

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 January 2012 (12.01.2012)

(10) International Publication Number
WO 2012/004653 A1

(51) International Patent Classification:

A61K 31/352 (2006.01) **A61P 35/00** (2006.01)
A61K 31/7048 (2006.01) **A61P 3/10** (2006.01)

(21) International Application Number:

PCT/IB201 1/001580

(22) International Filing Date:

7 July 2011 (07.07.2011)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

1590/DEL/2010 7 July 2010 (07.07.2010) IN

(71) Applicants (for all designated States except US):

COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH [IN/IN]; Anusandhan Bhawan, Rafi Marg, 110001 New Delhi (IN). **VISVA-BHATARI, SCHOOL OF LIFE SCIENCES** [IN/IN]; A CENTRAL UNIVERSITY, SANTINIKETAN, 731235 BIRBHUM (West Bengal) (IN).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BHATTACHARYA, Sushmita [IN/IN]; VISVA-BHARATI, SCHOOL OF LIFE SCIENCES, A CENTRAL UNIVERSITY, SANTINIKETAN, 731235 BIRBHUM (West Bengal) (IN). **DASGUPTA, Suman** [IN/IN]; VISVA-BHARATI, SCHOOL OF LIFE SCIENCES, A CENTRAL UNIVERSITY, SANTINIKETAN, 731235 BIRBHUM (West Bengal) (IN). **BARMA, Pomy** [IN/IN]; VISVA-BHARATI, SCHOOL OF LIFE SCIENCES, A CENTRAL UNIVERSITY, SANTINIKETAN, 731235 BIRBHUM (West Bengal) (IN). **BISWAS, Anindita** [IN/IN]; VISVA-BHARATI, SCHOOL OF LIFE SCIENCES, A CENTRAL UNIVERSITY, SANTINIKETAN, 731235 BIRBHUM (West Bengal) (IN). **PAL, Bikash Chandra** [IN/IN]; VISVA-BHARATI, SCHOOL OF LIFE SCIENCES, A CENTRAL UNIVERSITY, SANTINIKETAN, 731235 BIRBHUM (West Bengal) (IN). **BHATTACHARYA, Shelley** [IN/IN]; VISVA-BHARATI, SCHOOL OF LIFE SCIENCES, A CENTRAL UNIVERSITY, SANTINIKETAN, 731235 BIRBHUM (West Bengal) (IN). **BHATTACHARYA, Samir** [IN/IN]; VISVA-

BHARATI, SCHOOL OF LIFE SCIENCES, A CENTRAL UNIVERSITY, SANTINIKETAN, 731235 BIRBHUM (West Bengal) (IN). **BORDOLOI, Manobjyoti** [IN/IN]; North-East Institute of Science & Technology, 785006 Jorhat (Assam) (IN). **BARUA, Nabin Chandra** [IN/IN]; North-East Institute of Science & Technology, 785006 Jorhat (Assam) (IN). **RAO, Paruchuri Gangadhar** [IN/IN]; North-East Institute of Science & Technology, 785006 Jorhat (Assam) (IN).

(74) Agent: G., Arun, Kumar; K & S Partners, Intellectual Property Attorneys, 109, Sector - 44, 122003 Gurgaon, National Capital region (IN).

(81) Designated States (unless otherwise indicated, for every kind of national protection available):

AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available):

ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(Hi))

Published:

— with international search report (Art. 21(3))
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

[Continued on next page]

(54) Title: METHOD FOR INHIBITION OF NF-KB GENE EXPRESSION

(57) Abstract: The present invention discloses a method of inhibition of synthesis of NF- κ B by inhibiting its gene expression using isoflavones Daidzein and Daidzin. Daidzein, a non toxic dietary supplement isoflavone of the structure (1) and 7-O-e-glucopyranosyl daidzein of the structure (2) are novel inhibitors of NF- κ B which blocks the synthesis of NF- κ B by inhibiting its gene expression and have no toxic effect. Both daidzein of the structure (1) and daidzin of the structure (2) ameliorate palmitate induced overexpression of NF- κ B in skeletal muscle cells by eliminating the inhibition of NF- κ B on insulin stimulated glucose uptake. Daidzein inhibits NF- κ B expression in prostate and breast cancer cells that increased mortality of these cancer cells. Daidzin of the structure (2) has very good bioavailability over daidzein of structure (1). Daidzein of the structure (1) is not absorbed when orally fed to mice as it is eliminated from the gut through glucuronidation process catalyzed by UGT 1. Daidzin of the structure (2) is glucosylated daidzein and is protected from UGT1 mediated degradation and is hydrolysed to daidzein which is absorbed and remains for more than 4 hours in blood and is expected to be distributed to different tissues and organs. Daidzein of the structure (1) and daidzin of the structure (2) reduce palmitate stimulated increased synthesis of NF- κ B significantly. Daidzein inhibits formation of NF- κ B-DNA complex stimulated by palmitate and both daidzein and daidzin inhibited NF- κ B expression which leads to reduction of its DNA binding. Daidzein of the structure (1) was obtained from soy leaves through chromatography and both natural and synthetic daidzein and daidzin have same biological activities.



