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WESTERN BLOT EVALUATION OF LNCAP CELLS IN RESPONSE TO SELECTIVE PHYTOCHEMICAL TREATMENTS

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ABSTRACT: *Phytochemicals present in various plants and plant products instigate definite outcomes on cellular gene targets in living organisms. Many phytochemicals have been recognised for their potentials in chemoprevention and chemotherapy. This study is tasked to establish the effect of curcumin, DIM and EGCG on LNCaP cells to ascertain the effect on selected genes that have been previously reported to be implicated in prostate carcinogenesis. With the application of cell culture treatments and western blotting techniques, the androgen receptor (AR), Akt, Bax, BCL-2, β-catenin, p21, p53, phospho-Akt and surviving proteins were interrogated. Most interestingly is that the androgen receptor protein is observed to be uniquely down-regulated with the DIM treatment and paired combination treatments containing DIM. Selectively, DIM among the other investigated phytochemicals may possess the highest potential in the inhibition of the androgen receptor, a major key player in prostate carcinogenesis.*

KEYWORDS: Prostate Cancer, Androgen Receptor, Western Blotting, Phytochemicals, Chemoprevention.

INTRODUCTION

PCa is a global problem and the most common cancer affecting men in Nigeria and of black African ancestry [Akinremi *et al*., 2011]. The slow-growing PCa, usually referred to as "pussy cats", hardly kills the patient and many times the patients die with the disease. The aggressive PCa type also referred to as "tigers" affects majorly younger males, the disease progresses rapidly and can be fatal [Schroeder, 1993]. PCa is mostly diagnosed with an elevated prostate-specific antigen (PSA) and increased Gleason score (GS) [Jordan *et al*., 2016]. In the management of PCa, confined cases are treated with procedures that include radical prostatectomy, hormone therapy, radiotherapy, brachytherapy and surgical orchiectomy [Mottet *et al*., 2017; Cornford *et al*., 2017]. Advanced PCa cases are treated with androgen deprivation therapies (ADT), however most cases become hormone-refractory and regarded as castration-resistant prostate cancer (CRPC). Most fatalities of PCa are due to CRPC and subsequently metastasis [Li and Sarkar, 2016; Yang et al., 2017]. Tremendous advances have been made in PCa treatment and management strategies. However, due to the nature of PCa proliferation and progression, success has not been completely attained in the conclusive eradication of cancer cells during treatment and the ability to predict outcomes post-treatments

[Li and Sarkar, 2016; Yang et al., 2017]. The search for enhanced and effective regimen is still needed.

From epidemiological studies, the lowest incidence levels of PCa are observed in the Asian continent [Jordan et al., 2016]. Although cases in Asia are currently increasing due to sedentary lifestyles and unhealthy diet, some polyphenols have been attributed to be predominantly responsible for low PCa incidences [Jordan et al., 2016; Li and Sarkar, 2016]. These polyphenols include curcumin, DIM and EGCG . These polyphenols are sourced from turmeric, cruciferous vegetables and green tea respectively.

LITERATURE

Curcumin or diferuloylmethane is sourced from the roots of *Curcuma longa Linn*, commonly known as turmeric. Turmeric is used as a spice in Asian dishes, as a colouring agent and as food supplements. Its use as a food supplement is due to its numerous attributes in the field of medical science. Curcumin is well studied and its anticancer, antioxidative, anti-inflammatory and antimicrobial properties have been well reported [Jordan et al., 2016; Banerjee et al., 2017; Rivera et al., 2017; Yang et al., 2017]. 3,3'-Diindolylmethane (DIM) is a polyphenol metabolite of indole-3-carbinol (I3C), which is obtained from cruciferous vegetables such as broccoli, cabbages and brussel sprouts. DIM has been reported to possess anti-cancer properties and is currently being researched for effectiveness in the treatment of several cancers, including PCa [Zhu et al., 2016, Li and Sarkar, 2016]. (-)-Epigallocatechin-3-gallate (EGCG) is a polyphenol that is present in green tea, a widely consumed beverage in Asia []. With the green tea, polyphenols present include EGCG, (-)-epicatechin (EC), (-)-epigallocatechin (EGC) and (-)-epicatechin-3-gallate (ECG). However, EGCG is reported to be the most bioactive polyphenol [Miyata et al., 2019].

