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*Full Length Research*

# Assessment of the androgenic potencies of phytoestrogens with and without co-incubated testosterone by using TARM-Luc cell line

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**ABSTRACT:** The ability of the prostate gland to grow is a hormone-dependent process that is regulated by both androgenic and estrogenic factors. Phytoestrogens (PEs) are bioactive compounds with the estrogenic activity which may impact hormones and health in general. The TARM-Luc cell line was used in this study to investigate the androgenic potencies of phytoestrogens with and without co-incubated testosterone. The transcriptional activities (TAs) of 10 PEs (apigenin, daidzein, daidzin, equol, enterodiol, formononetin, genistein, genistin, glycitein, and matairesinol) were assessed as induction of the expressed luciferase activity, which is connected with the biological effects. These effects were compared to transcription caused by testosterone (50 nM) hormone (standards) *in vitro*. Androgenic receptors in the TARM-Luc cell line were used in the reporter gene assay tool. The standard curve for hormone standard was determined and the EC50 (M) for testosterone was ($3.7 \times 10^{-9}$ M). The order of androgenic potency of two PEs from their EC50s was apigenin ($4.5 \times 10^{-6}$ M) > glycitein ($4.8 \times 10^{-5}$ M). Dose-response curves in the TARM-Luc cell line, for the other eight PEs, were not established as most were anti-androgenic. The percent androgenic potency for apigenin was $8.2 \times 10^{-2}$ M and for glycitein was $7.7 \times 10^{-3}$ M relative to testosterone whose percent potency was arbitrarily assigned a value of 100. PEs inhibition or enhancement on the hormone testosterone (50 nM) -dependent transcription was dose-dependent and chemically specific. PEs enhanced testosterone hormone-dependent transcription and gave more additive and synergistic bioactive effects in a dose-dependent manner. Two PEs, apigenin and glycitein, were weak agonists in TARM-Luc cell line. The additive and synergistic effects of the PEs in combination with testosterone (50 nM) were a novel discovery in this study and relevant since it could have endocrine disrupting effects.

**Keywords:** Androgenic potencies, gene assays, phytoestrogens, testosterone, TARM-Luc Cell Line, transcriptive activity.

## INTRODUCTION

Phytoestrogens are naturally occurring polyphenolic, non-steroid substances produced in plants that have estrogenic effects. Phytoestrogens exert their estrogenic effects primarily through binding to estrogenic receptors (ERs) α and β, with a higher affinity for ERβ, and acting as agonists, partial agonists, and antagonists (Gorzkiewicz *et al*., 2021; Mostrom and Evans, 2018). Due to structural similarities with estrogen, phytoestrogens have weak pseudo-hormonal properties and the capacity to bind to estrogen receptors. Additionally, they might have anti-androgenic and anti-estrogenic effects (Memariani *et al*., 2020).

These effects are mediated by binding to estrogen receptors, by alterations in the concentrations of endogenous estrogens, and by binding to or stimulating the synthesis of sex hormone-binding globulin (Aronson,

2016). There are four main groups of phytoestrogens: isoflavonoids, flavonoids, stilbenes, and lignans. Of these the most commonly occurring are the flavonoids (of which the coumestans, prenylated flavonoids, and isoflavones have the greatest estrogenic effects) and the lignans (Lephart, 2021). The isoflavonoids include genistein, daidzein, coumestrol, and equol; the prenylated flavonoids include 8-prenylnaringenin, the stilbenes include resveratrol, and the lignans include enterodiol and enterolactone (Aronson, 2016). Phytoestrogens are widely present in a variety of vegetables, fruits, grains, and particularly in soybeans and associated products like bean curds/tofu and soy milk (Cai *et al*., 2021).

Phytoestrogens have been detected in maternal blood and urine, cord blood, and amniotic fluid (Padmanabhan *et al*., 2021). Dietary phytoestrogens are associated with lowered risk of menopausal symptoms such as hot flashes and osteoporosis, lowered risks of cancers (prostate cancer and uterine cancer), cardiovascular disease, obesity, metabolic syndrome, type 2 diabetes, brain function disorders, and protection of neural cells against injury evoked by various factors(Gorzkiewicz *et al*., 2021; Memariani *et al*., 2020).

Studies have found that PEs may act as Endocrine-Disrupting Chemicals (EDCs). EDCs are thought to be linked to immune system changes, abnormal growth patterns, and delays in neurodevelopment in children, as well as altered reproductive function in both males and females (Paterni *et al*., 2017). Other studies have found no evidence that PEs are EDCs. For example, there was no effect on the male reproductive system, no amelioration of metabolic and inflammatory parameters in androgen-deficient men who had prostate cancers and also no behavioural changes in children fed soya (Jing *et al.,* 2008; Napora *et al.,* 2011; Khalil *et al*, 2014). Difference *in vitro* tests have been used to assess PE endocrine activity. *In vitro* assays, such as reporter gene assays, there are advantages of being fast, high through-put, and versatile; especially in connection with the application of complex study designs and require no animal sacrifice (Willemsen *et al.,* 2004; Connolly *et al.,* 2009; Svobodová and Cajthaml, 2010; Van der Linden *et al*., 2014). It is crucial to keep in mind that EDCs can enter the body through the skin, inhalation of gases and particles in the air, and ingestion of food, water, or dust. Additionally, EDCs from pregnant women can be passed on through the placenta or breast milk to a developing fetus or children (Paterni *et al*., 2017).

The effect of PEs on the transcriptional activity of natural hormones is important as both the PEs and hormones will be in the same endocrine system upon PE consumption and metabolisation. They will hence interact with the receptors and extra-receptor pathways that influence endocrine action in the presence of the hormone. The ensuing competition and other interactions will then determine the effective transcription effect. The effect of Pes in vulnerable subpopulations such as babies has been of concern, especially in the context that infants, especially boys may have hormonal surges in infancy that can peak at adult levels (Forest *et al*., 1974; Raivio *et al*., 2003; Patisaul and Jefferson, 2010; Sirotkin, 2014). Hormone action enhancement or inhibition in the presence of potential EDs has been studied *in vitro* and *in vivo* (Ni *et al.,* 2010). Ni and colleagues employed an *in vitro* system that observed dose-dependent transcriptional activity of daidzein, alone or in combination with high (10nM) or low (1nM) concentrations of estrogen in a (Chinese Hamster Ovary) CHO-K1. Daidzein (5 nM to 50 µM), did not affect ERβ-mediated transcription induced by 1 nM estradiol, but it significantly inhibited ERβ-mediated transcription induced by 10 nM estradiol at 500 nM (Ni *et al.,* 2010).

Montani and colleagues did a meta-analysis of combinatorial effects with a bias towards the supramaximal (effect higher than for the natural hormone for that receptor) effect (Montani *et al.,* 2008). As all animals and humans have endogenous hormones at various levels all *in vivo* tests involving assessing endocrine disruptive effects of PEs reflect this interaction to some extent. On the other hand, in the later stages of childhood, an increase in androgens and a decrease in estrogen associated with dietary phytoestrogens have been noted in girls and boys, respectively (Domínguez-López*et al*., 2020).

On the other hand, androgens are steroid hormones with pleiotropic and diverse biochemical and physiological functions, and androgen deficiency has a detrimental effect on human health (Traish, 2017). The vital sex hormone testosterone, which is produced in the testicles, is essential for many bodily physiological and biological processes. The body's typical activities could be adversely impacted by its absence. The development of male secondary sexual characteristics, spermatogenesis, sexual performance, and male infertility are all negatively impacted by testosterone disturbances (Masuku *et al*., 2019).