WO 2012/004653 A1

— with sequence listing part of description (Rule 5.2(a))

“METHOD FOR INHIBITION OF NF-KB GENE EXPRESSION”

Field of Invention

The present invention relates to a method of inhibition of synthesis of **NF-κB** by inhibiting its gene expression using plant based isoflavones Daidzein and Daidzin derived from soybean (*Glycine max*) plant.

Background Art:

NF-KB is a well known transcription factor that plays a critical role in inflammatory diseases including diabetes and cancer. In cells, **NF-κB** remains as an inactive heterodimer having two polypeptide chains i.e. p50 and p65. The nuclear localisation signal domains of these two dimers remain blocked by another protein known as inhibitor of kappa B (**IκB**). Phosphorylation of **IκB** by inhibitor of kappa B kinase (**IKK**) leads to its proteosomal degradation and that allows translocation of active form of **NF-κB** to the nucleus where it binds to DNA and activates the promoter of a specific gene. **NF-KB** regulates plethora of gene expression and many of them are related to diabetes, cancer, arthritis and Alzheimer's diseases.

Hence, a potent inhibitor of **NF-κB** is of high demand. There are some **NF-κB** inhibitors available in the market and almost all of them are highly toxic and therefore cannot be used for therapeutic purpose. However all of these are activation inhibitors i.e. either it inhibits **IκBκ** or **IKK** phosphorylation. Magnolol, honokiol obtained from *Magnolia officinalis* inhibits **NF-κB** activation and downregulates NF-DB induced expression of gene products (Tse, A.K., Wan, C.K., Zhu, G.Y., Shen, X.L., Cheung, H.Y, Yang, M. and Fong, W.F. (2007) Magnolol suppresses NF-kappaB activation and NF-kappaB regulated gene expression through inhibition of IkappaB kinase activation. *Mol. Immunol.* 44, 2647-2658). Curcumin, a known potent **NF-κB** activation inhibitor is widely known. Acetyl salicylate also inhibits **NF-κB** by inhibiting **IKKκ**. There are also some proteasomal inhibitors like MG132 which inhibit **IκBκ** degradation and restrict **NF-KB** to cytosol. Dithiocarbamates are known inhibitors of **IKK** (Shreck, R., Meier, B., Mannel, D.N., Droge, W. and Baeuerle, P.A. (1992) Dithiocarbamate as potent inhibitors of nuclear factor kappa B activation in intact cells. *JEM* **175**, 1181-1194). SN-50, parthenolide and PDTC are also commonly used as potent **NF-κB** activation inhibitor. The research is still going on to find out an inhibitor that has no toxic effects. Here, we have come across a novel molecule isolated from natural source which

efficiently blocks NF- κ B pathway but in a new target, it inhibits NF- κ B gene transcription but had no toxic effect.

There are many known NF- κ B inhibitors; they inhibit NF- κ B activation. We have invented inhibitors of NF- κ B which blocks the synthesis of NF- κ B by inhibiting its gene expression. Daidzein, a non toxic isoflavone having molecular weight of 254, usually present in number of dietary supplements such as soybeans, legumes and peas. Daidzein inhibits NF- κ B gene expression that results in inhibition of protein expression and therefore, can be utilized for treating the various critical diseases, such as diabetes, cancer etc. NF- κ B is a transcription factor which was discovered by David Baltimore (Sen, R. and Baltimore, D (1986) Inducibility of κ immunoglobulin enhancer-binding protein NF- κ B by a post translational mechanism. *Cell* 47, 921-928. & Singh, H., Sen, R., Baltimore, D. and Sharp, P.A. (1986) A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature* 319, 154-158). Presently intensive research is going with NF- κ B as it regulates number of gene expressions which are associated with cancer, diabetes etc. This transcription factor also regulates transcription of most of the inflammatory genes. Therefore to search for a molecule which inhibits NF- κ B is very important where cancer and diabetes are posing a great threat to human health. We have reported earlier that lipid induced overexpression of NF- κ B in skeletal muscle cells is related to insulin resistance (Barma, P., Bhattacharya, S., Bhattacharya, A., Kundu, R., Dasgupta, S., Biswas, A., Bhattacharya, S., Roy, S.S. and Bhattacharya, S. (2009) Lipid induced overexpression of NF- κ B in skeletal muscle cells is linked to insulin resistance. *Biochim. Biophys. Acta.* 1792, 190-200). Daidzein ameliorates palmitate induced overexpression of NF- κ B in skeletal muscle cells. The problem with isoflavone as drug candidate is that they are glucuronidated in intestine and liver, and excreted out of the body through the urine and faecal matter. Absorption of daidzein poses a real problem to act as a drug because it has great limitation for bioavailability. Oral administration of daidzein does not lead to its availability in the serum suggesting its elimination from the gut through glucuronidation process due to the presence of UGT1 enzyme released from the gut cells (Hosoda, K., Furuta, T., Yokokawa, A., Ogura, K., Hiratsuka, A. and Ishii, K (2008). Plasma profiling of intact isoflavone metabolites by high-performance liquid chromatography and mass spectrometric identification of flavone glycosides daidzin and genistin in human plasma after administration of kinako. *Drug Metabolism and Disposition* 36, 1485-1495). We also faced the same

