1. Background

PCa remains the second leading cause of male cancer death with approximately 903,500 new cases and 258,400 deaths in 2008 worldwide (cancer facts and figures 2012). PCa remains the most common noncutaneous malignancy in American men with an estimated lifetime risk of disease of 16.6% for Caucasians, 18.1% for African-Americans and 3.5, 4.3% lifetime risk of death respectively (Klein and Thompson 2004).

It is a chronic disease that needs a long period for development from small lesion to become clinical manifestation (Nunez et al., 2008). There is an imbalance between prostate cell growth and apoptosis in PCa. This imbalance is complex and influenced by microenvironment around prostate such as growth factors, cytokines and steroid hormones. These factors stimulate proliferation and minimize cell apoptosis (Sciarra et al., 2008, Nelson et al., 2003). The role of inflammation in prostate diseases is suggested by the presence of inflammatory cells within the prostate of cancer patients (Novara et al., 2006). Their role has been noted since 1937, but Virchow already hypothesized that the origin of cancer was at sites of chronic inflammation in 1863. Many studies showed more than 92% incidences of inflammatory lesions in prostate tissue in PCa. Histopathology investigations also confirmed that inflammation is much more common in the transition and peripheral zones of the prostate that is a predilection for PCa (Elongchamps et al., 2008, Sutcliffe et al., 2008).

The molecular mechanisms responsible for inflammation mediated PCa is not yet clear. However, there are indications that this form of cancer transformation may not be solely due to infectious agents their selves, but rather, may be as a result of the presence of pathogen encoded Oncogenes (De Marzo et al., 2007). Emerging evidences suggest that both innate and adaptive immune response may have huge contributions during inflammation induced PCa transformation (De Marzo et al., 2007, Sciarra et al., 2007, Maeda et al., 1998, Shacter and Weitzman 2002).

In most cases, the initial trigger for prostatic inflammation remains unclear. Various potential sources exist for the initial event, including direct infection (viruses, fungi, mycobacteria and parasites), urine reflux inducing chemical and physical trauma, dietary factors (Ingested carcinogens can reach the prostate through the bloodstream or by urine reflux), hormones, exposure to environmental pollutants, genetic predisposition or a combination of two or more
of these factors. Furthermore, any of these can break in immune tolerance and the development of an inflammatory reaction to the prostate (De Marzo et al., 2007, Fleshner and Klotz 1999, Kogan-Sakin et al., 2009, Wong et al., 2009).

According to NCI 1991 report it has been established that the people who die after the age of 50-60 years they have PCa, but the disease does not appear and it is in dormant state. It might appear and reach to metastasis with time. So with increase in longevity it is going to emerge as a matter of major health concern. PCa metastasis usually occurs due to inflammation of prostate cells and/or mutations in Ar gene. ADT the only therapy available, blocks testosterone driven proliferation of PCa cells and causes apoptosis in majority of the PCa cells (Pienta and Bradley 2006). Elevated PSA level is the only confirmatory marker available today, but till date we do not have any early diagnostic tool. PCa has a long latent period and provides a better tool for chemopreventive intervention. Even today metastatic PCa remains an incurable disease and therapy can only delay progression.

Epidemiological data indicate that vegetables and fruits containing chemopreventive agents have shown protective effects against cancer. Studies show that people from low risk geographic populations such as the Chinese and Japanese, who changed their residence to a place in the Western Nations, had an increased chance of developing PCa (Muir et al., 1991). From these observations stems the hypothesis that environmental factors may act as late stage promoters involved in the transformation of PCa from a latent form to a more aggressive form. Life style and dietary habits have been identified as major risk factors in PCa growth and progression (Abdulla & Gruber 2000, Agarwal and Rao 2000). On the contrary many researchers have also reported evidences suggesting that, up to 30% of men in the general population aged above 50 years and above, irrespective of geographic origin have foci of prostate neoplasic growth (Muir et al., 1991, Klein et al., 2004).

The uses of chemopreventive agents of natural products have gained increased importance due to their minimal side effects and almost no toxicity. Many researchers have reported the chemopreventive role of Lycopene, phytoestrogen of Soya products, some metals like selenium against prostate cancer. Some investigators have reported the role of Selective estrogen receptor modulators (SERMS) in inhibiting PCa growth, but the results are not conclusive and much more research is needed in this area (Thompson 2007, Ellem et al., 2007).
Luteolin is a flavonoid of class flavones, which possess potent antioxidant, proapoptotic and COX-2 inhibition properties (Harris et al., 2006, Bagli et al., 2004). It can be taken as chemopreventive agent in PCa where inflammation is one of the important features in the induction of disease. It can further be strengthening having its proapoptotic efficacy. Secondly PCa cells respond to estrogen hormone but the administration of female hormone may cause adverse effects on other body organs hence cannot be used as an interceptor to PCa, therefore we intend to use site-specific phytoestogens SERMS in the present study (Pettersson and Gustafsson 2001). Diadzein is a SERM of plant origin present in soy, which is shown to exhibit estrogen like action.