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 TARM-Luc cell lines. The cell line is suitable for assessing the agonist or antagonistic action of the compound and hence revealing the mode of action of that ED. They can determine mixture effects, such as PEs and hormones that were undertaken in this study. 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 (Soto *et al*., 2006; Connolly *et al.,* 2009; Schug *et al*., 2013). Over the past few decades, there has been a significant increase in the study of phytoestrogens. However, additional research is still required to assess the safety of phytoestrogens, advantageous and harmful doses, gender differences in response to phytoestrogens, and the results of combining phytoestrogen application with other medications and dietary supplements (Nikolić*et al*., 2017).

This study investigated the androgenic potencies of phytoestrogens with and without co-incubated testosterone by using the TARM-Luc cell line.

## MATERIALS AND METHODS

### Chemicals and reagents

Dulbecco's Modified Eagles Medium (DMEM) + GlutaMAX$^{TM-1}$ (Cat. No. 31053-028), Penicillin 100U/ml/ Streptomycin 100 µg/ml (Cat. No.15070-063), General Foetal bovine serum (FBS) (Cat. No. 10270-106), hormone depletes 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). Methanol, $CH_3OH$, 32.04g/mol, 99.7%, Sigma Aldrich, flutamide (Cat. No. F9397). Testosterone (Cat. No. A8380) 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).

### Reporter gene assay

Androgen-responsive (TARM-Luc) reporter gene cell lines, cell culture and Luciferase assay were determined as described by Willemsen *et al*. (2004) and Puranik*et al*. (2019). Before 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). Cells were seeded at a concentration of $4 \times 10^5$ cells/ml, into white-walled 96 well plates with clear flat bottoms (Greiner Bio-One, Frickenhausen, Germany**)** at 100 µl/well and incubated for 24 h. 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 testosterone and PE concentrations used are shown in Table 1 below. Antagonist tests were carried out by incubating the PEs and (their metabolites) at the shown concentrations (Table 1) with 50 nM testosterone (TARM-Luc). The cells were incubated for 48 hrs. The supernatant was discarded and the cells were washed twice with phosphate-buffered saline (PBS) before 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).

**Table 1.** Concentrations used for reporter gene assays.

| Compound | Concentrations used |
|---|---|
| Testosterone | 50 pM - 50 nM |
| Apigenin | 0.25 nM - 80 µM |
| Daidzein | 0.25 nM - 50 µM |
| Daidzin | 0.1 nM - 20 µM |
| Enterodiol | 0.5 nM - 10 µM |
| Matairesinol | 0.5 nM - 80 µM |
| Equol | 0.25 nM - 20 µM |
| Formononetin | 0.5 nM - 115 µM |
| Genistein | 0.5 nM - 150 µM |
| Genistin | 0.1 nM - 20 µM |
| Glycitein | 1.0 nM - 100 µM |

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 using triplicate readings for a minimum of three experiments.

### Agonistic and antagonistic effect

The procedure used for evaluating the (ant) agonistic effect of PE extract was done as described by Willemsen *et al*. (2004) and Puranik *et al*. (2019), where standard testosterone hormones were used as positive controls on TARM-Luc cell lines respectively. For this test, cells that had been subcultured as for the reporter gene assays above were seeded in a 96-well Greiner bio-plate at 100 µl and a cell density of 10,000 cells/ml. They were incubated at 37°C/8% $CO_2$ in a humidified environment. The PE standards, negative controls and testosterone positive controls were added. For this test, 990 µl of assay media was spiked with an equal amount (5 µl each) of PE standard/extract and of methanol (blank) and vortexed. An aliquot of 100 µl was then added to each well. For each adjacent well with a particular PE, the 990 µl assay media was spiked with 5 µl PE standard and 5 µl hormone. This was to allow for a direct comparison of the effect of PE alone or in combination with the hormone. Two negative controls that were used are (i) A blank with methanol (1%) in assay media which is a solvent for the standards and (ii) A blank as above that had known antagonists; flutamide,

at a concentration of 2762 ng/ml for the androgen cell line at a concentration of 0.5% in assay media while the two positive controls that were used (i) The first positive control had hormone testosterone (50 nM) for the androgen cell line, and methanol, both hormone and methanol at 0.5% each in assay media and (ii) The second positive control had hormone, testosterone and the appropriate antagonist, flutamide, for the androgen cell line.

### Cytotoxicity assay

PE standard or hormone concentration as that used for reporter gene assays were tested for cytotoxicity. After the requisite cell exposure to hormone or PE for 48 h in the androgen cell line, 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 37ºC/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 (Mosmann, 1983; Masuku *et al*., 2020). 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 GraphPad Prism version 5.00 for Windows (GraphPad Software Inc., San Diego, California, USA). All data were expressed as mean induction ± SD of three independent experiments (n = 3) with each experimental point performed in triplicate. For gene assays, calibration curves of Testosterone, Apigenin, and Glycitein were fitted with a sigmoidal dose-response curve. The logarithm was used instead of the actual concentration as shown in the following equation:

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

Where X is the logarithm of the concentration, Y is the response, and $EC_{50}$ is the mid-point between the Top and Bottom which are the maximum response and the baseline response; respectively (Willemsen *et al.,* 2004). For Transcriptional activity (TA), values are mean % TA ± SD relative to testosterone (50 nM) of three independent experiments.

## RESULTS

### Non-normalised dose-response curves in TARM-Luc cells

The un-normalised dose-response curves of apigenin and glycitein against testosterone illustrated below clearly show the two phytoestrogens had weak androgenic activity (Figure 1). They were partial agonists as their maximum induction was never as high as that of testosterone.

Apigenin had an average maximum fold induction of 20 while glycitein had 15 against 30 for testosterone. This depicts that they have weak androgenic activity as higher concentrations in the micromolar region are required to register any androgenic activity compared to testosterone whose androgenicity is evident at the picomolar level. It was not possible to get any dose-response curves of eight PEs. Phytoestrogen enterodiol had slight androgenic activity (maximum induction at the tested concentrations ranging at 12-22% of testosterone hormone (50 nM) arbitrarily assigned a maximum induction of 100%). The other seven phytoestrogens daidzin, formononetin, equol, genistein, genistin, daidzein and matairesinol were antagonistic and registered < 10% maximum induction compared to the testosterone at the same concentration.

### Normalised dose-response curves in TARM-Luc cells

Two dose-response curves for two PEs, apigenin and glycitein were determined. They were weak agonists and induced androgenicity comparable to testosterone at higher concentrations (Figures 1 and Figure 2). In all these cases there was a dose-dependent increase at the tested concentrations. The $EC_{50}$ which is the concentration that gives half-maximal luciferase expression was calculated and reported for the curves (Table 2) and percent relative potencies of the PEs to testosterone.

The rank order as determined by the $EC_{50}$ (M) is as follows for the androgen cell line testosterone ($3.7 \times 10^{-9}$) < apigenin ($4.5 \times 10^{-6}$) < glycitein ($4.8 \times 10^{-5}$). It was not possible to get dose-response curves with the other PEs as they were mainly anti-androgenic.