problem. In *in vitro* cell line daidzein could effectively blocked NF- κ B transcription, since its absorption through the gut is a problem, its action on target tissue for inhibiting NF- κ B synthesis will not be effective to treat diseases where NF- κ B is involved. To solve this problem we thought to use daidzin which is glucosylated and that could protect it from UGT1 mediated degradation. To examine this hypothesis, we purified daidzin from soybean leaves which has appreciable content of this isoflavone glycoside. We orally administered daidzin solution in mice, serum was collected at different times, subjected to protein precipitation and supernatant was lyophilized to observe absorption of daidzin. Interestingly, it is the nonglycosidic compound, daidzein which could be detected in the serum. This suggests that daidzin is hydrolysed to daidzein and remained for more than 4 hours in blood. We also found the daidzein in liver and skeletal muscle. Daidzein decreases NF- κ B protein and gene expression and it simultaneously inhibits NF- κ B regulated genes also. Fetuin-A (K2-Heremans Schmid glycoprotein [Ahsg]), an endogenous inhibitor of insulin receptor tyrosine kinase phosphorylation abrogates insulin stimulated downstream signals (Auberger, P., Falquerho, L, Confreres, J.O., Pages, G., Le Cam, G., Rossi, B. and Le Cam, A. (1989) Characterization of a natural inhibitor of the insulin receptor tyrosine kinase: cDNA cloning, purification, and anti-mitogenic activity. *Cell* 58, 631-640.& Rauth, G., Poschke, O., Fink, E., Eulitz, M., Tippmer, S., Kellerer, M., Haring, H.U., Nawratil, P., Haasemann, M. and Jahnen-Dechent, W. (1992) The nucleotide and partial amino acid sequences of rat fetuin: Identity with the natural tyrosine kinase inhibitor of the rat insulin receptor. *Eur. J. Biochem.* 204, 523-529.). Fetuin-A is primarily synthesized in liver and secreted into serum. Fetuin-A gene is overexpressed in palmitate treated rat primary hepatocytes. We found that this overexpression of fetuin-A is mediated by NF-KB. NF-KB siRNA inhibits the palmitate induced fetuin-A overexpression whereas in forced expression of NF- κ B in hepatocytes induces fetuin-A expression in absence of palmitate. Daidzein also inhibits fetuin-A protein and gene expression as it down regulation of NF- κ B synthesis. As both NF- κ B and fetuin-A is related to insulin resistance and type 2 diabetes, inhibition of NF- κ B transcription by daidzein helps in amelioration of insulin action. Daidzein increases insulin stimulated glucose uptake, as it helps in insulin stimulated Glut-4 migration towards the plasma membrane, which is downregulated by palmitate. Based on the several reports which indicate that NF-KB activity is increased in different types of cancers, we checked whether in cancer cells daidzein can inhibits NF- κ B gene expression or not. We have found that in PC-3

(prostate cancer cell line) and MCF-7 (breast cancer cell line) cells daidzein significantly inhibits NF- κ B gene expression. Therefore we can consider NF- κ B as a universal NF- κ B transcription inhibitor.

5 Objectives of the invention

The main objective of the present investigation is to provide a method of inhibition of synthesis of NF- κ B by inhibiting its gene expression using plant based molecules Daidzein and Daidzin.

- Another objective of the present investigation is to provide a therapeutic active
10 compound that will inhibit NF- κ B transcription thus abrogate NF- κ B gene expression which will give another alternative to treat critical diseases like diabetes, cancer etc.

Summary of the invention

- Accordingly, the present invention provides a method for inhibiting NF- kappaB gene
15 expression which comprises administering to a vertebrate in need of treatment, a therapeutically effective amount of atleast one compound selected from the group consisting of isoflavone obtainable from a soyabean plant belonging to the genus Glycine, and pharmaceutically acceptable salts thereof.

- In an embodiment of the present invention, a method for inhibiting NF- kappaB gene
20 expression which comprises administering to a vertebrate in need of treatment, a therapeutically effective amount of atleast one compound selected from the group consisting of isoflavone obtainable from a soyabean plant belonging to the genus Glycine, and pharmaceutically acceptable salts thereof.

- In another embodiment of the present invention, isoflavone selected are daidzein and
25 daidzin extracted from soybean.

In another embodiment of the present invention, the cell lines used were selected from the group consisting of PC-3 Prostrate Cancer cell line, and MCF-7 Breast Cancer cell line.

- In another embodiment of the present invention, the NF-KappaB inhibition is used to
30 treat diseases selected from the group comprising of Breast Cancer, Prostate Cancer and Diabetes.

In another embodiment of the present invention, the effective amount of Daidzein used is about 3-5 μ g /ml.

