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Combinations of selected diet-derived agents show enhanced growth inhibition of LNCaP cell lines

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Abstract

Prostate cancer (PCa) is the most common male-specific cancer worldwide. More often, PCa is diagnosed early. With the slow development of some PCa cases, there is a wide window of opportunity for chemoprevention. Chemoprevention is intended to improve the quality of life, reduce morbidity and mortality in patients. Based on epidemiological studies, it is believed that dietary agents that are utilised specifically in a part of the world may be related to its low disease incidence profile. Healthier diets in Asia contain high levels of curcumin, diindolylmethane (DIM) and epigallocathechin-gallate (EGCG). These diet-derived agents may be responsible for the much lower cancer incidences in Asia. Our aim is to utilise cell models of PCa (LNCaP cell lines) and investigate the effect of curcumin, DIM, EGCG and their paired combinations on cellular growth and survival through the cell proliferation analysis, ATP and Clonogenic assays. Results from cell proliferation analysis showed significant time-dependent growth inhibition following treatment with curcumin, DIM, EGCG and their paired combinations respectively ($P < 0.0001$). However the paired combination of these diet-derived agents: curcumin and DIM (C+D), curcumin and EGCG (C+E) and, DIM and EGCG (D+E) did indicate enhanced inhibition to LNCaP cells viability and growth. Further studies need to be conducted for paired combinations of notable diet-derived agents as they may possess improved chemoprevention abilities in dealing with the progression of PCa.

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Introduction

PCa is the second most commonly diagnosed cancer in men worldwide (Torre *et al.*, 2015). The development of PCa demonstrates well-defined initiation, promotion and progression stages (Johnson *et al.*, 2008). However, with many prostate cancer cases having a long and slow period of carcinogenesis (Singh and Agarwal, 2006; Johnson *et al.*, 2008), it is ideal that chemoprevention is a recommended approach to tackling the disease. Chemoprevention as an intervention strategy has the capability to curb the progression of the disease, improve the quality of life of patients, reduce morbidity and decrease mortality (Betram *et al.*, 1987; Maru *et al.*, 2016). Epidemiological studies have indicated lower prevalence of prostate cancer in Asia and less developed countries while higher prostate cancer prevalence have been observed in western and more-developed countries (Manson *et al.*, 2007; Torre *et al.*, 2015). These variations in prostate cancer epidemiology may be relative to a high consumption of some dietary constituents in the regions with lower PCa incidences. In today's world, humans encounter and are surrounded by carcinogenic agents. It is indeed necessary that chemopreventive agents from dietary sources are well studied as potential regimens for the prevention, impediment or reduction of cancers (Lippman and Lee, 2006).

Curcumin otherwise known as diferuloylmethane, is the major natural polyphenol obtained from the rhizome known as turmeric or *Curcuma longa* or *Zingiberaceae*. Curcumin is a common feature in Asian cuisine and is widely used as a spice (Aggarwal *et al.*, 2003; Thangapazham, 2006; Howells *et al.*, 2011; Pulido-Moran *et al.*, 2016). Curcumin has a long history of use in Indian medicine. It possesses anti-oxidant, anti-tumour, anti-inflammatory, anti-bacterial, anti-viral and anti-spasmodic properties (Lin *et al.*, 2006; Montopoli *et al.*, 2009; Pulido-Moran *et al.*, 2016).

3,3'- Diindolylmethane (DIM) is a phytochemical obtained from cruciferous vegetables (Howells *et al.*, 2007). Cruciferous vegetables include Brussel sprouts, broccoli and cabbage. During cooking preparations, glucobrassicin which is a major constituent of cruciferous vegetables undergoes hydrolysis and releases Indole-3-carbinol (I3C). Further condensation of I3C at low pH in the stomach yields DIM (Nachshon-Kedmi *et al.*, 2003; Howells *et al.*, 2007). It has been identified that DIM exhibits increased chemopreventive activity in animal models than its parent compound I3C (Garikapaty *et al.*, 2006). However, both DIM and I3C have been identified to possess anti-tumour and chemopreventive actions in cell line and animal models (Howells *et al.*, 2002).