In this study, the effect of these well-known phytochemicals: curcumin, DIM, EGCG and their paired combinations will be investigated on the LNCaP cell line. The LNCaP cells are uniquely selected as in vitro models of PCa for their hormone sensitivity and possession of functional androgen receptors. The effect of curcumin, DIM, EGCG and the paired combinations of curcumin + DIM, curcumin + EGCG, and DIM + EGCG on LNCaP cells will be investigated and the effect on specific protein expressions will be interrogated. Previous evaluations of curcumin, DIM and EGCG have been previously published [Ibeawuchi-Onuoha, 2016; Ibeawuchi-Onuoha and Anyiam, 2017; Onuoha and Ikimi, 2019], and the findings as it concerns LNCaP growth, cell cycle and apoptosis were favourable in the search for an effective regime to chemoprevention and management of PCa. Based on the potential of the understudied diet-derived phytochemicals, it is necessary to investigate the effect on key proteins. This strategy will utilise the western blotting technique to evaluate protein expression. The intensity of blots will indicate reduced activity (low expression), increased activity (high expression) and unchanged activity of specific proteins.

METHODOLOGY

Reagents

Curcumin, Diindolylmethane (DIM) and Epigallocathechin-gallate (EGCG) were purchased from Sigma-Aldrich (Germany). Primary antibodies for androgen receptors (AR), Akt, Bax and Bcl-2 were obtained from Santa Cruz Biotechnology (California, USA). Primary antibodies for p53 and p21, were obtained from Dako (Denmark). Primary antibody for β-catenin was obtained from BD transduction laboratories™ (California, USA), primary antibody for phospho-Akt was obtained from Biosource (Paisley, UK), primary antibody for Survivin was obtained from Novus Biological Incorporated (Colorado, USA) while primary antibody for β-Actin was obtained from Abcam (Cambridge, UK). Secondary antibodies used were anti-Rabbit IgG and anti-Mouse IgG obtained from Sigma-Aldrich (Poole, UK), donkey anti-Goat IgG obtained from Santa Cruz Biotechnology (California, USA), Stabilized Goat anti-Mouse was obtained from Pierce (Illinois, USA).

Acrylamide, stripping buffer, resolving buffer, running buffer, transfer buffer and Whatman nitrocellulose membrane were obtained from Geneflow Ltd (Staffordshire, UK). Restore™ Stripping buffer was obtained from Pierce Biotechnology (Illinois, USA). The Bradford protein assay reagent was obtained from Biorad (Hemel Hempstead, UK), ECL hyperfilm and ECL chemiluminescence reagents were obtained from GE Healthcare, Amersham (Buckinghamshire, UK), pre-stained protein ladder was obtained from Fermentas (York, UK). Methanol and Chloroform was obtained from Fisher Scientific, UK and Isoton was purchased from Beckman Coulter Limited (Buckinghamshire, UK). Distilled water was obtained from the NANO pure diamond water purifier (Barnstead International, UK), PBS was prepared by dissolving one tablet of phosphate buffered saline tablet manufactured by Oxoid (Hampshire, UK) in 1 liter of distilled water, with an additional 0.1% Tween (Sigma-Aldrich, MO, USA) for PBST preparation. Non-fat dried skimmed milk manufactured by Marvel (Premier Brands limited, UK) was purchased from reputable retailers.

Cell Culture

The androgen-responsive human lymph node prostate carcinoma cell lines (LNCaP) were obtained from American Type Culture Collection (ATCC) (Virginia, USA). The cells were cultured in RPMI 1640 media from Invitrogen (Paisley, United Kingdom) and Sigma-Aldrich (Germany) with 10% foetal calf serum (FCS) from Invitrogen (Paisley, United Kingdom). The handling and maintenance of LNCaP cell lines have been previously described extensively in Onuoha and Ikimi, 2019.