- In another embodiment of the present invention, a method of treating human Prostrate
35 cancer, Breast cancer, and Diabetes comprising administering orally to a subject in

need thereof isoflavone selected from the group consisting of Diadzein and Diadzin in the range of 3-5 μ g /ml.

In another embodiment of the present invention, Use of Diadzein and Diadzin as an inhibitor of NF-kB gene expression.

- 5 In another embodiment of the present invention, Use of Diadzein and Diadzin for the treatment of human Prostate cancer, Breast cancer, and Diabetes.

In another embodiment of the present invention, a method for inhibiting NF- kappaB gene expression substantially as herein described with reference to the examples.

10 Brief description of the drawings

Figure 1: represents standard HPLC peak of daidzein.

Figure 2: represents standard HPLC peak of daidzin

15

Figure 3: represents that the oral administration of daidzin increases bioavailability of daidzein (Dn). (A) Time dependent increased bioavailability of Dn was observed in serum and (B) significant amount was detected in liver at 120 min.

- 20 **Figure 4:** represents Palmitate overexpresses NF-kB. Skeletal muscle cells were incubated with 0.75 mM palmitate for 4h in the presence insulin (100 nM) and insulin stimulated [3 H] 2- deoxy-glucose uptake was determined (Fig.4A). L6 myotubes were transfected with GFP-Glut4 plasmid followed by incubation with palmitate or palmitate plus daidzein; insulin was added prior to 30 minutes of termination of
- 25 incubation. Glut-4 translocation was observed under confocal microscope. (Fig. 4 B). Western blot was performed with palmitate incubated myotubes using anti-pNF-kBp65, NF-kBp65, pNF-kBp50 and NF-kBp50 antibodies. b-actin was used as loading control (Fig. 4C) Western blot was performed to determine NF-kB protein levels in a time and dose dependent manner (Fig.4D,E). Means $\pm$ SEM was calculated from five
- 30 independent experiments; * ($p < 0.001$, # $p < .01$) as compared with the control and palmitate.

- Figure 5:** represents that both Daidzein(Dn) and daidzin(Din) inhibits NF-kB protein and gene expression. Skeletal muscle cells were incubated with palmitate in the
- 35 presence or absence of daidzein. Changes in the NF-kB p65 and NF-kBp50 protein

level was determined through immunoblot using NF- κ B p65 or NF- κ B p50 antibodies. β actin was used as a loading control (Fig.5A). Skeletal muscle cells were incubated for 4h with insulin or palmitate or palmitate plus daidzein or without any of them (C-control). On termination of incubation RNA was extracted, followed by RT-PCR and real time PCR (Fig.5B). L6 myotubes were incubated with palmitate and palmitate+daidzin for 4h. Western blot and RT-PCR was performed using anti-NF- κ B specific antibody or NF- κ B specific primers (Fig. 5C).

Figure 6: represents purification of NF- κ B p65 protein. Control and palmitate incubated (6h) L6 skeletal muscle cell lysates were resolved on a 10% SDS-PAGE. A clear solid band at the 65 kDa region indicates overexpression of NF- κ B p65 protein. L6 skeletal muscle cells were incubated with palmitate and cell lysates after centrifugation was loaded on a Sephadex G-75 column. A control was run parallelly to see any expression of NF- κ B protein due to palmitate incubation. Separately collected fractions (such as A1, A2 and A3) from control and palmitate treated cell lysates were immunoblotted with anti-NF- κ B p65 antibody. The lanes A1 and A2 shows the immunoreactive bands of NF- κ B. The pooled fractions from gel filtration chromatography was lyophilized and loaded on a CNBr activated Sepharose 4B immunoaffinity column. P1 (unbound protein) and P2 (bound fraction after elution with 2M KI) peaks were collected and Western blot analysis shows P2 as immunoreactive band of NF- κ B

Figure 7: NF- κ Bp65 protein was delivered in L6 skeletal muscle cells and its incorporation was detected by immunofluorescence. NPT transduced cells were incubated with daidzein [3 H] 2-deoxyglucose uptake was determined (Fig.7A). Control and NF- κ Bp65 transduced cells (NPT) were subjected to Western blot analysis with anti-NF- κ B p65 antibody (Fig.7B). C) GFP-Glut 4 construct was transfected into the skeletal muscle cells and incubated with insulin (I) or I+NF- κ Bp65 protein or I+NF- κ Bp65 protein+daidzein or with none (C-control). On termination of incubation, GFP-Glut 4 localization was detected by using laser scanning confocal microscope. (Fig.7C) Values represent means $\pm$ SEM of three independent experiments, * p <0.01 (I vs I + NPT).

Figure 8: Increase of NF- κ B nuclear localization is inhibited by daidzein. L6 myotubes were incubated with palmitate or palmitate+daidzein. Immunofluorescence study was performed under fluorescence microscope.

Figure 9: represents the relationship between NF- κ B overexpression and activation. Nuclear extracts (NE) were prepared from L6 skeletal muscle cells incubated for 4h with palmitate, palmitate plus daidzein or SN50 or without any of them (control) followed by EMSA. Both wild type and mutant DNA sequences were used to determine the specificity of NF- κ B binding (Fig.9A). Levels of palmitate induced phosphorylation of IKK and I κ B were determined in skeletal muscle cells incubated in the presence of palmitate (P) or palmitate plus daidzein or without any of them (C) using anti-pIKK and anti-pI κ B antibodies as probes. Immunoblots for IKK and I κ B a protein served as loading controls.