Epigallocatechin-gallate (EGCG) is the main active polyphenolic compound obtained from both green tea and black tea (Chen and Dou, 2008). This compound makes up approximately 30-42% of the total dry weight of green tea (Khan, 2008). Assumptions have been made that correlate the tea-drinking culture of the Asians to the low rate of PCa incidence in Asia (Paschka, 1998).

Relatively some investigations have assessed the chemopreventive or chemotherapeutic efficacy of curcumin, DIM and EGCG on various cancers. However the aim of this study is investigate the anti-proliferative activities of curcumin, DIM, EGCG and their paired combinations on prostate cancer cellular models. With a review of previous methodologies and results, the respective IC_{50} values of curcumin, DIM and EGCG on LNCaP cell lines were revised and adopted from previous studies.

Materials and methods

Unless otherwise stated, all reagents were obtained from Sigma (Poole, United Kingdom). Curcumin, DIM and EGCG were obtained from Sigma-Aldrich (Germany).

Cell culture

The human lymph node prostate carcinoma cell lines LNCaP were obtained from American Type Culture Collection (ATCC) (Virginia, USA). The LNCaP cell lines were grown and maintained in RPMI (Roswell Park Memorial Institute) 1640 medium with 10% v/v foetal calf serum (FCS) (Invitrogen, Paisley, United Kingdom). The cell culture media was obtained from Invitrogen (Paisley, United Kingdom) and Sigma-Aldrich (Germany). LNCaP cells were resuscitated from liquid nitrogen

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by snap-thawing at 37ºC, re-suspended in 10 ml pre-warmed medium and centrifuged at 15,000 rpm for 5 minutes. Pellet formed was re-suspended in either 10 ml or 25 ml of fresh media with 10% FCS depending on the culture flask intended for use. This was cultured in a 37ºC incubator with 5% circulating CO_2 until the growing cells were approximately 70% confluent. For all protocols, LNCaP cells were always 70% confluent before been washed with PBS, trypsinised and centrifuged. Cell lines were not cultured with antibiotics as they tested negative to any infections. Cells were not used beyond eight passages following their generation.

Treatment of cell cultures

Curcumin, Diindolylmethane (DIM) and Epigallocathechin gallate (EGCG) were treatments utilised for the study and were all prepared in Dimethyl sulfoxide (DMSO), which constituted less than 0.1% v/v to the culture media used. Controls contained equivalent concentration of DMSO. The treatment of cells with curcumin, DIM and EGCG were designed in two Dose regimens. These concentration doses were obtained by adjusting IC_{50} values from previous published studies (Shenouda *et al.*, 2004; Garikapathy *et al.*, 2006; Kimura *et al.*, 2007; Valentini *et al.*, 2009).

Dose regimen 1: curcumin (5 μM), DIM (50 μM), EGCG (30 μM) and the paired combinations curcumin (5 μM) + DIM (50 μM), curcumin (5 μM) + EGCG (30 μM) and DIM (50 μM) + EGCG (30 μM).

Dose regimen 2: curcumin (2 μM), DIM (30 μM), EGCG (40 μM) and the paired combinations curcumin (2 μM) + DIM (30 μM), curcumin (2 μM) + EGCG (40 μM) and DIM (30 μM) + EGCG (40 μM).

Cell proliferation analysis

LNCaP cell count results were adjusted and 1 x 10^4 cells were seeded into 24 well plates. This was incubated for 48 hours at 37ºC and 5% CO_2. After the period of cell adherence, Seeded test LNCaP cells were treated with 1 ml of media containing treatments. Incubation was done for 72 hours and 144 hours respectively. Afterwards, cells were washed with PBS, trypsinised, neutralised with warm media and counted using the Beckman coulter 27 particle counter (Beckman Coulter, UK).

