



## IN VITRO ASSESSMENT OF DIET-DERIVED PHYTOCHEMICALS ON PSA SECRETION

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**ABSTRACT:** Prostate cancer (PCa) is increasingly a global public health burden. The prostate specific antigen (PSA) is the major biomarker for diagnosing and managing PCa. Due to the importance of PSA as a biomarker and as a downstream product of the androgen receptor (AR), it was imperative to expose LNCaP cells to selective diet-derived phytochemicals and investigate the effect on PSA protein secretion. The phytochemicals: Curcumin, 3,3'-Diindolylmethane (DIM) and Epigallocatechin-3-gallate (EGCG) were utilised singly and in paired combinations as in vitro treatments. The ELISA technique was utilised to assess secreted PSA levels. Results obtained indicated that DIM posed inhibitory effects to PSA production and secretion. Uniquely, DIM lowered PSA levels significantly when compared to curcumin and EGCG. This finding corroborates with previous reports where DIM was observed to inhibit AR protein expression. It is suggestive that DIM targets the androgen receptor and its downstream pathway that produces PSA. In conclusion, DIM may hold overwhelming potential chemoprevention and management of PCa, and can be harnessed for enhanced clinical applications.

**KEYWORDS:** Prostate Cancer, Prostate Specific Antigen, 3,3'-diindolylmethane, ELISA, Chemoprevention



## INTRODUCTION

PCa is a disease that affects many males in Nigeria [Akinremi *et al.*, 2011]. This disease exhibits two phenotypes, the slow-growing PCa type and the aggressive type. In many cases, the slow-growing type does not kill the patient, but dies with the patient, while the aggressive type can be fast-growing and fatal. Many patients with PSA levels above 4 ng/mL are advised for further examination which involves a DRE and prostate tissue biopsy [Farolfi *et al.*, 2018; Traynor *et al.*, 2020]. Currently, treatment modalities include surgical options such as radical prostatectomy, endocrine therapy like orchidectomy, and others such as radiotherapy, brachytherapy and chemotherapy [Ye *et al.*, 2020].

In desperate efforts for a specific biomarker for PCa, the PSA was discovered. Prior to the discovery of PSA, the prostatic acid phosphatase (PAP) was identified and used for PCa diagnosis and staging. The use of PAP was discontinued in the aftermath of PSA discovery. In 1979, Wang and colleagues isolated a 33-KD glycoprotein that is highly specific to the prostate than PAP. The PSA protein is secreted into the bloodstream and the levels can be detected and measured with low invasiveness [Traynor *et al.*, 2020]. PSA is a downstream target protein of the androgen receptor (AR). The production and secretion of PSA into the bloodstream indicates increased AR-induced proliferation of prostate cells [Bryce *et al.*, 2017]. Although normal prostate tissues and PCa tissues produce PSA, the presence of basal cells in normal prostate causes a retention of PSA, while the lack or reduced numbers of basal cells as observed in PCa causes a leak of PSA into the bloodstream, this thus results in increased levels of PSA during abnormalities associated with the prostate [Zheng *et al.*, 2020].

The PSA has a unique role and is a major diagnostic biomarker for PCa, it is a standard monitoring tool for PCa management [Bryce *et al.*, 2017; Traynor *et al.*, 2020]. Raised PSA levels does not always indicate PCa, but possibly a non-cancerous enlargement or inflammation of the prostate. PSA levels above 4 ng/mL may indicate progressive PCa, prostatitis or benign prostatic hyperplasia (BPH). After a radical prostatectomy procedure, PSA levels above 0.2 ng/mL may indicate biochemical recurrence, a poor post-operative outcome [Farolfi *et al.*, 2018; Tikkinen *et al.*, 2018; Traynor *et al.*, 2020].

Chemoprevention is a strategic use of nutritive or chemical agents that possess the capability to inhibit or delay or reverse the process of carcinogenesis [Wattenberg, 1985; Greenwald *et al.*, 1995]. Epidemiological studies have linked dietary factors to low incidences of PCa. The cultural consumption of unique phytochemicals in wholesome food has been reported as reasons for the lowest PCa incidences in Asia and Africa [Kubatka *et al.*, 2020]. Many phytochemicals are effective in inhibiting PCa cellular growth/proliferation [Ibeawuchi-Onuoha, 2016], triggering cell cycle arrest [Ibeawuchi-Onuoha and Anyiam, 2017] and instigating apoptosis [Onuoha and Ikimi, 2019].