Figure 10: Palmitate stimulation of NF- κ B expression is inhibited by daidzein (Dn). NF- κ B cis-reporter gene plasmid transfected L6 myotubes were incubated with palmitate (P) and palmitate+Dn. Luciferase activity was determined after 5h of incubation. Prior to palmitate incubation cells were pre-incubated for 1h with Dn. (Fig. 10A) Means $\pm$ SEM was calculated from three independent experiments. Myotubes incubated with(P) or without(C) palmitate and palmitate+Dn were subjected to FACS analysis using anti-NF- κ B specific antibodies (Fig. 10B).

Figure11: NF- κ B mediated fetuin-A expression is inhibited by daidzein (Dn). Hepatocytes were incubated without (Ctl) or with 0.75 mM palmitate (P) for 4h. Prior to palmitate incubation cells were pre-incubated for 1h with Dn. siRNA transfected cells were incubated with daidzein. Cells were lysed and immunoblotted with anti-Fetuin-A antibody. α -actin served as internal control. RNA extracted from the above incubations was subjected to RT-PCR and Real time PCR using Fetuin-A specific primers where *gapdh* served as internal control. Means $\pm$ SEM was calculated from three independent experiments.

Figure 12: Daidzein has protective effect against prostate cancer and breast cancer. PC-3 and MCF-7 cells were incubated with daidzein at a concentration of 5 μ g/ml. Cell mortality was determined under microscope. RNA extracted from the above incubations was subjected to RT-PCR using NF- κ B specific primers where *gapdh* served as internal control. Means $\pm$ SEM was calculated from three independent experiments (Fig. 12A&B).

Figure 13: Relationship between expression and activation of NF- κ B p65 in MCF-7 and PC-3 cells

MCF-7 and PC-3 cells were treated without (Con) or with Dn ($8\mu\text{g}/\text{ml}$) or transfected with NF- κ B p65 siRNA (p65KO) or scramble siRNA (Mock). Control cells contained DMSO as vehicle. Total RNA extracted from these cells was subjected to qPCR analysis using NF- κ B p65 gene specific primers. *gapdh* was used as κ B internal control. MCF-7 and PC-3 cells were incubated without or with Dn or transfected with NF- κ B p65 (p65KO) or scramble (Mock) siRNA followed by the incubation of [^3H]-leucine or [$\gamma^{32}\text{P}$]-ATP. NF- κ B p65 or pNF- κ B p65 was pull-down from the cell lysates and % incorporation of radiolabelled leucine or phosphate into NF- κ B p65 or pNF- κ B p65 was determined by radioactive counting in liquid scintillation counter. Figures are one of the representatives of five individual experiments. Data represented are means $\pm$ SEM, * $p < 0.01$ vs Con, # $p < 0.001$ vs Con.

Figure 14: Daidzein and daidzin inhibits cell survivability MTT assay was performed in a dose dependent manner with daidzein and daidzin in a dose dependent manner.

Detailed description of the invention

Daidzein, a non toxic dietary supplement isoflavone of the structure 1, is a novel inhibitor of NF- κ B which blocks the synthesis of NF- κ B by inhibiting its gene expression. Daidzein of the structure 1 inhibits NF- κ B gene transcription but had no toxic effect. Daidzin, glucopyranosyl daidzein of the structure 2 is also an inhibitor of NF- κ B which blocks the synthesis of NF- κ B by inhibiting its gene expression and has no toxic effect. Both daidzein of the structure 1 and daidzin of the structure 2 ameliorate palmitate induced overexpression of NF- κ B in skeletal muscle cells. Daidzein of the structure 1 and daidzin of the structure 2 increase insulin stimulated glucose uptake, as it helps in insulin stimulated Glut-4 migration towards the plasma membrane, which is downregulated by palmitate. Both daidzein of the structure 1 and daidzin of the structure 2 are inhibitors of PC-3 (prostate cancer cell line) and MCF-7 (breast cancer cell line) cells and are novel NF- κ B transcription inhibitor. Daidzin of the structure 2 has very good bioavailability over daidzein. Daidzein is present in dietary supplements, it is found in soyabeans, legumes and peas but it poses a problem of bioavailability. Daidzein of the structure 1 is not absorbed when orally fed to mice as it is eliminated from the gut through glucuronidation process due to the presence of UGT1 enzyme released from the gut cells. Daidzin of the structure 2 is glucosylated daidzein and is protected from UGT1 mediated degradation and is hydrolysed to

daidzein which is absorbed and remains for more than 4 hours in blood therefore expected to be distributed to different tissues and organs. Daidzein of the structure 1 and daidzin of the structure 2 reduce palmitate stimulated increased synthesis of NF- κ B significantly. Daidzein inhibits formation of NF- κ B -DNA complex stimulated by palmitate.