Here we intend to investigate the role of natural chemopreventive agents (Luteolin) and SERMS of plant origin (Diadzein) and elucidate the pathway so that these agents in future can be used as prophylactic and therapeutic tools in the treatment of PCa. The aim of the current study is to study the preventive role of diadzein and luteolin in NMU-testosterone induced PCa in Wistar rats.

2. Treatment regimen

2.1 Prostate cancer induction

PCa was induced by the method of Liao (2002) with some modifications. Rats were orally given cyproteron acetate (50 mg/kg b. wt) for 21 consecutive days in corn oil, followed by testosterone propionate (100 mg/kg b. wt/i.p) in corn oil from 23rd day to next three days. On 27th day animals received a single dose of NMU (50 mg/kg b. wt/i.p). After one week of NMU administration rats received daily testosterone propionate (2 mg/kg b. wt/i.p) in corn oil for next 125 days (Liao et al., 2002).

Rats were divided into six groups with 12 animals in each group. Group I served as control and PCa was induced in group II following Liao et al., 2002 method with slight modifications. In modulator treatment group III (daidzein 20 mg/kg, b.wt), group IV (daidzein 60 mg/kg, b.wt) and group V (luteolin 0.2 mg/kg, b.wt), group VI (luteolin 0.4 mg/kg, b.wt) modulators were given from 35th day to 160th day along with a promoter dose of hormone testosterone propionate 2 mg/kg, b.wt/i.p in corn oil.
2.2 Material and methods

The levels of inflammatory cytokines (IL 1β, IL 6, TNF α, IFN γ) and anti-inflammatory cytokines (IL 10) were measured by ELISA. The serum hormone levels of Testosterone, DHT, and PSA were measured by ELISA. The expression of Cox-2, p53, p21, Bax, Bel2, Casp-3, 8, and 9 was studied by real time RT-PCR using Gapdh as internal control. The protein expression of NF-kB, AR expression, the apoptosis protein expression of Caspase-3, 8, 9, BAX and anti-apoptosis protein expression of BCl2 was studied by IHC staining. The level of inflammation was further studied by Mast cells staining by use of Toluidine blue stain. The antioxidant enzyme status of SOD, GSH, GR, GPx, Catalase, and LPO were also estimated. For detailed methodology please refer to chapter II (material and methods).
3. Results

3.1 Effect of daidzein and luteolin on serum testosterone, DHT and PSA levels.

There was a significant increase in serum levels of Testosterone (p<0.001), DHT (p<0.001) and PSA (p<0.001) in group II when compared with control (group I). Administration of daidzein decreased the testosterone levels [group III] (p<0.01) and [group IV] (p<0.001) and luteolin also decrease [group V and VI] (p<0.001). But only higher dose of daidzein (group IV) and luteolin (group VI) showed a significant decrease in serum DHT levels (p<0.001). Both the doses of daidzein [group III and IV] (p<0.001) and luteolin [group V and VI] (p<0.001) significantly decreased the serum PSA levels dose dependently. (Figure 1)

Figure 1: Effect of daidzein and luteolin on serum hormone levels in NMU-testosterone induced PCa in Wistar rats: A. Serum Testosterone levels; B. Seum DHT levels; PCa induced group (II) resulted in increased in testosterone levels and DHT levels. Treatment of daidzein group (III & IV); luteolin group (IV and VI) showed dose dependent decrease in testosterone and DHT levles; C. Serum PSA levels: Serum PSA leves was elivated in PCa induced group (II). Daidzein group (III & IV) and luteolin group (V and IV) resulted in significant and dose dependentent decrease in serum PSA levels. Values are expressed in means ± S.E.M. (n = 12). Significant differences are indicated by ###p < 0.001 when compared with control animals (Group I), and ***p < 0.001, **p <0.01, *p <0.05 when compared with PCa induced animals (Group II).
3.2 Effect of daidzein and luteolin on inflammatory cytokines (IL 1β, IL 6, TNF α, IFNγ) and anti-inflammatory cytokines (IL 10).