In this study, Curcumin, DIM and EGCG were selected for *in vitro* investigation, to determine effect on secreted PSA levels of LNCaP cell lines. Curcumin is a polyphenol obtained from *Curcuma longa* Linn commonly called turmeric. Turmeric is a well-known food additive in Asian cuisine [Yang *et al.*, 2017]. DIM is a polyphenol metabolite from indole-3-carbinol (I3C) hydrolysis [Bradlow and Zeligs, 2010] and obtained from cruciferous vegetables such as cabbages, brussel sprouts and broccoli [Li and Sarkar, 2016]. EGCG is another polyphenol that is obtained from green tea and well-consumed in Asia and the green tea culture is becoming acceptable globally [Miyate *et al.*, 2019]. This study attempts to investigate the effect of



curcumin, DIM or EGCG and their paired combinations on PSA production and secretion of LNCaP cell lines.

## METHODOLOGY

Treatments used in this study included curcumin, DIM and EGCG. Treatments utilised in this study and Dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Germany). The human lymph node prostate carcinoma cell lines (LNCaP) were obtained from American Type Culture Collection (ATCC) (Virginia, USA). The cell culture media was obtained from Invitrogen (Paisley, UK) and Sigma-Aldrich (Germany).

### Cell Culture and Treatment

The human lymph node prostatic carcinoma cells, LNCaP were grown and maintained in RPMI (Roswell Park Memorial Institute) 1640 medium with 10% v/v foetal calf serum (FCS) (Invitrogen, Paisley, UK). All treatments were prepared in DMSO, which constituted less than 0.1% v:v to culture media used. Control treatments contained equivalent concentration of DMSO and treatment of cells was designed as thus: curcumin (5  $\mu$ m), DIM (50  $\mu$ M) and EGCG (30  $\mu$ M) and the combinations curcumin (5  $\mu$ m) + DIM (50  $\mu$ M), curcumin (5  $\mu$ M) + EGCG (30  $\mu$ M) and DIM (50  $\mu$ M) + EGCG (30  $\mu$ M) [Ibeawuchi-Onuoha, 2016]. Concentration doses were obtained by adjusting IC<sub>50</sub> values from previous published studies for this experimental design [Shenouda *et al.*, 2004; Garikapathy *et al.*, 2006; Kimura *et al.*, 2007; Valentini *et al.*, 2009].

LNCaP cells were washed with PBS, trypsinised, centrifuged, re-suspended in sterile warm 10% FCS media (RPMI 1640) and counted with a Coulter particle counter and size analyzer (Beckman coulter, Wycombe, UK). Cell count results were adjusted to obtain  $1 \times 10^4$  cells per well.  $1 \times 10^4$  cells were seeded into 24 well plates and incubated for 48 hours at 37°C and 5% CO<sub>2</sub>. After the period of cell adherence, 1 ml of media containing concentrations of curcumin, DIM, EGCG and the paired combinations of curcumin + DIM, curcumin + EGCG, DIM + EGCG were added to respective wells, in triplicates. Cells were incubated further for 144 hours at 37°C and 5% CO<sub>2</sub>. After the incubation period, supernatant media was aspirated and stored at -80°C prior to use for PSA-ELISA (Enzyme Linked Immunoassay Specific Antigen) assay.