Thus daidzein of the structure 1 and daidzin of the structure 2 have protective effect against prostate cancer and breast cancer by inhibiting the gene expression of NF- κ B in PC-3, breast cancer. These two compounds are also therapeutically active by inhibiting NF- κ B transcription which would be beneficial for treating critical diseases like diabetes, arthritis and Alzheimer's diseases etc. Daidzein of the structure 1 and daidzin of the structure 2 were obtained from soy leaves through chromatography and both natural and commercial/synthetic daidzein and daidzin have same biological activities.

The following examples are given by way of illustration and should not be construed to limit the scope of present invention.

Example 1

We have extracted and purified daidzin from soyabean leaf. Daidzin, an isoflavone was isolated and purified from *Glycine max* leaves through solvent fractionations, Diaion HP-20 and m-Bonadapak C-18 reverse phase column chromatography. Its structure was determined by ^1H NMR, ^{13}C NMR and Q-TOF-MS. The plant material of 100 g of fresh leaves was crushed in a mixer-cum-grinder and immersed in 200 ml of ethanol/water (70/30, v/v) overnight. The extract was obtained after filtration and the plant material was immersed again in raw ethanolic 200 ml of ethanol/water (70/30, v/v) overnight. The process is repeated three times. The combined extract was concentrated under reduced pressure at below 50°C. The concentrated extract was then defatted by extracting with hexane (100 ml x 3). The defatted aqueous layer was then extracted with chloroform followed by ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulphate and then distilled under reduced pressure at below 50°C. The gummy mass obtained was then chromatographed over a silica gel column using ethyl acetate, 50% methanolic ethyl acetate, methanol and 45% water in methanol. Fractions obtained by elution of 45% water in methanol were found to be daidzein and daidzin respectively. These two fractions were further purified by flash

chromatography with C18 RP column eluted with 45% water in methanol to get pure daidzein and daidzin. The isolated compounds were further confirmed by HPLC (Waters HPLC equipment) using Luna-C18 (2) column. Solvent system used was 45% methanol in water(v/v) in 35 min with flow rate 1 ml/min. 10 microliter sample was injected and chromatograms were recorded at 232 nm UV wherein daidzein appeared at retention time 5.6 minute in the chromatogram and daidzin appeared at retention time 8.76 minutes. And the structures were confirmed by IR, ^1H -NMR, ^{13}C NMR and Mass spectra.

10 Example 2:

Bioavailability: Bioavailability of daidzein and daidzin was tested by oral feeding of a solution of daidzein and daidzin to mice and collecting blood at 15 minutes, 30 minutes, 60 minutes, 120 minutes and 240 minutes followed by separation of serum. At first a standard curve was calibrated for HPLC estimation of daidzein and daidzin in blood by using standard and authenticated daidzein and daidzin. Later, we purified daidzein from soybean seeds by using authenticated daidzein as the standard. Analysis of daidzein was performed with Waters HPLC equipment using Zorbax C18 M45 column. Solvent system used was 0.1% H_2PO_4 in water =A and 0.1% H_2PO_4 in acetonitrile=B (gradient, 20 % B to 80% B in 25 min; 80% B to 20% B in 5 min) with flow rate 1 ml/min. All chromatograms were recorded at 232 nm UV. HPLC of chromatogram of daidzein, daidzin and a representative blood serum sample were shown at figure 1, figure 2 and figure 3 respectively. The peak of daidzein appears at retention time 5.6 minute in the chromatogram. The peak of daidzin appears at retention time 8.76 minutes. The blood serum sample does not show any peak at retention time around 8 minutes. However, the blood serum collected after 15 minutes of feeding showed a peak at retention time 5.227 minute indicating clearly that daidzin was not in the blood and daidzein, the hydrolyzed product of the isoflavone glycoside daidzin, was present in the blood serum. The hydrolyzed product of daidzin was observed for the serum sample collected at 4 hours, this shows that daidzein is available from circulatory source by the respective tissues and organs of the body for at least 4 hours which is an appreciable retention time in blood serum.

Bioavailability of daidzein:-

Since bioavailability of flavone compounds is a problem because of their rapid elimination through the intestine due to glucuronidation by UGT groups of enzymes

(King R. (1998) *Am. J. Clin. Nutr.* 68, 1496S-1499S), we examined the same for Daidzein. Daidzein has two naturally available forms, one is glycosylated (daidzin) and another aglycone (daidzein), we have used daidzein in our experiments as it is comparatively more active. However, its absorption through the intestine is less than daidzin. We have found that oral administration of glycosylated form could be detected in the serum and liver as daidzein (Dn) indicating cleavage of carbohydrate moiety in the intestine and that possibly provided protection from intestinal glucuronidation. This was also demonstrated in a previous report (King, R. and Bursill, D.B. (1998) *Am. J. Clin. Nutr.* 67, 862-872). Hence, bioavailability of Dn would not pose a problem for *in vivo* research and future therapeutic use.

