrated using knockout mouse models recently (Jin *et al*., 2020).

For ERα estradiol, had an EC_{50} (M) of 6.4 x 10^{-11} a value that is very comparable to ours. Genistein however had an EC_{50} (M) of 4.6 x 10^{-7} a value 10 x less than in our study. For the ERβ estradiol EC_{50} (M) was 3.9 x 10^{-11} which is comparable to our study. The order of the relative potency for the PEs in common with the ones in this study from their EC_{50} (M) were as follows: Genistein (3.4 x 10^{-9}) > Equol (1.7 x 10^{-8}) > Daidzein (2.8 x 10^{-6}) > Apigenin (1.4 x 10^{-6}) which was in contradiction with our order. This can be explained by the same factors explained earlier pertaining to different types of cells and isoforms, basal media, serum and promoter contexts amongst other considerations (Auld *et al.,* 2008; Montani *et al.,* 2008; Sotoca *et al.,* 2010).

The EC_{50} for 17β-estradiol obtained in the present study is comparable to one obtained in another study, approximately 1.5 x 10^{-11}(Frizzell *et al.*, 2011). This is most likely because the same cell line MMV-Luc cell line was used under the same conditions in both studies. Another study by Kang *et al*. (2008), which used Chinese Hamster Ovary (CHO) cells obtained a very different EC_{50} for 17β-estradiol, at the level of 6 x 10^{-2}M for similar reasons as those mentioned before regarding the use of different cells, serum and laboratory procedures. In a meta-analysis conducted by Montani (2008) differences in EC_{50}s for the same compound in various studies on estrogenicity may have been due to solvents used, type of cells, type of media, the health of the cells and passage number, exposure conditions, endpoint used to quantify potency, number of ERE (Estrogen response elements) amongst other considerations (Li, 2015; Montani *et al*., 2008; Pohjanvirta & Nasri, 2022; Wang *et al*., 2021).

As soon as EREs are bound to dimerized ERs, DNA bending and looping are then encouraged and controlled, enabling a specific interaction with the transcriptional machinery and co-regulator proteins (Jin *et al*., 2020).Competitive binding assays using cytosol or whole-cell preparations suffer from cross-talk with other nuclear receptors present in the homogenate such as the progesterone receptors. Recombinant (receptor protein) binding assays are better than whole cells as they do not usually suffer the artefact (Bovee *et al.*, 2008).Mammalian cells are however superior to yeast cells as they are closer or the same as humans and will carry out metabolism, uptake and membrane transport in a better and more relevant way for the benefit of human health (Nagel *et a*l., 1998). The yeast cell is disadvantageous because it is difficult for the cells to release the β-galactosidase or the luciferase reporter protein following exposure. As a result, large variations in the results have been noted (Bovee *et al.*, 2004; Pohjanvirta & Nasri, 2022).

On the other hand, studies have shown that analysing the distribution of ERα and ERβ in different tissues can reveal that these molecules have biological actions that are specific to different tissues. As a result, ERs typically exhibit high specificity for particular target organs. ERα, for instance, is found to be highly expressed in the uterus, ovarian theca cells, Leydig cells in the testes, breast, prostate stroma, epididymis, and liver; in contrast, ERβ is highly expressed in bone marrow, the brain, the prostate epithelium, the ovarian granulosa cells, and the testes (Jin *et al*., 2020).

CONCLUSIONS

MMV-Luc cell line is suitable for characterising PEs (apigenin, daidzin, daidzein, enterodiol, equol, formononetin, genistein, genistin, glycitein and matairesinol). The phytoestrogens were shown to have differential potential endocrine disruptive activity on their own or in the presence of 17β-estradiol (5 nM). Apigenin, equol, formononetin, daidzein and genistein were weak agonists but achieved superagonists status in the cell line at a concentration range (500 nM – 7 µM).These doses are in the range of possible plasma concentration (at least for equol, genistein and daidzein) in soya-fed milk babies and in populations that consume a lot of soya such as Asian vegetarians. These could have potential adverse ED effects or beneficial health implications such as increased inflammatory response, help fight obesity and cardiovascular diseases, may influence vascularization of boy infants and estrogenization of infant breast tissue, have transgenerational sexual malformations as well as affect future sperm production in boy infants.

Some of the effects have been observed in animal studies and may not be directly translated to humans. Genistin, daidzin, enterodiol and matairesinol were partial agonists due to the fact they did not achieve a fold induction equivalent to the full agonist fold induction at the tested concentrations. Glycitein was an antagonist to the MMV-Luc cell line and had a dose-

dependent effect on the transcriptional activity of 17β-estradiol (5nM). In this study dose-response curves in MMV-Luc cell lines for equol, formononetin, genistein, daidzein and apigenin, were established their EC_{50}s and determined their estrogenic potencies relative to 17β-estradiol.

The PEs that were more structurally similar to the estrogen hormone were more active compared to the more, unlike ones such as genistin and daidzin which are large molecules with a glucose group attached to them. This might have hindered them from interacting with the receptor. Enterodiol and matairesinal were also more structurally unlike the hormone. These results are of public health importance as phytoestrogens have been found to mimic hormones to various degrees in an estrogenic (MMV-Luc) cell line. This study has served to confirm the endocrine disruptive potential of PEs in the MMV-Luc cell line. It reiterates the need to re-evaluate the use of PEs in foods and particularly so in vulnerable sub-populations. Although the potential for endocrine disruption by PE was noted *in vitro*, this study would recommend further *in vivo* tests. This is because toxicokinetics involving metabolism and bioavailability affects the potencies of the PEs. Further *in vitro* assays may be designed to investigate the additive effects of phytoestrogens at physiologically relevant concentrations.

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Conflicts of Interest

The authors declare no conflicts of interest

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