ATP assay

LNCaP cells were counted and adjusted. Using a 96 well plate, the seeded cell density was 750 cells per well with 10% FCS media (RPMI 1640). This is allowed to culture and adhere for 48 hours. Afterwards, the cells were treated with Dose regimen 1 and Dose regimen 2 in triplicates. Treated cells were then incubated for 72 hours at 37ºC and 5% circulating CO_2.

Prior to analysis, 50 μl of mammalian cell lysis solution from the ATPlite™ Luminescence Assay Kit (Perkin Elmer, USA) was added to each sample well. The plate base was covered with white adhesive tape, the plate top was covered with transparent adhesive tape and left on a plate shaker for 5 minutes at 70 rpm. Afterwards, 50 μl of reconstituted lyophilized substrate solution (ATPlite™ Luminescence Assay System, 5000 Assay Kit, Perkin Elmer, USA) was added to each sample well and left on the plate shaker for 5 minutes at 70 rpm. Prior to reading the luminescence intensity of the plated samples, the plate was kept in the dark for 10 minutes and then analysed with the Fluostar Optima luminescence counter microplate reader (BMG Labtech, UK). The intensity of luminescence was directly proportional to the cell number.

Clonogenic assay

LNCaP cells were counted and adjusted. Cells were seeded in a 9 cm petri-dish at a cell density of 1 x 10^4 cells with 10% FCS media (RPMI 1640) and incubated for 48 hours, to allow for proper adherence to the petri-dish. Afterwards, cells were treated in triplicates of Dose regimen 1 and Dose regimen 2 respectively. Treated LNCaP cells were incubated for 24 hours at 37ºC and 5% CO_2. Subsequently, the media was aspirated, cells were gently scraped and re-suspended in 10 ml warm 10% FCS media (RPMI 1640).

To set-up the cells for cloning efficiency, 2 x 10^3 of the treated cells were then seeded into 9 cm petri-dishes and incubated for 2 - 4 weeks until clear and visible clones were observed. To evaluate the number of clones per treatment, the media was aspirated from the petri-dishes and plated cells were fixed with 100% methanol, left to dry and stained with 0.5% crystal violet solution and counted. The cloning efficiency was expressed as a percentage of the ratio of the number of colonies formed to the number of cells seeded.

Statistical Analysis

All experiments were measured against controls (DMSO) and most experiments were conducted in triplicates. The data were presented and analysed as mean $\pm$ S.D. Statistical programs used included the GraphPAD software (CA, USA) and Microsoft Excel. Statistical differences between experimental groups were compared for statistical significance using the repeated measures One-way ANOVA and Mann-Whitney U test separately. $P < 0.05$ was considered significant.

Results

Effects of curcumin, DIM, EGCG and their paired combinations on LNCaP cell growth and proliferation

To evaluate the effect of curcumin, DIM and EGCG on the growth, proliferation and viability of LNCaP, the IC_{50} values of curcumin, DIM and EGCG on LNCaP cell line were adopted and revised from previous studies (Shenouda *et al.*, 2004; Garikapathy *et al.*, 2006; Kimura *et al.*, 2007; Valentini *et al.*, 2009). These concentrations were used to evaluate the effect of these diet-derived agents on LNCaP proliferation via the Coulter cell counting technique.

The effect of Dose regimen 1 on LNCaP cells at 72 and 144 hours are shown in Figure 1. The percentage change in cell numbers when compared to DMSO (control) at 72 hours decreased significantly to 44%, 29.8%, 41.3%, 21.9%, 23.5% and 14.5% for curcumin, DIM, EGCG, curcumin + DIM, curcumin + EGCG and DIM + EGCG respectively ($P < 0.0001$) (Figure 1), while the percentage change in cell numbers to DMSO (control) at 144 hours decreased to 25.5%, 8.6%, 27.6%, 8.1%, 17.29%, 7.19% for curcumin, DIM, EGCG, curcumin + DIM, curcumin + EGCG and DIM + EGCG respectively ($P < 0.0001$) (Figure 1). Data on Figure 1 does show that there is a significant decrease in cell numbers with each treatment ($P < 0.0001$). Furthermore, there is an enhanced decrease in cell numbers with the paired combination treatments. This significant decrease in proliferation is further enhanced with the 144 hour treatments. This clearly indicates that the observed decrease in cell numbers with the diet-derived compounds is time-dependent as the change in cell numbers was significantly lower between both time-points (72 hours and 144 hours) for the same treatments.