Treatments of Cell Lines

Treatments used were curcumin, Diindolylmethane (DIM) and Epigallocathechin-gallate (EGCG). All treatments were prepared in dimethyl sulfoxide (DMSO) which constituted less than 0.1% v:v to culture media used. Control treatments contained equivalent concentration of DMSO and the treatment of cells was designed. The concentration doses were obtained by adjusting IC_{50} values from previous published works and studies for this experimental design (Shenouda *et al*., 2004; Garikapathy *et al*., 2006; Kimura *et al*., 2007; Valentini *et al*., 2009). The LNCaP cells were treated with concentrations of curcumin (5 μm), DIM (50 μM) and EGCG (30 μM) and the paired combinations were treated as thus: curcumin (5 μm) + DIM (50

μM), curcumin (5 μM) + EGCG (30 μM) and DIM (50 μM) + EGCG (30 μM) and incubated for 72 hours.

After the incubation period of 72 hours, all cell lysate preparation was carried out on ice. The media from each culture flask was aspirated, the adherent cells were washed twice with cold PBS (pH 7.2), trypsinized, centrifuged and pelleted. The cell pellets were kept on ice and lysed in 250 μl whole cell lysis buffer (200 mM Tris (pH 7.5), 1.5 M NaCl, 10 mM EDTA, 10% triton X-100, 25 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 10 mM sodium orthovanadate (1x RIPA buffer) and protease inhibitor cocktail in a 1:100 dilution). The cells were left to lyse on ice for 10 minutes, and afterwards were centrifuged at 4ºC, 13000 rpm for 5 minutes. The supernatant was removed and stored in eppendorfs at -20ºC until required.

Western Blotting Analysis

With the use of the Bradford protein assay reagent from Biorad (Hemel Hempstead, UK), 1 - 10 μg/ml of protein standard was measured serially and added to 1 ml of diluted Bradford reagent (1:5 in distilled H_2O). The samples were left standing for 5 minutes and the absorbance was read at 595 nm and used to produce a standard curve of Absorbance against Protein concentration (μg/ml). Thus the cellular protein were determined by adding 5 μl of cell lysate supernatant to 1 ml of diluted Bradford reagent (1:5 in distilled H_2O), the absorbance was read with the GeneQuant Pro (Amersham Biosciences, UK) at 595nm. Cellular protein was calculated from the standard curve equation and the appropriate volumes of supernatant samples were measured quantitatively in order to achieve equal loading of cellular proteins (30 μg) for each sample aliquot.

For the western blotting technique, the running gel and stacking gel were prepared and allowed to set. Aliquots of samples with the same amount of cellular protein were loaded unto the gel and left to run for 1 hour at 120V. After the sample running phase, the proteins on gel were transferred to Whatman nitrocellulose membrane (Geneflow, UK) for 2 hours at 100V. Membranes were then blocked in milk-PBST (5% fat-free Marvel milk in PBST w/v) for a minimum of 2 hours. To probe the blots, the membrane was incubated with primary antibody in a specified dilution and left on a rocker for 2 hours at 4ºC. Primary antibodies used were specific for Androgen receptor, p53, p21, survivin, β-catenin, Bax, BCL-2, Akt, phospho-Akt. Afterwards, the membrane was washed in PBST (5 x 5 minute washes) and then incubated with secondary antibody at a 1:2000 dilution for 1 hour on a rocking platform. After which the membrane was washed in PBST (5 x 5 minute washes). Electrochemiluminescence (ECL) reagent was poured over the membrane for 1 minute and then exposed to ECL-hyperfilm from 20 seconds to 30 minutes.

To confirm equal loading of cell samples, β-actin was used as the standardized protein. The blots were stripped of antibody and incubated in a Restore stripping buffer (Pierce, IL, USA) on a shaking platform for 15 minutes, then the membrane was washed 5 times in PBST and probed with β-actin antibody.