There was an increase in the levels of IL 1β (p<0.05), IL 6 (p<0.001), TNF α (p<0.001), IFN γ (p<0.001) and decreased levels of IL 10 (p<0.001) when compared with control (group I). Daidzein administration resulted in restoration of the cytokines levels to normal significantly and dose dependently, group III [IL 1β (p<0.001), IL 6 (p<0.05), TNF α (p<0.001), IFN γ (p<0.001) and IL 10 (p<0.001)] ; group IV [IL 1β (p<0.05), IL 6 (p<0.001), TNF α (p<0.001), IFN γ (p<0.001) and IL 10 (p<0.001)]. Administration of luteolin at both the doses group V [IL 1β (p<0.05), IL 6 (p<0.01), TNF α (p<0.001), IFN γ (p<0.001) and IL 10 (p<0.001)]; group VI [IL 1β (p<0.01), IL 6 (p<0.01), TNF α (p<0.001), IFN γ (p<0.001) and IL 10 (p<0.001)] showed significant restoration of cytokine level to normal. (Figure 2)

**Figure 2:** Effect of daidzein and luteolin on serum cytokine levels in NMU-testosterone induced PCa in Wistar rats: There was an increase in inflammatory cytokines, decrease in anti-inflammatory cytokines in PCa induced groups II. Daidzein group (III & IV) and luteolin (group V & VI) administration restored the cytokine levels to normal. Values are expressed in means ± S.E.M. (n =12). Significant differences are indicated by ###p<0.001, #p<0.05 when compared with control animals (Group I), and *p < 0.05, **p < 0.01 ***p < 0.001 when compared with PCa induced animals (Group II).
3.3 Effect of daidzein and luteolin on mast cell infiltration.

In order to study the inflammatory infiltration cells in the prostate toluidine blue staining was done to visualize the mast cells (violet colour). PCa induced group II showed massive Mast cell infiltration. Higher dose of daidzein and luteolin showed lesser degree of Mast cell infiltration. (Figure 3)

![Image of mast cell infiltration](image)

**Figure 3: Effect of daidzein and luteolin on mast cell infiltration in NMU-testosterone induced PCa in Wistar rats:** There was an increase in mast cell infiltration in PCa induced (group II). Daidzein and luteolin both the compounds showed a decrease in mast cell infiltration. Higher dose of luteolin proved to be more effective in retarding mast cell infiltration. (40X)
3.4 Effect of daidzein and luteolin on AR

As androgen receptors play an important role in prostate growth and development we studied the expression of AR on daidzein and luteolin treatments on NMU-Testosterone induced PCa. The $Ar$ mRNA expression was increased significantly by 9.1 fold in PCa group II ($p<0.001$) when compared with control. Daidzein administered groups showed down regulation of $Ar$ expression significantly and dose dependently [3.9 fold group III and 2.8 fold group IV ($p<0.001$)]. Similarly luteolin also exhibited a marked down regulation $Ar$ expression [1.8 fold group V, and 1.12 fold group VI ($p<0.0001$)]. We also crosscheck the AR receptor expression at protein by studying IHC staining; the findings of mRNA expression are in similar pattern shown by IHC staining. (Figure 4)

3.5 Effect of daidzein and luteolin on NF-kB expression on NMU-Testosterone treated wistar rats

We also studied the expression of NF-kB expression to see its activation and translocation. The mRNA expression of $Nfkb$ showed a 7.2 fold up-regulation in PCa group II ($p<0.001$) when compared with control. In daidzein lower dose (group III) $Nfkb$ levels were 2.0 fold regulated ($p<0.001$) and higher dose of daidzein proved to be much effective by $Nfkb$ expression by 1.2 fold ($p<0.001$). In case of luteolin treatment both the doses proved to be more effective, the $Nfkb$ mRNA expressions was 2.7 fold in group IV ($p<0.001$) and 1.86 fold in group V ($p<0.001$). The protein expression of NF-kB showed similar results as mRNA expression but, the protein expression of NF-kB in both the luteolin treated group the signal intensity was almost similar in group V and group VI. (Figure 5)
Figure 4: Effect of daidzein and luteolin on AR expression in NMU-testosterone induced PCa in Wistar rats: A. Ar mRNA expression. Daidzein (group III & IV) and luteolin (group V & VI) showed down regulation in Ar mRNA. B. AR protein expression: PCa (group II) showed more AR positive cells. Both the doses of luteolin (goup V & VI) and higher dose of daidzein (group IV) showed less AR positive cells. Values are expressed as mean ± SD and n=12. Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and *p < 0.001 when compared with PCa induced animals (Group II). (AR positive cells ▶ and ◻ AR negative cells)
Figure 5: Effect of daidzein and luteolin on NF-κB expression in NMU-testosterone induced PCa in Wistar rats: A. *Nfkb* mRNA expression. Daidzein (group III & IV) and luteolin (group V & VI) showed down regulation in *Nfkb* mRNA. B. **NF-κB protein expression**: PCa (group II) showed more NF-κB positive cells. Both the higher doses of luteolin (group VI) and daidzein (group IV) suppressed NF-κB activation. Values are expressed as mean ±SD and n=12. Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and *p < 0.001 when compared with PCa induced animals (Group II). (NF-κB positive cells and NF-κB negative cells)(40X)
3.6 Effect of daidzein and luteolin on apoptosis markers: caspase 3, 8, 9