Dose-response standard curves of (a) Glycitein and (b) Testosterone, apigenin and glycitein in the TARM-Luc Cell Line are shown in Figure 3.

### Enhancement or inhibitive effect on testosterone in TARM-Luc cell line

The combinatorial effects of the hormone testosterone (50 nM) with PEs in TARM-Luc cell lines were established at the tested concentration. The percentage maximum agonist affect transcriptional activity of the PEs compared to transcriptional activity for the hormone testosterone (50 nM), which were arbitrarily assigned a value of 100% were

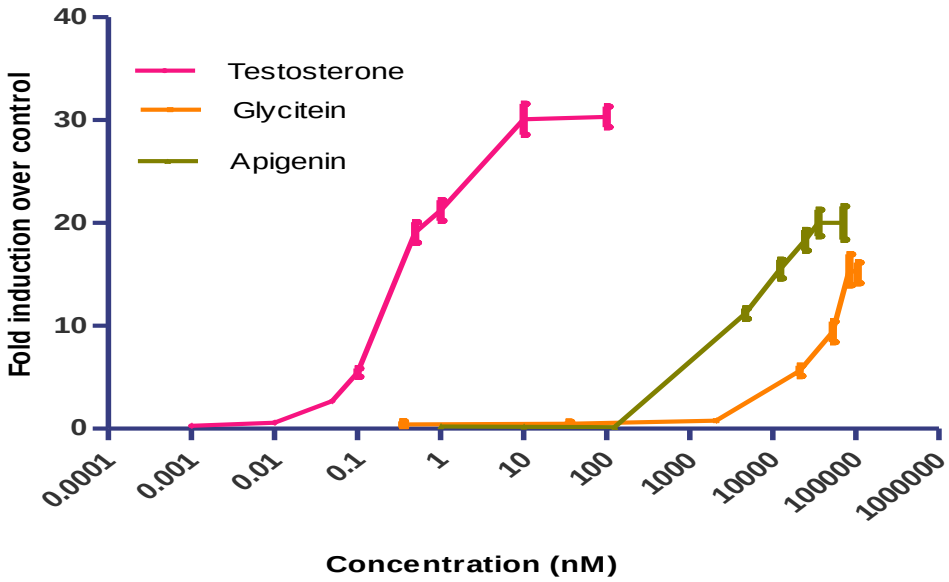



**Figure 1.** Dose-response standard curves of testosterone, apigenin and glycitein. Mean induction ± SD concerning untreated cells. The TARM-Luc cell line was treated with increasing concentrations of the hormone or compound in at least three independent experiments (n=3) with each experimental point performed in triplicate). The luciferase activity was measured after 48 h.

**Table 2**. The EC50 or molar concentration of hormone or PE eliciting half maximal luciferase expression in the TARM-Luc cell line and % potency relative to testosterone.

| Compound | $EC_{50}$ (M) | Relative potency (%) |
|---|---|---|
| Testosterone | $3.7 X10^{-9}$ | 100 |
| Apigenin | $4.5 X 10^{-6}$ | 0.082 |
| Glycitein | $4.8 X 10^{-5}$ | 0.0077 |

reported using a key adapted from Willemsen *et al.* (2004) with some modifications. This was to enable the strength of the agonism to be evaluated at different molar concentrations. The transcriptional effect of the PE and the inhibitive or enhancement capability of that PE on hormone-dependent transcription in the TARM-Luc cell line were evaluated. The combinatorial effect of the PE and the natural hormone was determined in TARM-Luc cell lines for the ten PEs as shown in Figures 4 to 8. Actual values are shown in Table 3 which has values for the transcri-ptional activity of two compounds expressed as mean ± SD % TA of the hormone testosterone (50 nM) (which were arbitrarily assigned 100%) in TARM-Luc cell lines.

**Apigenin and Daidzin**

In the TARM-Luc cell line apigenin (Figure 4a) had an undetectable effect (121 nM-2.3 µM) to the medium agonistic androgenic effect that peaked at 37 µM. Testosterone hormone transcription activity was not affected at the lowest 121 nM concentration of apigenin tested. It however induced additively or synergistically the agonistic TA of testosterone at the higher concentrations (1.15 -25 µM) of apigenin as seen in Table 3. When the TARM –Luc cell line was exposed to Daidzin (Figure 4b) no agonist effect was observed up to a concentration of 3.9 µM to a barely medium agonistic androgenic effect (11.5 % of hormone(5 nM) maximum induction) at the tested concentrations. Daidzin in the presence of 50nM testosterone had an inhibiting effect on the activity of the hormone which was < 25% inhibition on the hormone activity observed. This according to the key was not registered as significantly antagonistic as a clear-cut point was set at a reduction below 75% for the dose to be considered categorically antagonistic (Table 3).

**Daidzein and Enterodiol**

Daidzein (Figure 5a) in the TARM-Luc cell line, was antagonistic or had no agonistic effect in the cell line as it