RESULTS

The effects of curcumin, DIM and EGCG and the paired combinations of curcumin + DIM, curcumin + EGCG and DIM + EGCG were reported in previous studies (Ibeawuchi-Onuoha, 2016; Ibeawuchi-Onuoha and Anyiam, 2017; Onuoha and Ikimi, 2019). The under-studied diet-derived agents were reported to possess the potential to inhibit growth, instigate cell cycle arrest and promote apoptosis (Ibeawuchi-Onuoha, 2016; Ibeawuchi-Onuoha and Anyiam, 2017; Onuoha and Ikimi, 2019). Many cellular proteins are involved in the several mechanisms that control cell growth, cell cycle and apoptosis. The deregulation of these cellular proteins may be crucial in the onset and progression of carcinogenesis. With the reported effect of diet-derived phytochemicals on LNCaP cell lines (Ibeawuchi-Onuoha, 2016; Ibeawuchi-Onuoha and Anyiam, 2017; Onuoha and Ikimi, 2019), it is important that the effects of these diet-derived phytochemicals on key cellular proteins are investigated. This study attempts to investigate the individual effect of curcumin, DIM and EGCG and the paired combinations of curcumin + DIM, curcumin + EGCG and DIM + EGCG on key cellular proteins that are frequently implicated in carcinogenesis.

Important cellular proteins analysed were: the androgen receptor (AR), Akt, Bax, BCL-2, β-catenin, p21, p53, phospho-Akt and survivin. The expressions of the androgen receptor (AR), Akt, Bax, BCL-2, β-catenin, p21, p53, phospho-Akt and survival were analysed by the western blotting technique. The LNCaP cells were treated with concentrations of curcumin (5 μm), DIM (50 μM) and EGCG (30 μM) and the combinations curcumin (5 μm) + DIM (50 μM), curcumin (5 μM) + EGCG (30 μM) and DIM (50 μM) + EGCG (30 μM) respectively for 72 hours. Results obtained show that investigated proteins were differentially affected following treatment.

Results obtained from western blotting analysis are shown in Figure 1-4. From Figure 1, the effect of the different treatments on the androgen receptor protein was observed at variable levels. The respective treatments with DIM, the paired combinations of curcumin+DIM and DIM+EGCG were observed to inhibit the expression of the androgen receptor. From Figure 2, the proteins BCL-2 and Bax were poorly expressed by the LNCaP cell lines, however, the respective treatments with DIM, Curcumin+DIM and DIM+EGCG were observed to inhibit the protein expression of BCL-2 and Bax. From Figure 3, the p53 protein is observed to be highly expressed in the treated samples. The treatments with DIM, EGCG, curcumin+EGCG and DIM+EGCG were observed to comparatively increase the expression of p53. While with β-catenin, which is well expressed by LNCaP cells, the treatments DIM and the paired combinations DIM+EGCG were observed to inhibit β-catenin protein expression. From Figure 4, the treatments with curcumin, DIM, EGCG and the paired combinations curcumin+EGCG and DIM+EGCG were observed to inhibit p21 protein expression. With Phospho-Akt, the protein expression was observably unchanged with the respective treatments. Concerning the Akt protein, a slightly reduced expression was observed with the treatments DIM, Curcumin+DIM and DIM+EGCG. For the protein Survivin, the least expression was observed with the treatments EGCG and the paired combination curcumin+DIM. However, survival was also down-regulated in the negative control.