The induction of apoptosis was studied in terms of activation of Caspase 3, 8, and 9 expressions. The mRNA expression studied by Real Time RT-PCR showed a marked decrease in mRNA expression of Casp 3 (-2.43 fold), Casp 8 (-1.4 fold), and Casp 9 (-1.8 fold) ($p<0.001$) in PCa indication group II when compared to control. Daidzein at both the doses up regulated the Caspase expression in group III [Casp 3 (1.92 fold), Casp 8 (1.6 fold), Casp 9 (3.5 fold)] ($p<0.001$) and group IV [Casp 3 (2.5 fold), Casp 8 (4.3 fold), Casp 9 (7.3 fold)] ($p<0.001$) significantly induction mRNA expression respectively. But in luteolin treated groups very less induction of Casp 8 expression at both the doses group V (1.01 fold) and group VI (1.15 fold) respectively were seen. Luteolin significantly showed higher expression of Casp 3 [group V (2.0 fold) and group VI (4.1 fold)] ($p<0.001$) and Casp 9 [group V (1.8 fold) and group VI (4.6 fold)] ($p<0.001$) at both the doses respectively. We have also cross checked the expression of these Caspases at protein level by IHC. We found similar pattern of expression as revealed by mRNA expression in the PCa tissue. (Figure 6A, B, C)

3.7 Effect of daidzein and luteolin on expression of BCl₂, BAX and BCl₂/BAX ratio

We studied the expression of Bcl₂ to study the resistance of cells for apoptosis. PCa induced group II showed up-regulation of mRNA expression by 13 fold ($p<0.001$) as compared to control. Daidzein and luteolin showed significant and dose dependent down regulation of mRNA expression [group III, 2 fold, group IV, -1.07 fold ($p<0.001$)] and [group V, -1.0 fold, group VI, -2.89 fold ($p<0.001$)] respectively. The IHC staining of BCl₂ showed higher intensity and more positive stained cells in PCa treated group II when compared with control. Daidzein and luteolin at both the doses showed significant and dose dependent decreased in BCl-2 positive stained cells dose dependently. On the other hand the mRNA expression of Bax was down regulated by 1.4 fold in PCa induced group II when compared to control. Daidzein administration up-regulated the mRNA expression of group III by 2.4 fold and 7.5 fold in group IV ($p<0.001$). Luteolin also up-regulated Bax mRNA by 3.2 fold in group V and 4 fold in group VI ($p<0.001$). Bax/Bcl₂ ratio was studied in order to see the cell characteristics leading to apoptosis/resistance to cell death. The ratio of Bax/Bcl₂ was <1 (0.1) in PCa induced group II. In both dadzein treated group the Bax/Bcl₂ was >1; group III, 1.14 and 6.97 for group IV.
Luteolin administered groups showed higher ratio as compared to daidzein treatment. Group V, 3.2 and group VI, 1.3 as compared with group II. (Figure 7A, B, C)

Figure 6A: Effect of daidzein and luteolin on Caspase 3 expression in NMU-testosterone induced PCa in Wistar rats: A. Casp 3 mRNA expression. Daidzein (group III & IV) and luteolin (group V & VI) up-regulated Casp 3 mRNA. B. Caspase 3 protein expression: PCa (group II) decreased caspase 3 positive cells. Both the higher doses of luteolin (group VI) and daidzein (group IV) enhanced caspase 3 activation. Inducing apoptosis in daidzein and luteolin treated group. Values are expressed as mean ± SD and n=12. Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and *p < 0.001 when compared with PCa induced animals (Group II). (Casp.3 positive cells ▶ and Casp. 3 ▼ negative cells)(40X)
Figure 6B: Effect of daidzein and luteolin on Caspase 8 expression in NMU-testosterone induced PCa in Wistar rats: A. Casp 8 mRNA expression. Daidzein (group III & IV) up-regulated Casp 8 mRNA, but luteolin (group V & VI) showed only slight induction in Casp 8 mRNA at both the doses (group V & VI). B. Caspase 8 protein expression: PCa (group II) decreased Caspase 8 positive cells. Daidzein (group IV) enhanced caspase 8 activation. But luteolin at both the doses (group V & VI) showed slight activation in Caspase 8. Values are expressed as mean±SD and n=12. Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and *p < 0.001 when compared with PCa induced animals (Group II). (Caspase 8 positive cells ➔ and Caspase 8 negative cells) (40X)
Figure 6C: Effect of daidzein and luteolin on Caspase 9 expression in NMU-testosterone induced PCa in Wistar rats: 

A. Casp 9 mRNA expression. Daidzein (group III & IV) up-regulated Casp 9 mRNA and luteolin (group V & VI) showed induction in Casp 9 mRNA at both the doses (group V & VI). 