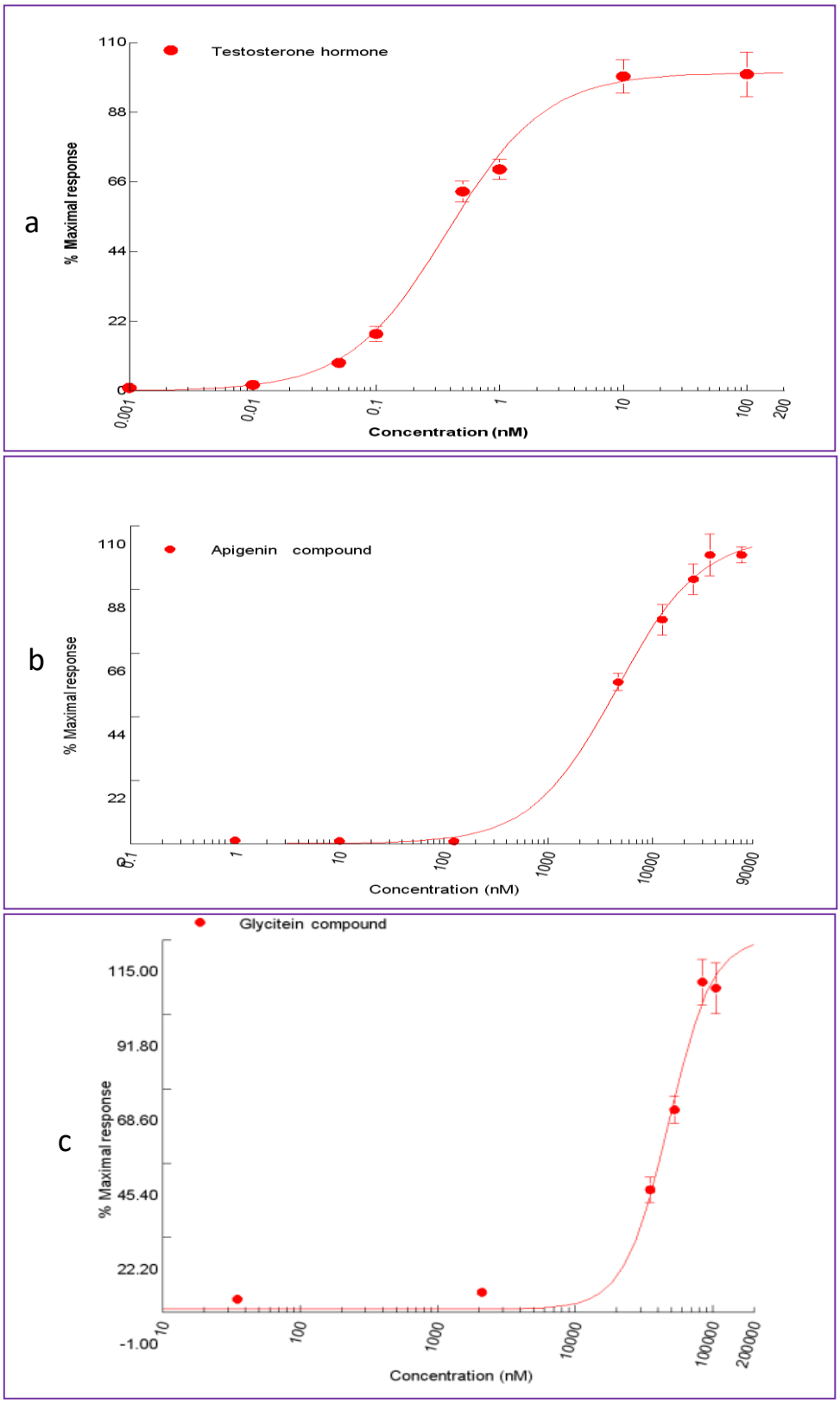



**Figure 2.** Dose-response standard curves of (a) Testosterone (b) Apigenin (c) Glycitein in TARM-Luc Cell Line. The TARM-Luc cell line was treated with increasing concentration of the compound and luciferase activity measured after 24 h. Values are % maximal luciferase activity ± SEM (normalised to 100%). Means of at least three independent experiments (n =3) with each experimental point performed in triplicate.

d

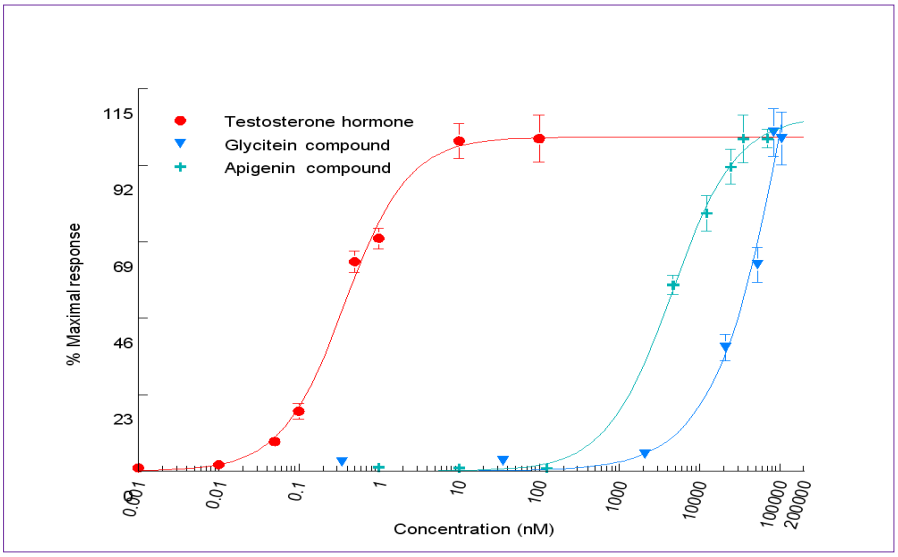
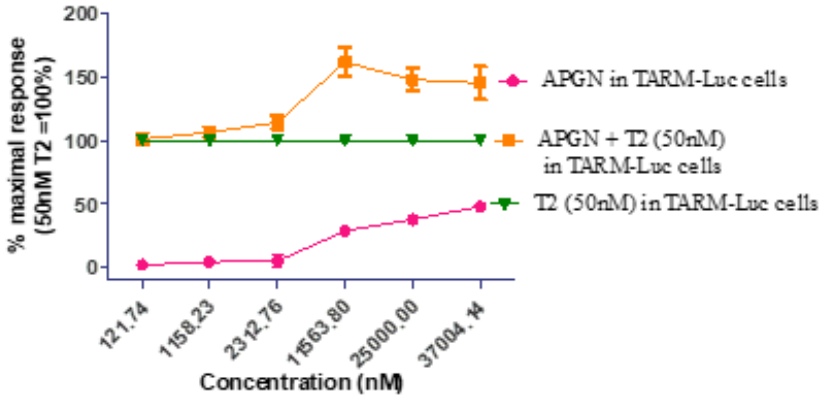



**Figure 3.** Dose-response standard curve of (d) Testosterone, Apigenin and Glycitein in TARM-Luc Cell Line. The TARM-Luc cell line was treated with increasing concentration of the hormone or compound and luciferase activity measured after 24 h. Values are % maximal luciferase activity ± SEM (normalised to 100%). Means of at least three independent experiments n =3 with each experimental point performed in triplicate.

a




b

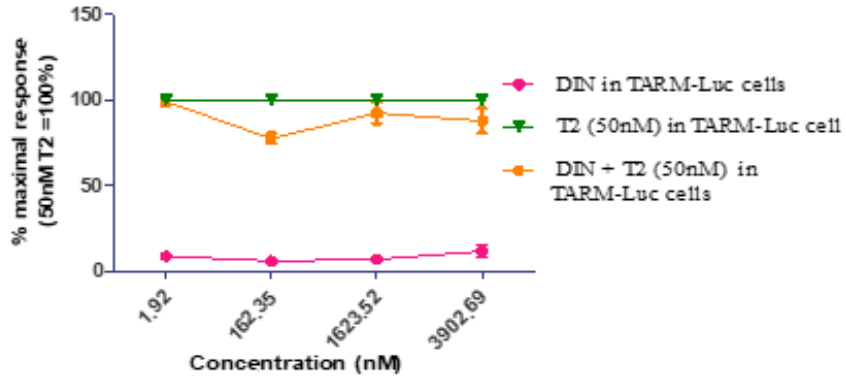



**Figure 4.** Per cent maximum Transcriptional activity (TA) of increasing concentrations of apigenin (APGN) and daidzin (DIN) in the absence or the presence of testosterone (50 nM) in TARM cell lines. Values are per cent luciferase activity relative to the maximum of hormone (arbitrarily taken as 100%) ± SEM (means from three independent experiments (n=3) with each experimental point performed in triplicate). The incubation period in the TARM-Luc cell line was 48 h.