Example 3

There are many reports about the role of NF- κ B in insulin resistance but the detailed mechanism is not well known. Recent evidences indicate that lipid induced elevation of NF-KB activity may play an important role in insulin resistance and type 2 diabetes. Activation of inhibitor of kappa B kinase (IKK) phosphorylates I κ B, an inhibitor of NF- κ B. Phosphorylation of I κ B leads to its proteosomal degradation resulting the release of NF-KB for its translocation to nucleus. Excessive activity of I κ B and NF- κ B has been shown to be linked with insulin resistance while inhibition of NF- κ B activation ameliorates it (Cai, D., Yuan, M., Frantz, D.F., Melendez, P.A., Hansen, L, Lee, J. and Shoelson, S.E. (2005) Local and systemic insulin resistance resulting from hepatic activation of IKK- β and NF- κ B. *Nat. Med.* 11, 183-190.) Earlier we have reported that overexpression of NF- κ B due to palmitate is associated with insulin resistance (Barma et al, 2009). NF- κ B is a transcription factor that not only regulates diabetes but it is related to most of the inflammatory diseases like cancer, arthritis and Alzheimer's disease. Therefore there is a need to find out a potent inhibitor of NF- κ B. We have isolated a isoflavone glycoside from soyabean leaves, known as daidzin. In intestinal cells it is hydrolysed to form daidzein. We found that both daidzin and daidzein can efficiently inhibit NF- κ B gene transcription and protein synthesis. Soy isoflavones daidzein and genistein are currently being investigated in clinical studies. It has been reported that genistein has raised concerns over its potential negative effects on immune function, brain function and DNA repair (Kim, D.J., Seok, S.H., Baek, M.W., Lee, H.Y., Na, Y.R., Park, S.H., Lee, H.K., Dutta, N.K., Kawakami, K. and Park, J.H. (2009) Developmental toxicity and brain aromatase induction by high genistein

concentrations in zebrafish embryos. *Toxicology Mechanisms and Methods* 19,251-256). Daidzein does not share these side effects and is more effective. Daidzein is present in dietary supplements, it is found in soyabeans, legumes and peas but it poses a problem of bioavailability. This problem could be avoided by using daidzin.

5

Example 4

To monitor inhibition of NF- κ B expression we have used L6 skeletal muscle cell line and conducted the assays where insulin activity is inhibited by NF- κ B. It is well-known that NF- κ B inhibits insulin activity. L6 myotubes or skeletal muscle cell were incubated with or without palmitate or palmitate plus daidzein. 100nM insulin and [3 H2-DOG] were added to these incubations before termination of incubation of cells. Insulin stimulated glucose uptake was significantly reduced by palmitate while daidzein co-incubation strikingly reduced palmitate adverse effect (Fig. 4 A). We transfected L6 myotubes with GFP-Glut4 plasmid followed by incubation with palmitate or palmitate plus daidzein; insulin was added prior to 30 minutes of termination of incubation (Fig. 4 B). Palmitate significantly increased both NF- κ B p65 and NF- κ B p50 protein levels (Fig. 4C). Palmitate induced increased NF- κ B gene and protein expression was concentration and time dependent (Fig 4 D,E).

20 Example 5

Determination of NF- κ B content in cells through Western blot showed that there was a significant decrease of palmitate stimulated NF- κ B due to daidzein (Fig.5 A). Both semiquantitative RT-PCR and quantitative Real Time PCR analysis showed that in palmitate incubated L6 myotubes NF- κ B gene expression was significantly increased which was prevented by daidzein (Fig.5 B). These results suggest that daidzein inhibits NF- κ B gene expression. Hence, daidzein inhibition of NF- κ B protein overexpression due to palmitate is effected via the inhibition of NF- κ B gene transcription. Daidzin, the flavone glycoside of daidzein also reduced palmitate induced NF- κ B gene expression and protein synthesis (Fig 2C).

30

Example 6

L6 myotubes were incubated without or with palmitate, lysed by ultrasonication and then centrifuged. The supernatant was collected and loaded on Sephadex G75 column. The elution profile from these cells showed an increase of NF- κ B p65 protein

due to palmitate (A2) treatment in comparison to control (A1). The eluted fractions from these incubations were subjected to immunoaffinity chromatography by anti-NF- κ Bp65 antibody for further purification. The eluted fraction (P2) from immunoaffinity chromatography shows the cross reactivity with anti-NF- κ B antibody in Western blot analysis (Fig.6). We have found that palmitate induced increased synthesis of NF-KB was prevented by daidzein.

Example 7

We transduced purified NF- κ B p65 protein to L6 skeletal muscle cells then incubated them with insulin and [3H]-2DOG to observe whether excess of NF- κ B p65 protein entry into the cell could affect insulin activity. Glucose uptake decreased in NF- κ B p65 protein transduced cells which were prevented by daidzein (Fig. 7A). Western blot analysis confirmed the increased level of NF- κ B p65 within the cell due to NF- κ B p65 protein transduction (Fig. 7B). NF- κ B p65 protein was transduced to a batch of GFP-Glut 4 transfected skeletal muscle cells and then subjected to insulin incubation. On termination of incubation, cells on the cover slips were fixed in paraformaldehyde (3.5%). The cover slips were examined for translocation of GFP-Glut 4 under laser scanning confocal microscope. GFP-Glut 4 translocation to plasma membrane in response to insulin was abrogated in NF- κ B p65 transduced cells which were prevented by daidzein (Fig. 7 C).