B. Caspase 9 protein expression: PCa (group II) decreased Caspase 9 positive cells. Daidzein (group IV) enhanced caspase 9 activation. But luteolin at both the doses (group V & VI) showed activation in Caspase 9. Values are expressed as mean±SD and n=12. Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and *p < 0.001 when compared with PCa induced animals (Group II). (Caspase 9 positive cells ➔ and ➔ Caspase 9 negative cells)(40X)
Figure 7A: Effect of daidzein and luteolin on BAX expression in NMU-testosterone induced PCa in Wistar rats: A. Bax mRNA expression. Daidzein (group III & IV) up-regulated Bax mRNA and luteolin (group V & VI) also induced Bax mRNA at both the doses (group V & VI).

B. BAX protein expression: PCa (group II) decreased BAX positive cells. Daidzein (group IV) enhanced BAX activation. Luteolin at both the doses (group V & VI) also activated BAX. Values are expressed as mean±SD and n=12. Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and ***p < 0.001, *p < 0.05 when compared with PCa induced animals (Group II). (BAX positive cells and BAX negative cells) (40X)
Chemoprevention of PCa by daidzein and luteolin

Figure 7B: Effect of daidzein and luteolin on BCL2 expression in NMU-testosterone induced PCa in Wistar rats: A. Bcl2 mRNA expression. Daidzein (group III & IV) and luteolin (group V & VI) down regulated Bcl2 mRNA expression. B. BCL2 protein expression: PCa (group II) increased BCL2 positive cells. Daidzein (group IV) decreased BCL2 activation. Luteolin at both the doses (group V & VI) also decreased BCL2 expression. Values are expressed as mean±SD and n=12. Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and *p < 0.001 when compared with PCa induced animals (Group II). (BAX positive ➞ cells and BAX negative ➞ cells)(40X)
Figure 7C: Effect of daidzein and luteolin on Bax/Bcl\textsubscript{2} ratio in NMU-testosterone induced PCa in Wistar rats: The Bax/Bcl\textsubscript{2} in PCa (group II) was <1 indicating an increased resistance to apoptosis. Higher dose of daidzein group IV induced apoptosis. Lower dose of luteolin proved effective in inducing apoptosis. Values are expressed as mean ± SD and n=12.
3.8 Effect of daidzein and luteolin on mRNA expression of Cox-2, p53, p21

To study the induction of apoptosis we studied the expression of Cox-2, p53 and p21. There was an increased in expression of mRNA 6.5 fold in Cox-2 (p<0.001), 1.4 fold in p53 (p<0.001) and 0.64 fold in p21 (p<0.001) expression in PCa induced group II when compared with control. Daidzein administrated groups showed a decrease in Cox-2 by 2.3 fold (p<0.001), induced p53 expression by 2.7 fold (p<0.05) and 2.0 fold p21 expression up-regulation (p<0.001) in group III; Cox-2 was 0.4 fold (p<0.001) down regulated; p53 expression was up-regulated by 3.4 fold (p<0.001) and 3.2 fold in p21 up-regulated (p<0.001) group IV. Luteolin at both the doses showed down regulation of Cox-2 by 1.9 fold, up-regulation in p53 mRNA expression by 1.62 fold (p<0.001) and p21 by 3.0 fold (p<0.001) in group V. In group VI, Cox-2 was down regulation by 1.2 fold (p<0.001) and there was an up-regulation in p53 mRNA expression by 4.3 fold (p<0.001) and p21 expression up-regulation by 4.7 fold respectively (p<0.001). (Figure 8)

Figure 8: Effect of daidzein and luteolin on mRNA expression of Cox-2, p53, p21 in NMU-testosterone induced PCa in Wistar rats: A Cox-2 expression: PCa induced group II there was an induction in Cox-2 expression and both daidzein and luteolin dose dependently suppressed the Cox-2 activation. B. p53 expression, C. p21 expression: PCa induced group II led to slight increase in p53 and p21 expression. Daidzein and luteolin administration led to marked increase in p53 and p21 expression which led to induction of cell to apoptosis. Both the compounds induced p53 and p21 mediated apoptosis. Significant differences are indicated by mean ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001 when compared with control animals (Group I), and *p < 0.05, **p < 0.01, ***p < 0.001 when compared with PCa induced animals (Group II).
3.9 Effect of daidzein and luteolin on expression of anti-oxidation armature in NMU-testosterone induced PCa in Wistar rats

PCa induced group II cause the depletion of SOD, Catalase, reduced glutathione, its dependent enzymes GR, GPx and increased the lipid peroxidation as compared to control (p<0.001). Daidzein at both the doses restored the levels to normal, but, only higher dose of daidzein (group IV) restored the SOD levels to normal (p<0.001). Similarly administration of luteolin dose dependently showed the significant restoration of the antioxidant enzymes to normal (p<0.001). (Table 2)