The effect of Dose regimen 2 on LNCaP proliferation was evaluated for 72 and 144 hours and displayed in Figure 2. Figure 2 showed that treatment of LNCaP cells with curcumin (2 μM), DIM (30 μM), EGCG (40 μM) and the paired combinations curcumin (2 μM) + DIM (30 μM), curcumin (2 μM) + EGCG (40 μM) and DIM (30 μM) + EGCG (40 μM) for 72 hours and 144 hours respectively had significant effects on cell numbers and did decreased LNCaP growth and proliferation ($P < 0.0001$). At 72 hours, the percentage change to the control (DMSO) was significantly decreased and the percentage changes obtained were 71.8%, 53%, 59.5%, 38.4%, 36.5% and 23.5% respectively ($P < 0.0001$). At 144 hours, the percentage change to the control (DMSO) were 50.7%, 16.4%, 18.5%, 11.6%, 19.4% and 11% respectively, which also decreased significantly ($P < 0.0001$). The observed inhibitory effect to LNCaP cell numbers was time-dependent as the change in cell numbers was significantly lower between both time-points (72 hours and 144 hours) for the same treatments.

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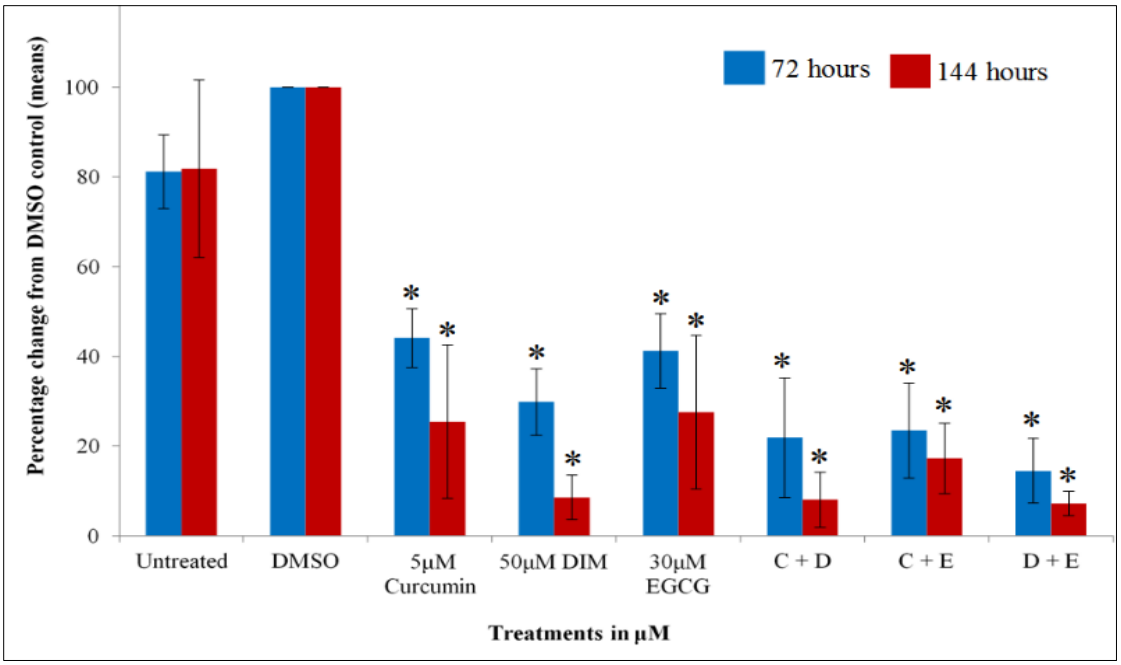

Figure 1: Dosing regimen 1 for 72 hours and 144 hours: The effect of curcumin (C), DIM (D), EGCG (E) and their paired combinations on LNCaP cell growth and proliferation expressed as percentage change to the control (DMSO). Columns are means of triplicates, while the bars are ± Standard deviation (SD). (*) indicates $P < 0.001$ when compared to DMSO (control) as determined by the repeated measures one-way ANOVA followed by a post-hoc Tukey test.