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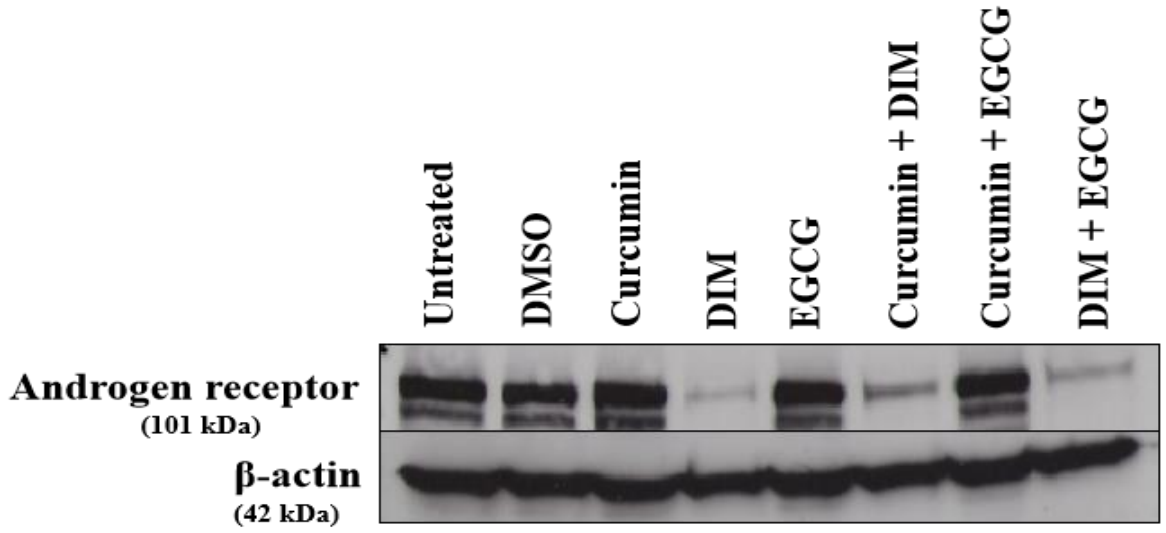

Figure 1: Effect of curcumin, DIM, EGCG and paired combinations on the levels of androgen receptor in LNCaP cell line Blots are representative of at least three experiments. β-actin protein expression also displayed to show equal loading of cell lysate sample (30 µg).

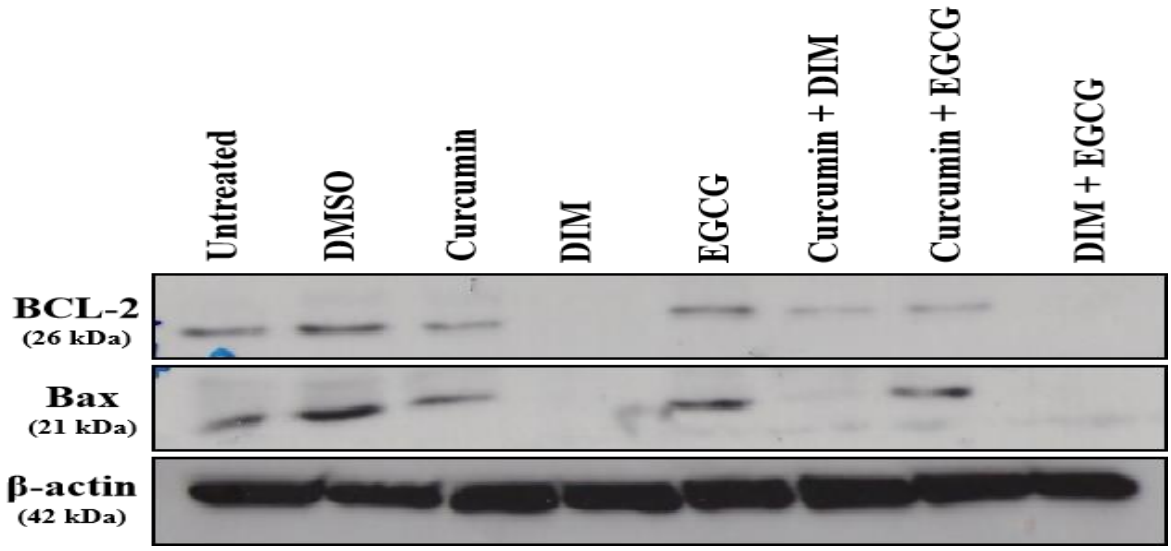

Figure 2: Effect of curcumin, DIM, EGCG and their paired combinations on the levels of BCL-2 and Bax in LNCaP cell line Blots are representative of at least three experiments. β-actin protein expression also displayed to show equal loading of cell lysate sample (30 µg).