Table 2: Effect of daidzein and luteolin on anti-oxidation armature in NMU-testosterone induced prostate cancer in Wistar rats:

<table>
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<th></th>
<th>GR</th>
<th>GPx</th>
<th>GSH</th>
<th>SOD</th>
<th>CAT</th>
<th>LPO</th>
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<td>Group I</td>
<td>306.49±2.3</td>
<td>105.98±4.4</td>
<td>343.80±1.3</td>
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<td>216.41±0.5#</td>
<td>1.84±0.10#</td>
<td>39.62±0.3#</td>
<td>89.92±0.8#</td>
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<tr>
<td>Group III</td>
<td>190.08 ± 1.4*</td>
<td>60.10±1.95*</td>
<td>235.93 ± 0.1*</td>
<td>2.65 ± 0.2NS</td>
<td>99.10±1.0*</td>
<td>70.38±0.9*</td>
</tr>
<tr>
<td>Group IV</td>
<td>229.96 ± 1.5*</td>
<td>71.79 ± 1.2*</td>
<td>272.12 ± 0.3*</td>
<td>5.55 ± 0.2*</td>
<td>111.9±0.5*</td>
<td>49.41±0.6*</td>
</tr>
<tr>
<td>Group V</td>
<td>211.2±3.2*</td>
<td>70.95±0.8*</td>
<td>239.93±0.2*</td>
<td>4.86±0.1*</td>
<td>102.8±1.0*</td>
<td>58.89±0.6*</td>
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<tr>
<td>Group VI</td>
<td>252.81±1.9*</td>
<td>77.28±0.70*</td>
<td>283.73±0.22*</td>
<td>8.02±0.23*</td>
<td>121.76±0.2*</td>
<td>46.02±1.0*</td>
</tr>
</tbody>
</table>

PCa induced group resulted in a marked decrease in GR, GPx, Catalase activities, GSH levels, increased lipid peroxidation and their levels were restored to normal, dose dependently after daidzein and luteolin administration. Values are expressed in means ± S.E.M. (n =8). GR as nmol NADPH oxidized/min/mg protein, GPx as nmol NADPH oxidized/min/mg protein, Catalase as nmol H2O2 consumed/min/mg protein, GSH is expressed as nmol GSH/gm tissue, LPO is expressed as nmol TBARS formed/hr/g tissue, SOD is expressed as U/mg protein. Values expressed in mean ± SE Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and * p < 0.001 when compared with prostate Cancer (Group II).
3.10 Effect of daidzein and luteolin on histopathological alterations.

In order to study the changes in the prostate gland induced by NMU-testosterone in prostate cancer the H & E staining of prostate tissue was carried out. The photomicrographs (40x) revealed high grade PIN with secretary cell proliferation as well as stromal hyperplasia in prostate cancer induced group II. Administration of daidzein showed low grade PIN with stromal cell hyperplasia and higher dose of daidzein showed low grade PIN, atrophy prostate with degenerative changes in the acine. While luteolin treated group also showed low grade PIN, but at lower dose stromal hyperplasia was seen. While the higher dose of luteolin treatment showed decreased in secretory cell proliferation with low grade PIN, atrophy with degenerative changes in acine of prostate are seen. (Figure 9)
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Chapter V

Jamia Hamdard, Department of Medical Elementology and Toxicology

Figure 9: Effect of daidzein and luteolin on histopathology changes: Group I 40x prostate section of rat control group showing acini and normal prostate architecture, secretory cells (→), stroma cells (→). Group II (PCa induced) group showing high grade PIN. Group III PCa+ daidzein at a dose of 20mg/kg b.wt showed a low grade PIN. Group IV PCa+ daidzein 60 mg/kg b.wt showed low grade PIN with prostate atrophy. Group V luteolin treated 0.2 mg/kg b.wt showed low grade PIN and with some stromal hyperplasia and group VI luteolin 0.4 mg/kg b.wt treatment resulted in low grade PIN, with prostate atrophy and degenerative changes. (40x)
4. Discussion

Inflammation is a complex phenomenon consisting of a humoral (cytokines) and cellular (leukocytes, monocytes and macrophages) components (Narayanan et al., 2009, Sciarra et al 2008). Inflammation is usually a self-limited event, with initial pro-inflammatory cytokines and growth factor release and angiogenesis followed by anti-inflammatory cytokine-mediated resolution (Sandhu et al., 2008).

In normal tissues, anti-inflammatory cytokines are synchronically up-regulated after the pro-inflammatory cytokines are produced, leading to inflammation resolution (Caruso et al., 2009). In chronic inflammation, mainly composed of chronically activated T cells and mononuclear phagocytes (monocytes and macrophages), there are persistence of promoters or a failure in mechanisms required to resolve inflammation. This will release more pro growth cytokines as well as various growth factors and attract additional immune cells to the inflammation site which amplifies the inflammatory response (Wong et al., 2009, Mechergui et al., 2009).