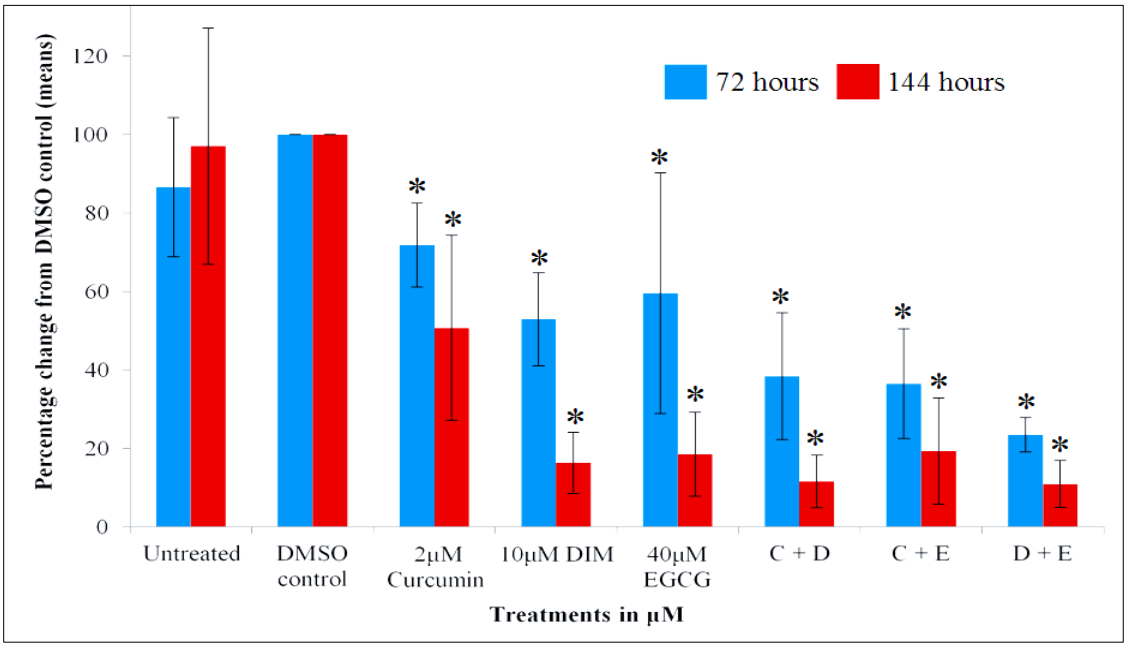

Figure 2: Dosing regimen 2 for 72 hours and 144 hours: The effect of curcumin (C), DIM (D), EGCG (E) and their paired combinations on LNCaP cell growth and proliferation expressed as percentage change to the control (DMSO). Columns are means of triplicates, while the bars are ± Standard deviation (SD). (*) indicates $P < 0.001$ when compared to DMSO (control) as determined by the repeated measures one-way ANOVA followed by a post-hoc Tukey test.

Effects of curcumin, DIM, EGCG and their paired combinations on ATP synthesis of LNCaP cells

The effect of curcumin, DIM, EGCG and their paired combination on ATP production of LNCaP cells was also investigated. Following relevant treatments, the ATPlite™ Luminescence Assay System, 5000 Assay Kit (MA, USA) was utilised. The assumption used in this analysis is that increased ATP levels should correlate to high cell viability, while decreased ATP levels should correlate to low cell numbers and reduced cell viability. LNCaP cells were treated respectively with curcumin, DIM and EGCG and their paired combinations for 72 hours. With Dose regimen 1 (Figure 3), only the sole treatment of DIM (50 µM) was significantly different from the DMSO (control) ($P < 0.001$). With Dose regimen 2 (Figure 3), the individual means were different from each other, however there was no significant difference between the means of the treated and the DMSO control. There was no interpretable trend in comparison with the DMSO control.