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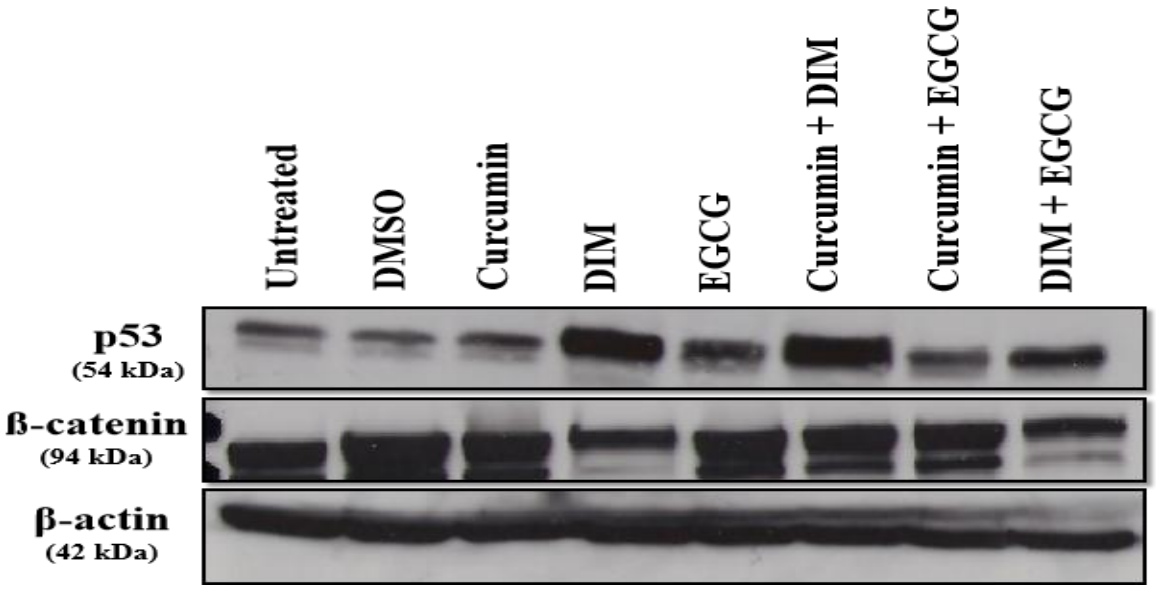

Figure 3: Effect of curcumin, DIM, EGCG and their paired combinations on the levels of p53 and β-catenin in LNCaP cell line Blots are representative of at least three experiments. β-actin protein expression also displayed to show equal loading of cell lysate sample (30 µg).