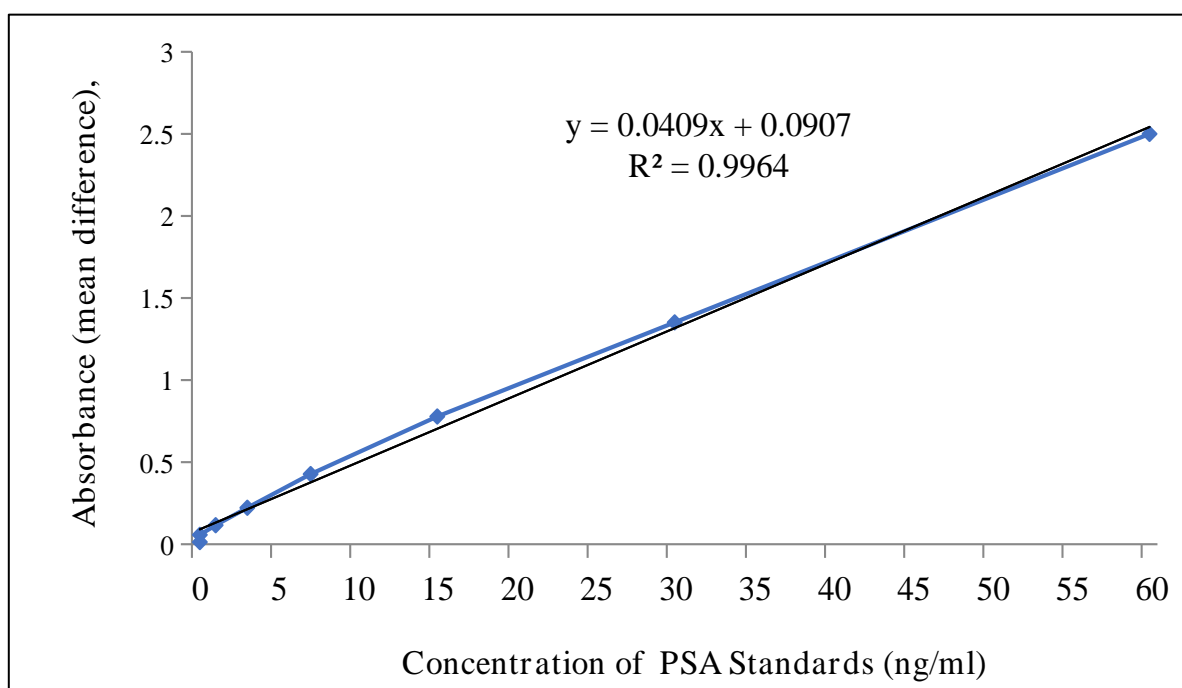
### PSA-ELISA assay

The PSA levels were evaluated with the use of the Quantikine® Human KLK3/PSA immunoassay kit (RnD systems, Minneapolis, USA). Samples and reagents were brought to room temperature before use. 100  $\mu$ l of cell culture supernatant media samples were added into each well in triplicates in 96 well plates. Absorbance of the plates were read at 450 nm and 540 nm wavelengths, within 30 minutes using a microplate reader. A graph of absorbance and concentration of the standards was determined and the unknown PSA concentration was extrapolated and deduced from the Standard curve.

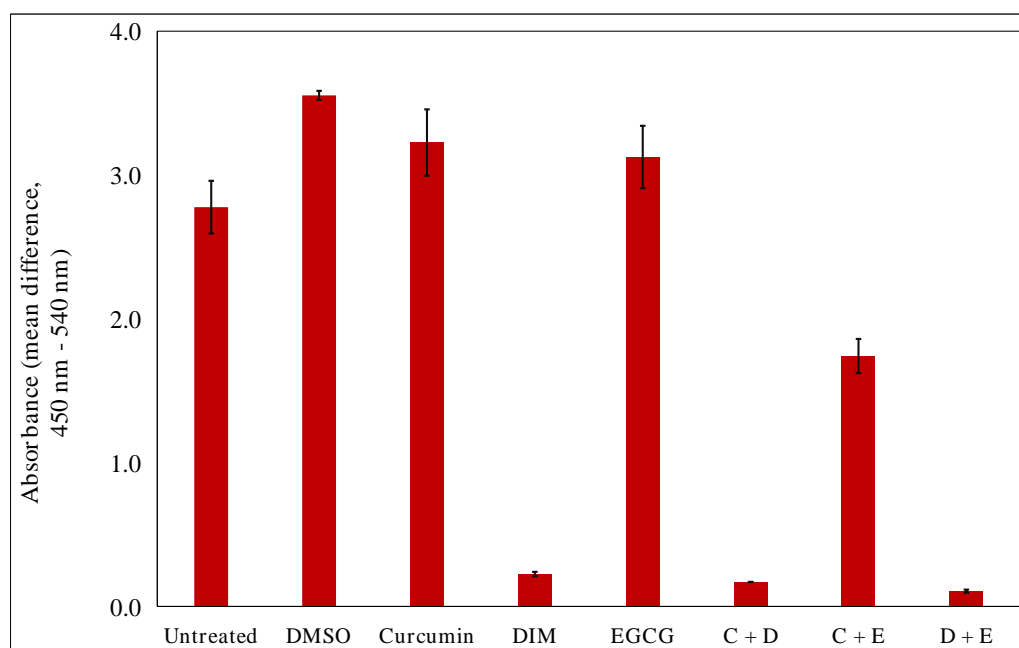
## RESULTS

The effect of curcumin, DIM, EGCG and their combinations were examined on PSA secretion levels from LNCaP cells with the ELISA technique (Human KLK3/PSA Immunoassay, R&D Systems, Abingdon, UK). All treated and controls were assayed in triplicates. The Standard from kit were also assayed in triplicates. Absorbance readings were obtained at 450 nm and 540 nm. Using the kit's standard operating protocol, the mean difference of absorbance values (450 nm - 540 nm) for the standards were plotted against the standard concentrations of 0, 0.94, 1.88, 3.75, 7.5, 15, 30 and 60 ng/ml to generate a standard curve (Figure 1).