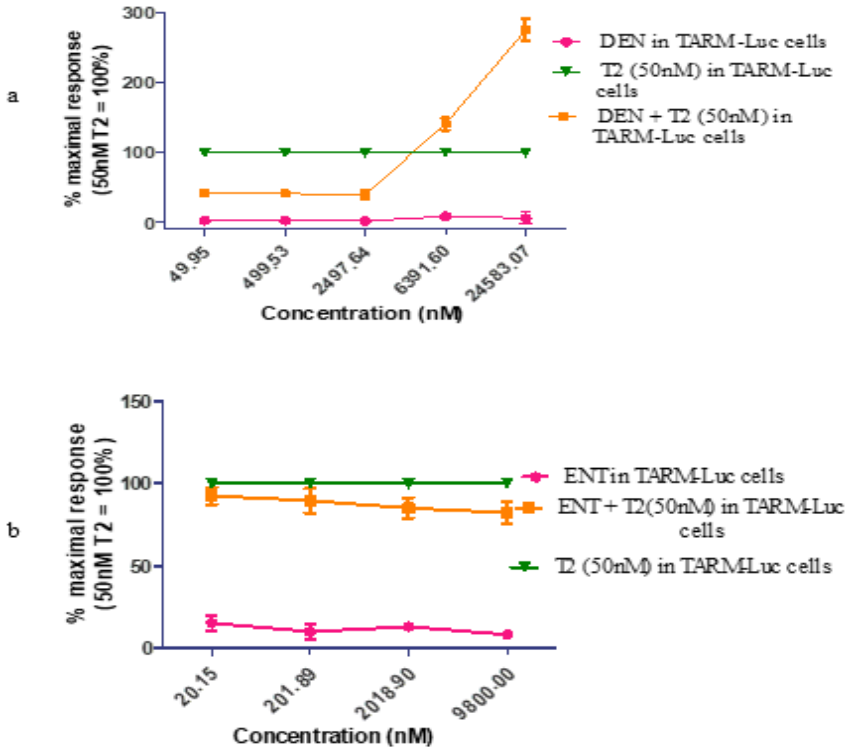



**Figure 5.** Per cent maximum Transcriptional activity (TA) of increasing concentrations of Daidzein (DEN) and enterodiol (ENT) in the absence or the presence of testosterone (50nM) in TARM -Luc cell lines. Values are per cent luciferase activity relative to the maximum of hormone (arbitrarily taken as 100%) ± SEM (means from three independent experiments (n=3) with each experimental point performed in triplicate). The incubation period was 48 h.

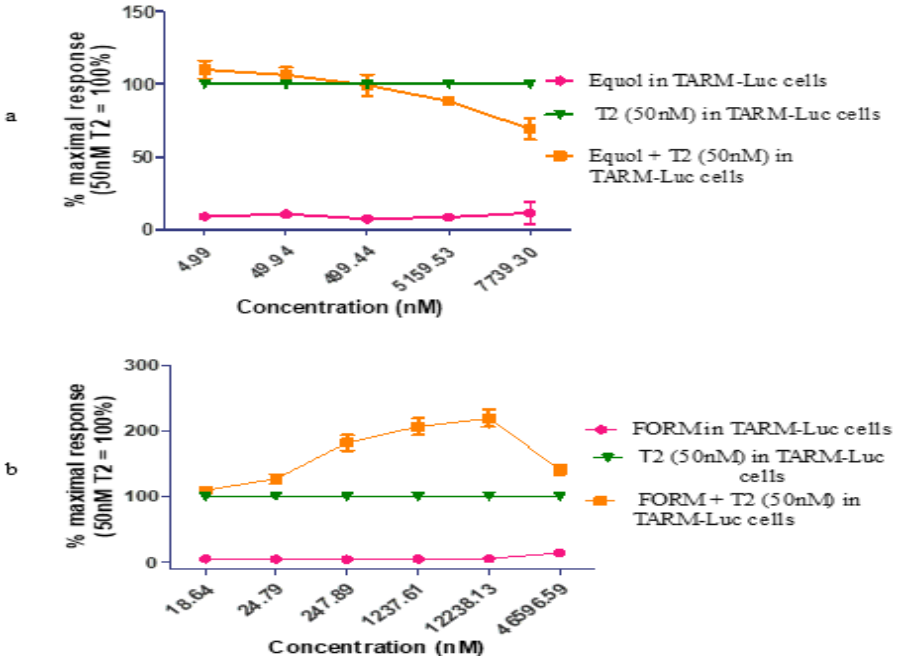



**Figure 6.** Per cent maximum Transcriptional activity (TA) of increasing concentrations of equol and formononetin (FORM) in the absence or the presence of testosterone (50 nM) in TARM -Luc cell lines. Values are per cent luciferase activity relative to the maximum of hormone (arbitrarily taken as 100%) ± SEM (means from three independent experiments (n=3) with each experimental point performed in triplicate). The incubation period was 48 h.

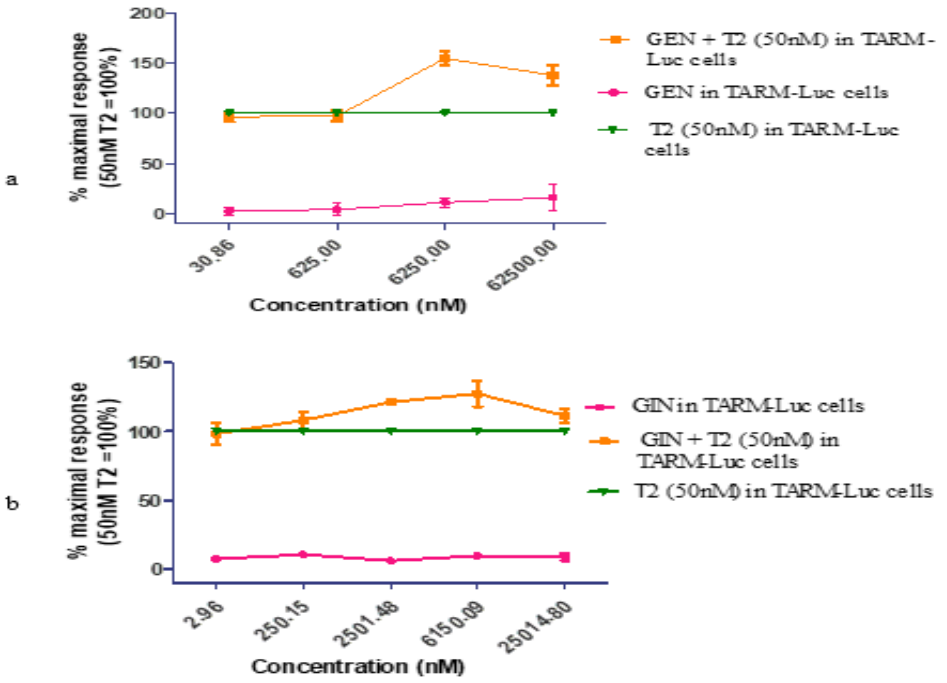



**Figure 7**. Per cent maximum Transcriptional activity (TA) of increasing concentrations of genistein (GEN) and genistin (GIN) in the absence or the presence of testosterone (50 nM) in TARM -Luc cell lines. Values are per cent luciferase activity relative to the maximum of hormone (arbitrarily taken as 100%) ± SEM (means from three independent experiments (n=3) with each experimental point performed in triplicate). The incubation period was 48 h.