Prostate has a fully active immunologic response and involves a broad spectrum of immune responses against foreign antigens and contains scattered stromal and intraepithelial endogenous inflammatory cells such as T and B lymphocytes, macrophages and mast cells (Kramer et al., 2002, de Marzo et al., 2007). PCa induction led to massive infiltration of mast cells as seen by toluidene blue staining. T-cells increase with age, which correlates with the incidence of prostate inflammation during the aging process (Harsch et al., 2005). T-cells are known to release factors that stimulate matrix formation and secreting potent epithelial and stromal mitogens which could promote prostate stromal and epithelial proliferation/hyperplasia (Penna et al., 2009). Stromal – epithelial prostate interactions play a pivotal regulatory role in the maintenance of homeostasis in health and development of disease (Harsch et al., 2005, Kogan-Sakin et al., 2009). The histopathology photomicrographas reveles a high grade PIN, secretory cell and stromal cell proliferation. Daidzein and luteolin being anti proliferative and anti-inflammatory properties retarded the proliferation of prostate.

Prostate cell itself can induce inflammation reaction because it can express Antigen Presenting Cell (APCs) and all of the Toll-like receptors (TLRs). These expressions can produce pro-inflammatory cytokine and activate immune responses (Nickel 2007, Mechergui et al., 2009, Kramer et al., 2002, Penna et al., 2009, Begley et al., 2008) Konig et al found a different
expression pattern of the TLR in PCa tissue, increased expression was obtained for TLR 1, 2, and 3 in PCa tissue (Konig et al., 2004). Daidzein and luteolin attenuated the secretion of pro-inflammatory cytokines; this may be due to T-Cell suppression. Because T-cells in the prostatic stromal and epithelial cells simultaneously secrete higher pro-inflammatory cytokines such as interleukins (IL-1, IL-1α, IL-2, IL-4, IL-6, IL-7, IL-8, and IL-17), the CXC-type chemokine and their receptors in PCa tissues compared to normal prostate tissue (Kogan-Sakin et al., 2009, Mechergui et al., 2009, Lucia and Lambert 2008, Steiner et al., 2003). These cytokines were thought to induce fibromuscular growth and proliferation of prostatic stromal or epithelial cell by an autocrine or paracrine loop or via induction of COX-2 expression (Nickel et al., 2007, Penna et al., 2009, Steiner et al., 2003, Lucia and Torkko 2004, Bouraoui et al., 2008). IL-17 up-regulates the secretion of other pro-inflammatory cytokines, such as IL-8 and IL-6 as well as of TGF-β. IL-6 is recognized as one of the potent growth factors for prostatic epithelial and stromal cells, (Kramer et al., 2002, Novara et al., 2006).

Mechergui (2009) and Konig (2004) found IL-6 and IL-8 were over expressed in PCa tissue (Mechergui et al., 2009, Konig et al., 2004). IL-6 regulates the growth of prostate carcinoma and activates the androgen receptor dependant gene in prostate cancer cells in the absence of androgen (Mechergui et al., 2009).

COX-2 is up-regulated in a variety of malignancies including prostate cancer, throughout the tumorigenic process from early hyperplasia to metastatic disease (Wang et al., 2004, Caruso et al., 2009). Hsu et al indicate that normal prostate epithelial cells do not express significant levels of COX-2 (Hsu et al., 2000). Many studies showed over expression of COX–2 in prostate cancer. COX-2 over expression was also higher in Prostatic Intraepithelial Neoplasia (PIN) and poorly differentiated tumors (Sandhu et al., 2008, Konig et al., 2004, Fernandez et al., 2008, Wang et al., 2004). We found a decrease in chronic inflammation in daidzein and luteolin treated group, this may have blocked COX-2 activation. Moreover these groups also exhibited decrease PIN and PSA levels. Many reports state that, chronic inflammation induces COX-2 production. (Sciarra et al., 2008, Coussens et al., 2002, Caruso et al., 2009, Fernandez et al., 2008). This may lead to increases production of Prostaglandin (PG) E2 (an adhesion to the extracellular matrix), concentrations of Bcl-2 (pro apoptotic genes), and reduces the E-cadherin protein (with consequent loss of cell-to-cell adhesion). COX –2 also modulates production of angiogenic factors to induce angiogenesis. COX-2 also known to increases the carcinogenic potential of
cells through the oxidation of pro-carcinogens to carcinogens, increased cell growth, decreased apoptosis, as well as decreased immune response to abnormal or cancer cells matrix-metalloproteinase overexpression with an associated increase in invasiveness (Novara et al., 2006, Sarkar et al., 2007, Roberts et al., 2002, Pathak et al., 2005).