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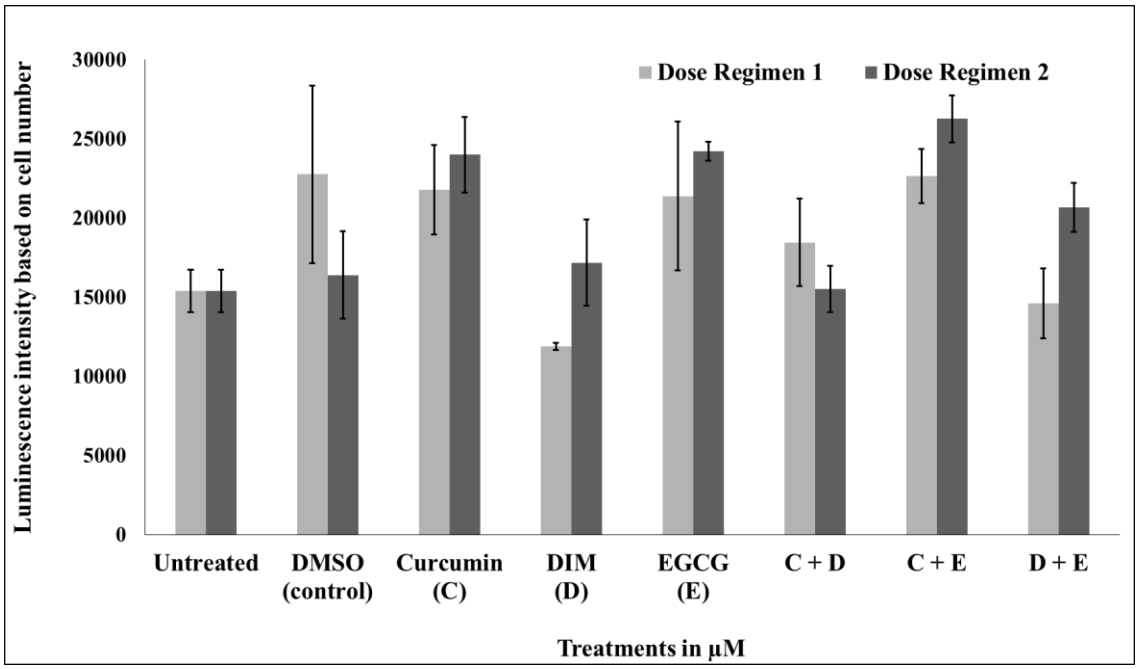

Figure 3: Effect of curcumin (C), DIM (D), EGCG (E) and their paired combinations on ATP generation of LNCaP cells. LNCaP cells were treated with Dosing regimen 1 and 2 respectively for 72 hours in 96-well plate. Columns are means of triplicates and the bars are the Standard deviation of triplicate readings.

Effects of curcumin, DIM, EGCG and their paired combinations on the cloning efficiency of LNCaP cells

To further evaluate the extent of LNCaP cell damage and survival after treatment, 24 hour-treated LNCaP cells were re-seeded and the clones formed were stained and counted. This experiment was carried out to determine the ability of the LNCaP cell lines to recover from treatment. The effectiveness of the treatments should correlate with decreased cloning efficiency of the sample. The data produced from the treatment of LNCaP cells with Dose regimen 1 of curcumin, DIM and EGCG and their paired combinations is shown in Figure 4. The result shows significant differences between the means, but there was no significant decrease or difference when the treatment means were compared with DMSO control ($P < 0.05$). Treatment with Dose regimen 2 generated the result shown in Figure 4. The result also shows a significant difference among the different mean posts, however there was no significant difference or decrease of treatment means in comparison to DMSO control.