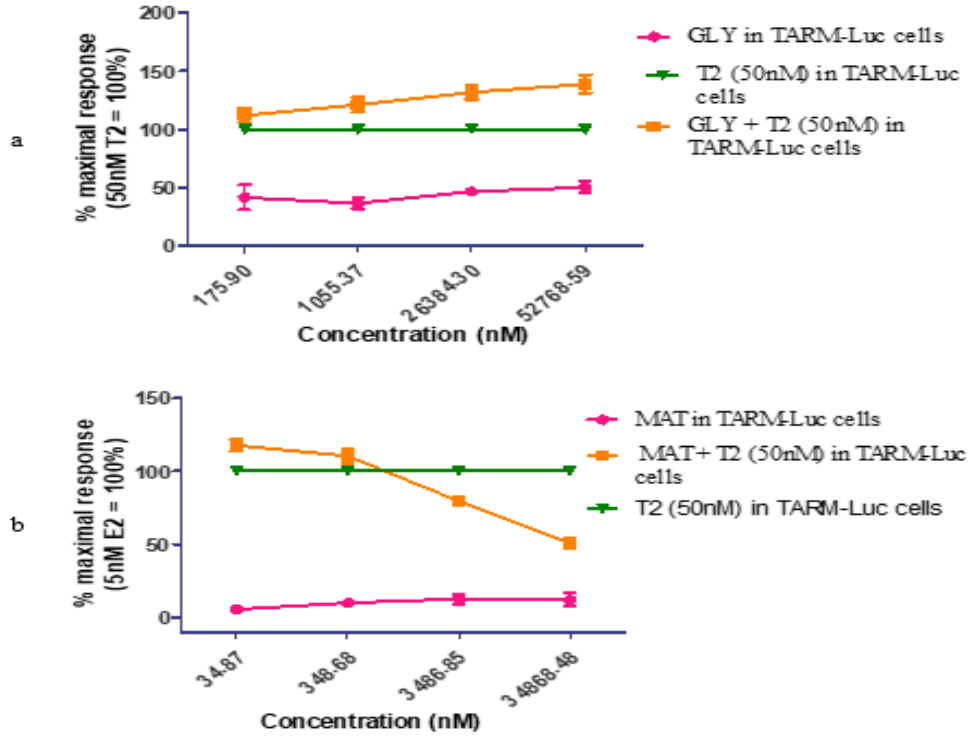



**Figure 8.** Per cent maximum Transcriptional activity (TA) of increasing concentrations of glycitein (GLY) and matairesinol (MAT) in the absence or the presence of testosterone (50 nM) in TARM -Luc cell lines. Values are per cent luciferase activity relative to the maximum of hormone (arbitrarily taken as 100%) ± SEM (means from three independent experiments (n=3) with each experimental point performed in triplicate). The incubation period was 48 h.

**Table 3.** Percent transcription activity of phytoestrogens in the presence and in the absence of hormone in TARM-Luc cell lines (Values are mean % TA ± SD relative to testosterone (50 nM) of three independent experiments), the luciferase activity measured after 48 h.

| Compound | Concentration (nM) | % TA of the compound (Relative to the Maximum of T2 arbitrarily taken as 100%) | % TA of the Compound + hormone (Relative to Maximum of T2 arbitrarily taken as 100%) |
|---|---|---|---|
| Apigenin | 121.74 | 2.13 ± 0.59* | 101 ± 5.99** |
| | 1158.23 | 4.51 ± 3.54* | 106 ± 5.89*** |
| | 2312.76 | 5.11 ± 7.71* | 114.31 ± 7.36*** |
| | 11563.8 | 28.98 ± 4.92*** | 161.83 ± 16.93*** |
| | 25000 | 37.89 ± 4.94*** | 148.08.01 ± 13.25*** |
| | 37004.14 | 48.08 ± 2.33*** | 145 ± 18.40*** |
| Daidzin | 1.92 | 8.74 ± 3.19* | 99.23 ± 3.45** |
| | 162.35 | 5.90 ± 1.69* | 77.58 ± 4.28** |
| | 1623.52 | 6.83 ± 2.36* | 92.45 ± 8.83** |
| | 3902.69 | 11.50 ± 5.30** | 87.85± 10.14** |
| Daidzein | 49.95 | 2.56 ± 4.50* | 41.80 ± 5.86* |
| | 499.53 | 2.60 ± 5.94* | 40.96 ± 2.61* |
| | 2497.64 | 1.85 ± 3.43* | 39.51± 7.80* |
| | 6391.6 | 8.35 ± 2.79* | 140.67 ± 12.51*** |
| | 24583.07 | 5.88 ± 4.47* | 274.59 ± 22.08*** |
| Enterodiol | 20.15 | 22.00 ± 6.25** | 92.50 ± 7.36** |
| | 201.89 | 17.00 ± 2.00** | 89.50 ± 10.30** |
| | 2018.9 | 16.00 ± 9.00** | 85.00 ± 8.83** |
| | 9800 | 12.00 ± 3.25** | 82.25 ± 9.57** |
| Equol | 4.99 | 8.94 ± 3.17* | 112 ± 6.72** |
| | 49.94 | 10.70 ± 3.83** | 106.45 ± 7.21** |
| | 499.44 | 7.28 ± 1.10* | 99.39 ± 10.30** |
| | 5159.53 | 8.60 ± 2.36* | 88.31 ± 2.57** |
| | 7739.3 | 11.50 ± 3.64** | 69.31 ± 6.30* |
| Formononetin | 18.64 | 5.22 ± 3.81* | 109.19 ± 6.94*** |
| | 24.79 | 4.35 ± 3.91* | 126.69 ± 9.67*** |
| | 247.89 | 4.04 ± 2.50* | 181.57 ± 6.93*** |
| | 1237.61 | 4.54 ± 2.18* | 206.18 ± 17.84*** |
| | 12238.13 | 5.47 ± 3.21* | 218.78 ± 18.40*** |
| | 46596.59 | 13.97 ± 4.64** | 140.75 ± 1.04*** |
| Genistein | 18.64 | 5.22 ± 5.81* | 109.19 ± 6.94*** |
| | 24.79 | 4.35 ± 5.91* | 126.69 ± 9.67*** |
| | 247.89 | 4.04 ± 4.50* | 181.57 ± 16.93*** |
| | 1237.61 | 4.54 ± 7.18* | 206.18 ± 17.84*** |
| | 12238.13 | 5.47 ± 7.21* | 218.78 ± 18.40*** |
| | 46596.59 | 13.97 ± 4.64** | 140.75 ± 11.04*** |
| Genistin | 2.96 | 7.28 ± 1.69* | 98.37 ± 11.04** |
| | 250.15 | 10.45 ± 2.50** | 108.00 ± 8.83*** |
| | 2501.48 | 5.95 ± 2.21* | 121.33 ± 2.05*** |
| | 6150.09 | 9.50 ± 2.94* | 127.50 ± 13.25*** |
| | 25014.8 | 8.65 ± 3.68* | 111.40 ± 7.07*** |

**Table 3.** Contd.

| Compound | Concentration (nM) | % TA of the compound (Relative to the Maximum of T2 arbitrarily taken as 100%) | % TA of the Compound + hormone (Relative to Maximum of T2 arbitrarily taken as 100%) |
|---|---|---|---|
| Glyciten | 175.9 | 41.48 ± 15.27*** | 111.92 ± 8.90** |
| | 1055.37 | 36.63 ± 6.90*** | 121.24 ± 8.90** |
| | 26384.3 | 46.59 ± 4.67*** | 131.67 ± 8.50** |
| | 52768.59 | 50.13 ± 6.96*** | 138.88 ± 10.98** |
| Matairesinol | 34.87 | 5.51 ± 1.54* | 117.77 ± 5.52*** |
| | 348.68 | 9.90 ± 1.47* | 110.50 ± 7.36*** |
| | 3486.85 | 12.58 ± 4.93** | 79.47 ± 4.34** |
| | 34868.48 | 11.89 ± 6.49** | 50.94 ± 4.96* |

**For the middle column**, * no effect < 10% of the hormone (Black); **Slight agonistic activity, value > 10 < 25% of the hormone (purple); ***medium agonistic activity, Value >25<75 of the hormone (blue). **For the last column for combinatorial (PE + T2) effects** *value <75% PE dose considered antagonistic to hormone (Black); **value > 75% agonistic activity of hormone not clearly additive where additive means PE activity and hormone activity is the sum of the individual activity, ***> 100 and Synergistic meaning the value > sum of (% PE activity + (100) for T2 activity).

registered less than 10% agonistic activity of the testosterone (50 nM) at all tested concentrations. Daidzein in the presence of testosterone hormone (Table 3) had a mixed reaction to the transcriptional capabilities of testosterone. At lower concentrations, it was an antagonist (< 2.5 µM); it exhibited superagonist status which was at 140% that of testosterone (50 nM), at 6 µM and peaked at 25 µM at 275% agonistic activity compared to that of testosterone (50 nM). In the TARM-Luc cell line enterodiol (Figure 5b) slight agonistic activity (12-22%) that of testosterone (50 nM) hormone arbitrarily assigned 100%. Enterodiol suppressed the agonistic activity of testosterone in a linear trend by a maximum of 18% at the 10 µM level (Table 3).