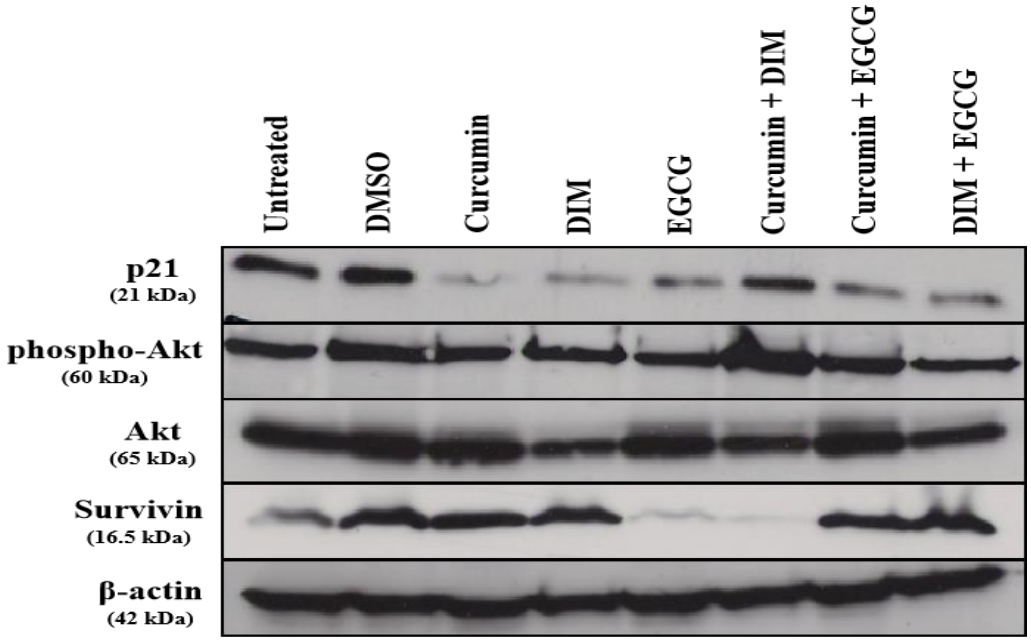

Figure 4: Effect of curcumin, DIM, EGCG and their paired combinations on the levels of p21, phospho-Akt, Akt and Survivin in LNCaP cell line Blots are representative of at least three experiments. β-actin protein expression also displayed to show equal loading of cell lysate sample (30 µg).

DISCUSSION

The expressions of specific proteins: androgen receptor (AR), BCL-2, Bax, p53, β-catenin, p21, phospho-Akt, Akt and survival were analysed by western blotting. From the results obtained, the investigated proteins were either activated (highly expressed), down-regulated (poorly expressed), not-expressed or unchanged following treatments. Treatments used were curcumin, DIM and EGCG and the paired combinations of curcumin + DIM, curcumin + EGCG and DIM + EGCG.

The expression of AR was at its lowest with the treatments: DIM, curcumin+DIM and DIM+EGCG. Reduced expression of AR indicates functional inhibition. AR is very important in the development of the normal prostate, proliferation of prostate cancer and well expressed by the LNCaP cell lines [Zhang et al., 2018]. From the negative and positive controls, the AR expression is high (Figure 1), which indicates the important role AR plays in survival of LNCaP cells. The reduced AR expression with treatments of DIM, curcumin+DIM and DIM+EGCG indicate that the observed effect could be attributed to the presence of DIM, as a sole treatment and in combination. The result of DIM inhibitory effect on AR correlates with previous reports by Bhuiyan et al, 2006 and Chinnakannu et al, 2009.

The BCL-2 family proteins are important regulators of apoptosis, made up of proteins that function as inhibitors and inducers of programmed cell death [Gupta et al., 2002; Hardwick and Soane, 2013]. Functionally, BCL-2 is anti-apoptotic while the Bax group (consisting of Bax, Bak and Bid genes) are pro-apoptotic [Gupta et al., 2002; Rubio et al., 2005]. The expression of BCL-2 and Bax were observed to be lowest with treatment applications of DIM, curcumin+DIM and DIM+EGCG respectively. It was reported that the expression of BCL-2, an anti-apoptotic gene is frequently increased in prostate cancer and the expression of the anti-apoptotic gene Bax remains constant or unchanged [Rubio et al., 2005]. In this study, the reduction of BCL-2 expression by DIM, curcumin+DIM and DIM+EGCG respectively indicated these treatments had an effect on the anti-apoptotic function of BCL-2, which negatively impacted on survival and increased apoptosis of LNCaP cell lines. Furthermore, the effect of DIM, curcumin+DIM and DIM+EGCG treatments on the Bax gene was not expected. From observation, the expression of Bax was greatly reduced than controls. It was anticipated that Bax, a pro-apoptotic gene, would display increased expression with potential treatments due to the divergent and complementary roles of BCL-2 and Bax.