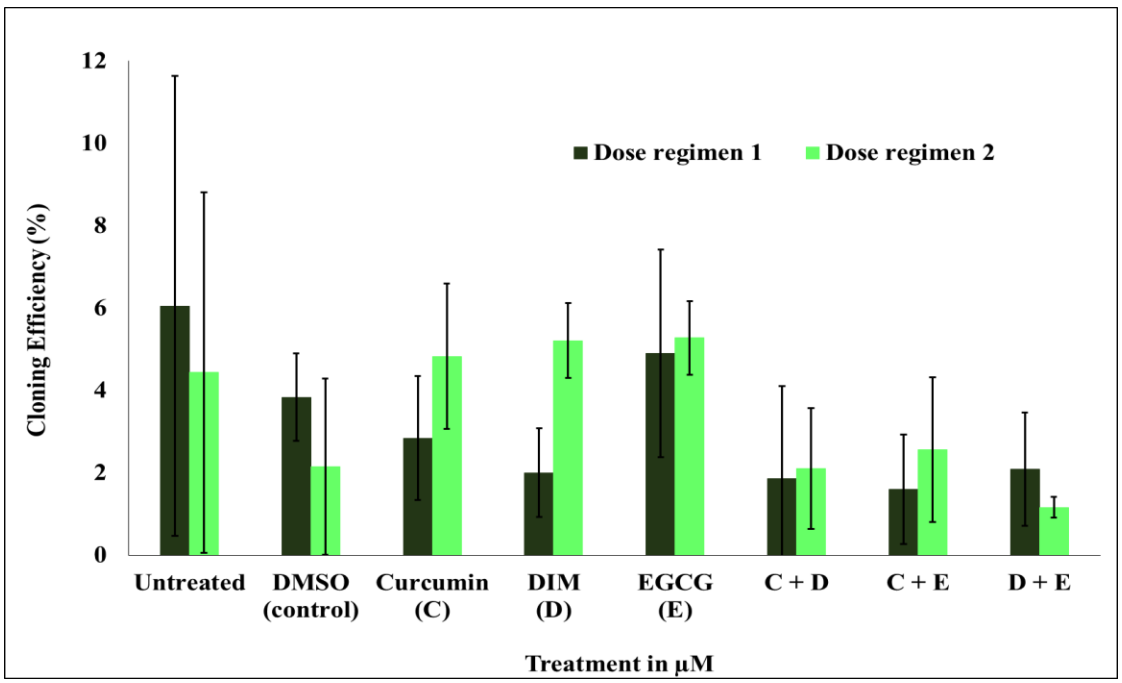

Figure 4: Effects of curcumin, DIM and EGCG and their paired combinations on the Cloning efficiency of LNCaP cells. The columns are means of triplicates and the bars are ± standard deviation of triplicate samples.

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Discussion

Chemoprevention can be described as the utilisation of effective agents which may be either natural or synthetic, and can be utilised for the prevention, impediment or reduction of various cancer types (Howells and Manson, 2005; Lippman and Lee, 2006). Due to the failings of many chemotherapeutic agents in prostate cancer and synthetic chemopreventive agents (Singh and Agarwal, 2006), there is a growing demand for more chemopreventive agents from natural sources with little or no toxicity (Lippman and Lee, 2006). Based on epidemiological studies on well-known cancers in Asia, Europe and the USA; overall cancer incidence were higher in Western Europe and the USA. High cancer incidences were found to be associated with unhealthy diet, geographical location and environment (Manson *et al.*, 2007). Cancer incidences were least in Asian countries and these were attributed to healthy diets that contained high levels of polyphenols and phytochemicals (Howells and Manson, 2005; Manson *et al.*, 2007). Many polyphenols and phytochemicals have been observed to possess anti-carcinogenic properties (Manson *et al.*, 2007). The dietary constituents namely curcumin, Diindolylmethane (DIM) and Epigallocathechin gallate (EGCG) are the focus in this study. They have been widely researched and shown to be effective in the inhibition of many cancers.