## Equol and Formononetin

Equol and Formononetin are shown in Figure 6a below and in Table 3. When TARM-Luc cell lines were exposed to equol at varying concentrations equol was highly antiandrogenic as it had less than 10% of testosterone activity at the tested concentration. In the presence of testosterone, there was no effect on hormone action at concentrations < 5 µM). The concentrations beyond 5 µM beyond equol had an antagonistic effect (11% that of 50 nM testosterone) probably due to cell cytotoxicity which translated into reduced luciferase expression for the combination.

Exposing TARM- Luc cell line to formononetin (Figure 6b and Table 3) resulted in an antagonistic effect or lack of androgenic effect (agonist effect of less than 10% of hormone) for 12 µM and medium agonist effect (13.97% of hormone) at the highest concentration about 47µM with formononetin was observed. Formononetin in the presence of the testosterone (50 nM) hormone however had a superagonist effect observed at all concentrations of 24 nM (127%) which peaked at 12.25 µM (218%). Beyond that (about 47 µM) agonistic activity fell (at higher concentration) probably due to cytotoxicity to the cells.

## Geneistin and Genistein

When the TARM-Luccell line was exposed to genistein, like almost all PEs it was antagonistic or had no androgenic effects recording a 4-5% agonistic effect of testosterone (50 nM). It was weakly agonistic (14% agonistic effect of hormone) at 47 µM. In the presence of testosterone hormone, however, genisteins' combinatorial transcriptional activity was additively agonistic or synergistic beyond 625 nM and additively at 47 µM (Figure 7).

Genistin was anti-androgenic or antagonistic in the TARM-Luc cell line exhibiting 6-10% maximal induction relative to testosterone (50 nM), which was arbitrarily assigned a 100% induction. In the presence of testosterone, genistin had no effect at the lowest concentration tested (2.96 nM); had an agonistic effect (at 250 nM) and an agonistic effect that was additive or synergistic at 2.5- 25 µM (Figure 7).

## Glycitein and Matairesinol

When the TARM –Luccell line was exposed to glycitein, it had medium agonist activity (36 -50%) maximum induction relative to testosterone (50 nM) which was arbitrarily assigned 100% induction, at all tested concentrations. Glycitein effect on testosterone hormone (50 nM);

transcriptional activity was agonistic that was neither of additive nor synergistic proportions but had an increasing trend from lowest concentration to highest concentration tested (Table 3 and Figure 8). In TARM Luc cell line matairesinol was anti-androgenic or no agonist effect was observed at 35-350 nM with maximal agonist activity of 5 and 10% of testosterone (50 nM) induction arbitrarily assigned a maximal induction of 100%. It was of slight agonist proportions at concentrations of 3.5-35 µM at 12-12.5% of the testosterone. Matiresinol at the two lower concentrations (35 and 350 nM) enhanced testosterone hormone transcriptional activity synergistically and additively respectively, in the TARM-Luc cell line. At a higher concentration (3.5 µM) there was an inhibitive effect on the testosterone bioactivity as the combined maximal induction was 80% which was further inhibited to definitely antagonistic proportions (51%) at a concentration of 35 µM. It was observed that at higher concentrations matairesinol inhibited both hormone activities in the cell line.

## DISCUSSION

### Fold Inductions of phytoestrogens in TARM-Luc cell lines

#### *Fold inductions in TARM Luc cell lines in non-normalised curves*

Results from the Androgen-responsive (AR) reporter gene assay exhibited that eight PEs are antiandrogenic and two weakly androgenic at high (around micromolar) concentrations. Androgenicity was measured as the luciferase activity expressed over control in TARM-Luc cells. To track changes in protein levels and gene expression, reporter proteins are crucial tools in the study of biological processes. Reporter proteins called luciferases allow for quick and extremely sensitive detection with an exceptional dynamic range (Masser*et al*., 2016).

Apigenin and glycitein had some androgenic and at very high (micromolar range) concentrations of the PEs were required to show any androgenic compared to the testosterone which was detectable at the picomolar level. In Figure 4 apigenin had an average maximum fold induction of 20 (67%) while glycitein had 15 (50%) against 30(100%) for testosterone. For the other eight PEs (daidzein, daidzin, equol, formononetin, genistein, genistin, matairesinol and enterodiol), the fold induction was barely ≤ 14% of testosterone. A study by Willemsen *et al.,* 2004 failed to identify any PE as AR agonist at the tested concentrations. In the current study of Park and colleagues, they found the antiandrogenic effect in male fish and the antiestrogenic effects in female fish. They conclude that these reactions may eventually interfere with reproductive performance (Park *et al*., 2022).

Ain *et al*. (2021) discovered that plant extract had antiandrogenic properties that were supported by the disruption of the seminiferous epithelium's normal arrangement, which reduced tubular diameter and interfered with normal spermatogenesis. Similar findings were made in the current study by Sharath and colleagues, who discovered that the polyphenol-rich extract had strong antioxidant and antiandrogenic potential *in vitro*. This may be due to the extract containing more -OH groups, as suggested by the broad peak for -OH in FTIR spectra (Sharath *et al*., 2022).

One explanation for the anti-androgenicity is that PEs (such as genistein) down-regulate androgen receptors by modulating HDAC6-HSP90 cochaperone function. The AR is normally stabilised by chaperone Hsp90 (heat shock protein) activity. It was found that genistein and other inhibitors inhibit the Hsp90 deacetylase HDAC6, which increases the ubiquitination of the AR. Only HDAC6 possesses both a ubiquitin-binding site and two catalytic sites. These suggested that, like other inhibitors, it had the potential to be used in the chemotherapy of prostate cancer (Basak *et al*., 2008; Garmpis *et al*., 2019).

When HDAC6 is inhibited, chaperone proteins like HSP90, aggresomes, and proteasomes are also inhibited. HDAC6 inhibitors consequently interfere with the proper degradation of proteins, which causes an accumulation of polyubiquitinated proteins and the activation of the apoptotic and autophagic pathways (Basak *et al.,* 2008; Movafagh and Munson, 2019). Once more, HDAC6 has been linked to a variety of tasks, including intracellular transport, the release of neurotransmitters, and aggregate formation (LoPresti, 2021).