The well-known tumour suppressor gene, p53 was interrogated in this study (Figure 3). p53 induces the genes, p21 and Bax that instigate cell cycle arrest and cell death respectively [Singh et al., 2017]. The loss of p53 leads to non-regulation of cell cycle control and apoptosis, while the mutation of p53 leads to progression of cancers [Yang et al., 2019]. The p53 is observed to be either lost or mutated in many cancers, including PCa [Yang et al., 2019]. It was observed that the p53 protein was well expressed in the treated samples than in the control samples. p53 was well-expressed with treatments of DIM, curcumin+DIM and DIM+EGCG. The increased expressions of p53 indicates that selective treatments may possess the potential to induce functional p53 activity.

Since p21 is a transcriptional target of p53, the effect of the diet-derived agents were interrogated on p21 protein expression (Figure 4). In the event of DNA damage, p21 acts as a tumour suppressor gene, as it effects cell cycle arrest, suppresses proliferation of tumours by binding and inhibiting the cyclin-dependent kinases (CDKs) in the G1 phase [Eastham *et al*.,

1995; Abbas and Dutta, 2009; Karimian *et al*., 2016]. In addition, p21 has been reported to behave as an oncogene by promoting cell growth [Abbas and Dutta, 2009]. p21 was observed to be well expressed in the control samples and poorly expressed in the treated samples (Figure 4). Since p21 was well expressed in the untreated LNCaP cells, the observed result of p21 does indicate that p21 performs an oncogenic role in the progression of the cancer cell line through increased stimulation of the cell cycle. The obvious reduced effect of p21 expression by the respective treatments indicate possible effectiveness in declination of cell cycle activity.

β-catenin protein expression was queried in this study for its functionality in cell-cell interactions and cellular signalling. β-catenin is usually complexed to E-cadherin, and involved in the maintenance of cell membrane, inter-cellular junctions and normal tissue architecture [Whitaker *et al*., 2008]. PCa progression and metastasis is frequently associated with loss of β-catenin. Akt and its phosphorylated and active form, phospho-Akt were also investigated for their unique role in cell survival. Akt is otherwise known as protein kinase B (PKB). To ensure cell survival, the phosphorylation of pro-apoptotic proteins are inhibited by phospho-Akt via the PI3k/serine-threonine kinase Akt signalling pathway [Malik et al., 2002; Ayala et al., 2004; Le Page et al., 2006]. Increased expression of Akt/phospho-Akt indicates PCa progression. From results obtained (Figure 3 and 4), β-catenin, Akt and phospho-Akt expressions were unchanged with treatments indicating the ineffectiveness of the respective treatments in altering the expressions.

A pro-survival protein, survival was investigated in this study. Survivin belongs to the family of "inhibitor to apoptosis protein" (IAP) and is uniquely expressed differently in normal and prostate cancer tissues [Zhang et al., 2010; Hennings et al., 2020]. Expectedly, survival as an inhibitor of apoptosis should be down-regulated by effective inducers of apoptosis. The result obtained (Figure 4) showed that survival was unevenly expressed in the controls and across the different treatments. In addition, results obtained from the study (Figure 4) does indicate that survival is well-expressed in LNCaP cells, and that the treatments EGCG and the combination pair, curcumin+DIM were observed to lower survival expression.

Implication to Research and Practice

With the comparative analysis of these well-known polyphenols: curcumin, DIM and EGCG and the *in vitro* effect on selected proteins, the inhibitory effect of AR by DIM is notable. DIM is widely reported to target and inhibit multiple pathways that promote PCa progression [Draz et al., 2017]. Our results also recommend that curcumin and EGCG possess some level of potential, however the leads provided by this study should stimulate further research to confirm targeted signalling pathways.

CONCLUSION

The use of western blot technique validates that the investigated proteins are well-expressed by the *in vitro* model of androgen-sensitive LNCaP cells. With the expression of these proteins, any agents or treatments that can alter cellular survival and proliferation can be qualitatively established by a reduction in expression. Interestingly, due to the key role of the AR on PCa survival and progression, the inhibitory role of DIM on AR is notable and should be further studied.

Further Research

The potential of DIM will be investigated further in experimental animals and the efficacy of DIM will be ascertained under *in vivo* conditions.

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