Curcumin, DIM and EGCG are found in dietary sources. Curcumin is the major constituent of turmeric, while DIM and EGCG are sourced from cruciferous vegetables and green tea respectively. It has been reported that curcumin, DIM and EGCG hold some potentials as chemopreventive agents of prostate cancer (Sarkar and Li, 2004).

The inhibitory effect of curcumin, DIM, EGCG and their paired combinations: curcumin and DIM, curcumin and EGCG, and DIM and EGCG were investigated in this study. The results obtained from the growth and proliferation analysis illustrated that all dietary agents decreased the relative LNCaP cell numbers. In addition, all dietary agents reduced the proliferation and growth rate of the LNCaP cells in a dose and time dependent approach. With the Dose regimen 1, the percentage change in cell numbers in comparison to DMSO (control) for all treatments were within the range of 14.5% to 44% following 72 hours treatment and the range of 7.19% to 27.6% following a 144 hour treatment. With the Dose regimen 2, the percentage change in cell numbers for all treatments were within the range of 23.5% to 71.8% following a 72 hours treatment and the range of 11% to 50.7% following a 144 hour treatment. DIM exhibited the greatest growth inhibitory effect as a sole treatment, while the paired combination of DIM and EGCG showed the highest inhibitory action comparatively. The results obtained does show that the concentrations of the respective diet-derived agents used in this study are in agreement with previous studies where curcumin was reported to possess an IC_{50} range of 2.2 μM to 7.8 μM and shown to possess inhibitory growth rate effect on LNCaP cell lines and (Valentini *et al.*, 2009). DIM was also shown to inhibit LNCaP growth and was termed an effective anti-proliferative agent in LNCaP cell lines with an IC_{50} of 50 μM (Garikapathy *et al.*, 2006). The results also showed consistency to studies conducted with EGCG. Some reports stated that EGCG did possess inhibitory effects on LNCaP growth with an IC_{50} of 28.1 μM (Kimura *et al.*, 2007), while another study reported that EGCG had an IC_{50} of 100μM (Shenouda *et al.*, 2004). The combination dietary agents gave notable inhibitory effects on LNCaP cell growth and this was in agreement with reports, which stated that a combination of agents with high chemopreventive properties, with similar or different mechanism of action and with little or no toxicity would be ideal in the search for better chemoprevention (Lieberman *et al.*, 2001).

In order to evaluate cell viability and proliferation after treatment, the ATP luminescence based assay was conducted and shown in figure 3. There was an observable decrease in cell numbers for the treated LNCaP cells but this decrease was not significantly lower when compared to DMSO (control). This experiment produced some false-positive results in four separate experiments and the dietary agents may have been responsible for influencing the luminescence spectra negatively.

In addition, the level of cytotoxicity of the treatments and the ability of the LNCaP cells to recover from a 24 hour treatment were investigated by the clonogenic assay (Figure 4) and the result showed that the effect was dose dependent. The cells pre-treated with dose regimen 1 produced less clones and had lower cloning efficiency except for the EGCG (30μM) treatment. The results obtained in figure 4 did not show conformity with the growth inhibition data and this could be attributed to the clumpy and sticky nature of the LNCaP cells which could affect the seeding density of LNCaP cells, which may produce a false-positive and yield a high cloning efficiency.

It is important that the potential of curcumin, DIM and EGCG on LNCaP cell proliferation and survival were investigated since uncontrolled proliferation and immortality are hallmarks of cancer cells. Results reported from this study does indicate that the investigated diet-derived agents did have variable inhibitory effects on cellular function, cellular proliferation and survival of LNCaP cell lines. With further studies, curcumin, DIM and EGCG may hold stronger prospects in chemoprevention since these diet-derived agents were able to obstruct cellular proliferation, this capability is crucial in chemoprevention.

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