Chronic Inflammation also produces a free radical substance/oxidative stress and various reactive oxygen species (ROS) (Sciarra et al., 2008, Coussens et al., 2002, Caruso et al., 2009, Fernandez et al., 2008, Untergasser et al., 2005, Rigas and Sun 2008). This oxidative stress can induce vascular tissue damage, protein structure and function damage, genomic damages and cause post-translational modifications including those involved in DNA repair and apoptosis (Sciarr et al., 2008). These can lead to oxidative DNA damage in point mutations, deletions, or rearrangements and reduce DNA repair. These oxidative stresses also alter the stem cell population. Genomic alteration in cellular DNA results in modulating the imbalance between cell proliferation and cell death. A change in the normal regulation of programmed cell death lead to hyperplastic or precancerous transformation (MacLennan et al., 2006, Fleshner and Klotz 1999, Wong et al., 2009, Sandhu 2008, Uemura et al., 2008). All of these active factors production also induce repetitive tissue damage and repair with the release cytokines, growth factors and oncogenes, leading to increase epithelial or stromal cell proliferation (MacLennan et al., 2006).

Normally, oxidative stresses is removed by natural protective mechanism, the superoxide dismutase enzyme system, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) enzyme (Sciarr et al., 2008). Polyphenols have been well demonstrates to be free radical scevengers. Both daidzein and luteolin has shown restoration of antioxidant enzymes induced by NMU-Testosterone to normal, this may have prevented the DNA damage in prostate cells, maintaining the homeostasis of cell proliferation/cell death. There was an increase in caspase activation 3, 8, and 9 and increased BAX/BCl2 ratio (>1), resulting in induction of cell death in DNA damaged cells in daidzein and luteolinn administered group. Similar findings are reported by other groups. (Tang et al., 2012, Kim et al., 2007, Chang et al., 2005)

Human prostate tissue is also vulnerable to oxidative DNA damage due to more rapid cell turnover and fewer DNA repair enzymes. Balance between oxidative stress and antioxidant component of the cells have a role in developing prostate disease (Khandrika et al., 2009). There
are increases in oxidative stress and decreased antioxidant mechanisms in prostate disease. Khandrika et al., also found increasing ROS generation for more aggressive phenotype in PCa cell lines (Khandrika et al., 2009). Prostate cancer cell expresses lower levels of antioxidant enzymes or almost total inactivation of pro-oxidant scavenging enzyme (Pathak et al., 2005, Goodwin et al., 2008, Aydin et al., 2006, Jung et al., 2007, Yilmaz et al., 2008, Fleshner and Kucuk et al., 2001). Age was one of contributing factor changing the oxidant/antioxidant balance is shifted towards oxidative stress. Production of ROS and free radicals in mitochondria was increased during aging. There are also a down regulation, and/or underexpression of antioxidant with increasing age (Khandrika et al., 2009, Sikka 2003, Ripple et al., 1997).

Oxidative stress then can activate the transcription factor NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) by TNF-α/AP-1 transduction pathway and NIK transduction pathway. NF-kB is known as a master inflammatory transcriptional regulator and is highly active in macrophages. Targets of NF-kB include genes regulating immune response, inflammation, cell proliferation, cell migration, and apoptosis. The nuclear translocation of NF-kB can activate target genes involved in carcinogenesis (Sarkar et al., 2007, Nunez et al., 2008). NF-kB potentially can lead to the amplification of the inflammatory response in the tumor environment. Dysregulation of the transcription factor NF-kB has been proposed to be one putative molecular mechanism leading to chronic inflammation and cancer. In a chronically inflamed tumor environment, it is difficult to distinguish whether aberrant NF-kB activation originates from tumor cells or from immune infiltrates. Wong (2009) found exposure of prostate epithelial cells to proinflammatory soluble mediators directly activated NF-kB and induced local production of proinflammatory cytokines in the prostate epithelial cells (Wong et al., 2009). NF-kB was found a significant increase in the PIN and adenocarcinoma (Wong et al., 2009, Narayanan et al., 2009). The IL-1β-induced NF-kB pattern of intraprostatic chemoattractive signals might have a capability for maintaining the chronic inflammation and proliferative inflammatory atrophy (PIA) in the prostate, which are recognized as putative precursor lesions in the development of prostate cancer (Vykhovanets et al., 2009).

We conclude that, diet and age being major confounding factors in PCa. Its long latency period provides a better opportunity to introduce dietary agents as alternate therapy to retard and delay the onset of the PCa. We found that administration of daidzein and luteolin, both belonging to flavonoid group of polyphenols showed retardation in oxidative stress, inflammation which
finally attenuated the cell proliferation via p53/p21 activation, which led to the execution of serine protease (Caspases 3/8/9) for induction of apoptosis in DNA damaged cells. So, we can say the both these flavonoides may be used as dietary supplements for delaying the onset of PCa.