### $EC_{50}$s and relative potencies of PEs in the TARM-Luc cell line

Androgens are steroid hormones that are essential for the establishment and upkeep of the male phenotype and sexual function. Other non-reproductive organs affected by these hormones include bone and skeletal muscle (Patrão*et al*., 2009). Furthermore, Androgens are hormones that enable male sexual development and maturation. They also maintain male reproductive function and sperm formation. Chemicals that can mimic androgens, block them or in any way, modulate their metabolism and can affect their function. Two PEs, apigenin and glycitein were found to be weakly androgenic at the tested concentrations.The $EC_{50}$s (Table 2) for the androgen cell line were testosterone ($3.7 \times 10^{-9}$ M), apigenin ($4.5 \times 10^{-6}$ M) and glycitein ($4.8 \times 10^{-5}$ M).

The $EC_{50}$ for testosterone $3.7 \times 10^{-9}$ M is comparable to that of $8 \times 10^{-10}$ M (Willemsen *et al.,* 2004) where the same cell lines were used. They also exposed the cells to PEs for 24 hrs. In our study, our optimum exposure time was 48 h. This might have accounted for the observed differences as the cells might have changed as observed

in their androgenic activity. The $EC_{50}$ for testosterone reported in other studies using Yeast Receptor Assay and Chinese Hamster Ovary (CHO) cells was different from our findings (Kang *et al.,* 2008). A meta-analysis conducted by Montani and colleagues on differences in $EC_{50}$s for the same compound in various studies on estrogenicity which may be related to androgenic was due to various reasons (Montani *et al.,* 2008). Hormones and health may be impacted by dietary phytoestrogens. The effects of dietary phytoestrogens are influenced by the exposure (phytoestrogen type, matrix, concentration, and bioavailability), the consumer's ethnicity, hormone levels (related to age, sex, and physiological condition), and their current state of health (Domínguez-López *et al*., 2020).

Mammalian cells have however been reported as superior to yeast cells. This is because they are closer or similar to human cells concerning metabolism, uptake and membrane transport hence relevant to human health (Nagel *et al*., 1998). The yeast cell has limitations because the release of the β-galactosidase or the luciferase reporter protein is problematic following exposure. As a result, large variations in the results have been noted with the use of yeast cells (Bovee *et al.,* 2004). However, Masser and colleagues created stable and destabilized NLuc genes that were codon-optimized for yeast expression, and they describe the properties and potential applications of this brand-new, highly sensitive luciferase reporter in yeast (Masser *et al*., 2016).

**Enhancement or inhibitive effects of phytoestrogens on testosterone–dependent transcription TARM-Luc cell line**

Testosterone (T), either directly or through its conversion into the more potent metabolite 5α-dihydrotestosterone (5α-DHT) or via aromatization into estradiol (E2), modulates crucial biochemical signalling pathways of human physiology and is essential for the development and/or maintenance of functions in a variety of tissues and organs (Traish, 2017). The importance of assessing PEs' effect on the TA of hormones is gaining currency as the appreciation of the fact that in the body PEs interact with the hormones in the cell context and the effective transactivation may be affected by the hormones (Ososki *et al.,* 2003). Additionally, phytoestrogens have been shown to influence sex hormones via ER-independent mechanisms of action, such as by changing the levels of hormone-binding globulin (SHBG) (Domínguez-López *et al*., 2020).

When PEs were coincubated with testosterone hormone, a range of combinatorial bioactivity effects depending on the concentration were observed. These observations ranged from antagonistic at a lower concentrations, no effect, and agonist (additive or synergistic). This depicted that the PE have potential endocrine disruptive activity in the androgenic TARM-Luc cell line as they could cause a change in the activity of testosterone (50 nM), especially in a synergistic manner. This is the only study as far as we are aware that has studied the transcriptional activity effect of PEs on the hormone testosterone (50 nM) with so many individual phytoestrogens at a wide concentration range.

According to the latest research by Kudo and colleagues, both traditional androgens and 11-oxygenated androgens stimulate androgen signalling in prostate cancer (PC) cells. The development of castration-resistant prostate cancer has been linked to the intratumoral conversion of adrenal androgen precursors to active androgens because 5 -dihydrotestosterone (DHT) levels in castrated PC patients do not completely decrease (CRPC). Dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), 4-androstene-3, 17-dione (A4), and 11-hydroxy-4-androstene-3, 17-dione (11OHA4) are androgen precursors that are pooled at high concentrations in the human adrenal gland, and 11-oxygenated androgens are alternative sources of intratumoral androgens (Kudo *et al*., 2022).

On the other hand, it has been reported that dietary phytoestrogens have an impact on human health when consumed over a lifetime. For instance, Domnguez-López and colleagues discovered that pregnant women's insulin metabolism improved. Children with hypothyroidism have been found to have goitrogenic effects, and alterations in sex hormones have been observed in late childhood. Although there have been reports of positive goitrogenic effects, improved glycemic control, and decreased cardiovascular risk markers in postmenopausal people, the effects on hormones are inconsistent in premenopausal and postmenopausal women. Goitrogenic effects and a decrease in insulin in non-alcoholic fatty liver patients in adult men (Domínguez-López *et al*., 2020).

**Conclusions**

The TARM-Luc cell line was suitable for assessing potential endocrine disruptive effects of PEs in the presence and the absence of testosterone (50 nM) androgenic character of PEs. The PEs studied could mimic hormone action and hence have the potential for endocrine disruption. This study determined the dose-dependent androgenic character of all 10 PEs at various concentrations. The dose response curves and $EC_{50}$s for two (glycitein and apigenin) in TARM-Luc cell lines were determined. The other eight were antagonistic at all concentrations tested. The conclusion was that all PEs tested were mainly antagonistic at lower concentrations or all concentrations. Two of them (apigenin and glycitein) were weak agonists in the TARM-Luc cell line at higher concentrations (micromolar region). The study found that 8/10 of the PEs enhanced it at one or more of the tested concentrations after assessing the dose-dependent effect of the PEs on the testosterone (50 nM) hormone TA.

Daidzin and enterodiol did not enhance testosterone (50 nM) while the effect of matairesinol TA on the hormone was almost negligible. The enhancement or inhibition effect of PEs on the transcriptional activity of hormones provided insight into dose-dependent ant (agonist) character of the PEs with the hormone standards at the tested concentrations.

These results are of public health importance as phytoestrogens have been found to mimic hormones to various degrees in an androgenic (TARM-Luc) cell line. Likewise, the bioactivity of the hormones is suppressed or amplified by the presence of the PEs. This observation would be of particular importance especially regarding the phytoestrogens enhancing effect on testosterone as relates to the consumption of isoflavones (such as genistein, daidzein, and glycitein). This is because they form about 90% of active isoflavones in soybean and consumption in soya-based food and infant formulas result in concentrations (micromolar levels) due to bioaccumulation, especially with frequent feeding. The superagonist character of apigenin, formononetin, genistein and genistin in the presence of testosterone in the TARM-Luc cell line indicates the mode of action was non-genomic. This may warrant further investigation. This study has served to confirm the endocrine disruptive potential of PEs in the presence and absence of hormones in the TARM-Luc cell line. It emphasizes the need to re-evaluate the use of PEs in foods and especially in children. The *in vivo* tests are recommended to further confirm the androgenic potential. Further *in vitro* assays may be designed to investigate the additive effects of phytoestrogens at physiologically relevant concentrations. In addition, further tests may be conducted to characterise the mode of action of the PEs (in the presence or absence of hormone), super-agonist action, and TARM-Luc cell.

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## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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