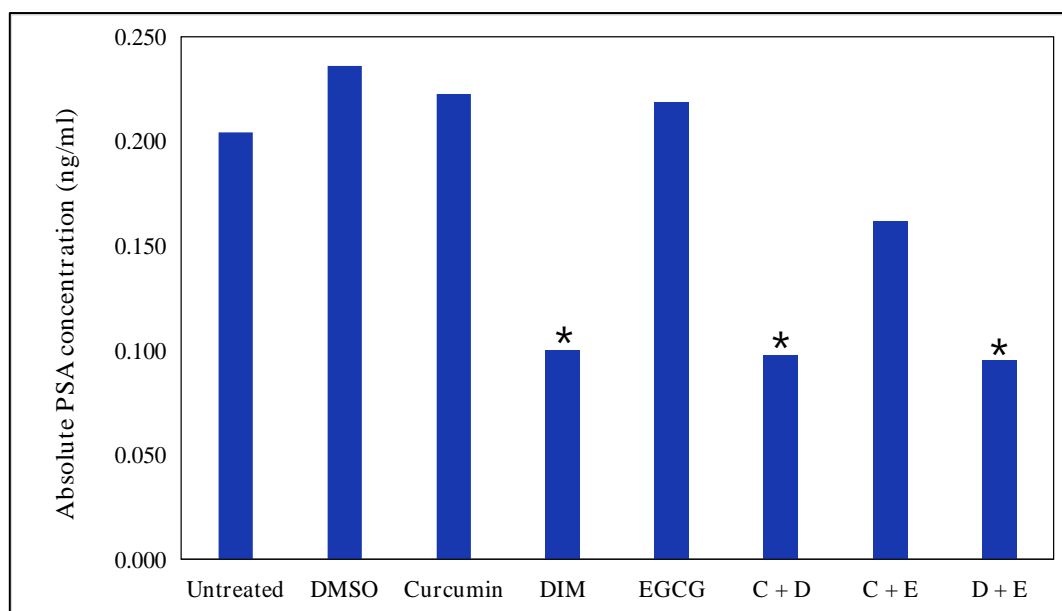
In addition, the mean difference of absorbance values (450 nm - 540 nm) were obtained for treated and untreated media samples (Figure 2). From Figure 1 and generated equation, The relative PSA concentrations were deduced. The relationship between investigated treatments and absolute PSA concentrations was displayed in Figure 3. After 144 hours, there was a significant decrease in PSA levels following the treatments of DIM, C + D and D + E ( $P < 0.05$ ).



**Figure 1: Standard curve generated for the determination of absolute PSA concentration. Mean difference of absorbance for each standard concentration (0, 0.94, 1.88, 3.75, 7.5, 15, 30 ng/ml) to obtain a linear graph.**



**Figure 2: Mean difference of absorbance of treated and control assayed samples. Media samples were employed for this assay and analysed in triplicates. Columns are mean differences of absorbance while bars are  $\pm$  standard deviation.**



**Figure 3: Absolute PSA concentration of treated and control assayed samples. Effect of curcumin, DIM, EGCG and their paired combinations on the secreted levels of PSA by LNCaP cells. (\*) $P < 0.05$  when compared to DMSO (control), as determined by the repeated measures one-way ANOVA followed by a post-hoc Tukey test.**



## DISCUSSION

This study aimed at evaluating the effect of selected diet-derived phytochemicals on levels of secreted PSA proteins from LNCaP cells. The main finding of this study is that the *in vitro* treatments of DIM, C + D and D + E respectively on LNCaP cells caused significant lower secretions of PSA. DIM stood out as the polyphenol with the most potential in the inhibition of PSA.

PSA belongs to the human kallikrein family of serine proteases and are initially synthesised as preproenzymes, and needs to be activated in two steps. PSA is activated firstly with the proteolytic removal of a short amino acid chain at the N-terminus and then with subsequent activation by some proteases, either the matrix metalloproteinases (MMPs) or urinary plasminogen activator (uPA). Functionally, PSA inhibits the coagulation of the seminal fluid [Saxena *et al.*, 2012]. The growth and development of normal prostate and PCa is largely dependent on AR, its role is crucial in prostate organogenesis [Zhang *et al.*, 2018]. AR is responsible for PSA secretion, and PSA remains a very important diagnostic biomarker.

PSA is uniquely investigated in this study because it is prostate-specific and regulated by androgens. Increased levels of PSA indicate abnormalities of the prostate. Since DIM and paired combination treatments containing DIM were capable of inhibiting or lowering PSA levels, this may indicate effectiveness of DIM to prostate carcinogenesis and development. The observation from this study corroborates with previous findings: the polyphenol DIM, was found to ideally inhibit LNCaP cell growth and proliferation maximally when compared with curcumin and EGCG [Ibeawuchi-Onuoha, 2016], in a different study that utilised the western blotting technique, the polyphenol DIM played an inhibitory role on AR protein expression [Onuoha and Anyiam, 2021]. With DIM's capabilities to inhibit LNCaP growth and target the AR and potentially inhibit AR function, it is suggestive that DIM inhibits PSA secretion because it targets AR, the up-stream protein of PSA.

## CONCLUSION

The comparative investigation of curcumin, DIM and EGCG phytochemicals on LNCaP cells indicate that DIM has the most capabilities to inhibit AR and its downstream protein, PSA. The future of DIM as a potential chemopreventive regime lies in its pharmaceutical acceptability and development.





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