Example 8

Palmitate activation leads to the increase in NF- κ B nuclear localization. We tracked NF-KB in skeletal muscle cells by immunofluorescence study using FITC conjugated anti-NF- κ B p65 antibody. Palmitate enhanced NF- κ B level both in the cytoplasm and nuclear region was inhibited by daidzein (Fig.8).

Example 9

Palmitate induced increase of NF- κ B translocation to the nucleus coincides with the enhanced binding of NF- κ B with DNA. This was examined by performing EMSA. NF-KB -DNA complex formation was stimulated by palmitate whereas daidzein and SN-50 reduced the formation of this protein-DNA complex (Fig.9 A). Palmitate induces NF-KB binding to its κ B enhancer element and regulates its own gene expression (Barma et al, 2009) whereas daidzein inhibits this binding and thus inactivates its gene

expression. This suggests NF- κ B has an autoregulatory loop, which controls its own gene expression. Palmitate induced phosphorylation of IKK and I κ B κ was not affected by daidzein suggesting that daidzein has no effect on the NF- κ B activation pathway (Fig.9 B).

5

Example 10

- 10 Daidzein inhibits palmitate induced NF- κ B promoter activity. Cells were incubated without (control) or without palmitate and palmitate plus daidzein. NF- κ B promoter activity increased remarkably due to palmitate incubation and daidzein reduces the palmitate stimulatory effect (Fig.10 A). We also observed that NF- κ B protein level in both control and palmitate treated cells by performing FACS analysis. Flow cytometric
- 15 analysis showed that increased level of NF- κ B in response to palmitate was significantly inhibited by daidzein (Fig.10 B).

Example 11

- NF-KB regulates transcription of many inflammatory genes. It regulates transcription of
- 20 fetuin-A, an inhibitor of insulin receptor tyrosine kinase. Primary hepatocytes were incubated without or with palmitate or palmitate plus daidzein. Palmitate significantly induced fetuin-A gene expression. Addition of NF- κ B siRNA or Daidzein, inhibits fetuin-A synthesis by inhibiting NF- κ B expression (Fig. 11). By preventing NF- κ B gene expression daidzein can modulate NF- κ B downstream signalling.

25

Example 12

- Daidzein has also protective effect against prostate cancer and breast cancer. Daidzein at concentration of 5 μ g/ml reduce the gene expression of NF- κ B in PC-3, androgen independent prostate cancer cell line (Fig.12 A) and MCF-7, breast cancer
- 30 cell line (Fig.12 B).

Methods

The detailed methodologies utilized to obtained results in connection with the role of daidzein (Dn) in inhibiting NF-kB gene transcription are provided to understand our

claim. Kindly note that although there are several reports on the inhibition of active NF-kB, which is a phosphorylated form of NF-kB by this isoflavone, inhibition of NF-kB gene and protein synthesis has no earlier report. Our novelty lies here.

5 1. Reagents

All tissue culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, USA. [³H]-leucine (specific activity 1000 Ci/mmol), was obtained from GE Healthcare, Kowloon, Hong Kong. [γ -³²P]-ATP (Specific activity: 6000 Ci/mmol) was procured from BRIT, India. The primary antibodies for NF-kBp65, pNF-kBp65, 10 pIKK α / β , pI κ B α , and β -actin were purchased from Santa Cruz Biotechnology Inc., California, USA. Control and NF-kBp65 (h) siRNA were also purchased from Santa Cruz Biotechnology Inc., California, USA. Fluorescence iso-thiocyanate (FITC) conjugated goat anti-rabbit and alkaline phosphatase conjugated secondary antibodies were purchased from Sigma Chemical Co., St. Louis MO, USA. All other chemicals 15 and reagents were purchased from Sigma Chemical Co., St. Louis MO, USA.

2. Cell lines and cell culture

The breast cancer cell line MCF-7, the prostate cancer cell line PC-3 and L-6 skeletal muscle cell line were gifted by Dr. Partha P. Banerjee, Georgetown University 20 Medical Center, USA (ATCC Deposit No.MCF-7, HTB-22, PC-3 CRL-1435, L6-CRL-1458.). These cells were cultured in DMEM containing Earle's salts and non-essential amino acids supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μ g/ ml) in a humidified 95% O₂/5% CO₂ atmosphere at 37°C.

25 3. Cell culture treatments

Confluent cells were subcultured by trypsinization and subsequently seeded in six well culture plates containing DMEM with essential supplements. Confluent MCF-7 cells were incubated with 3Dg/ml daidzein (Dn) and PC3 cells were incubated with 5 μ g/ml daidzein. Daidzein was dissolved in DMSO. Control cells were treated with 30 equal amounts of DMSO in the media. At the end of incubation, cells were lysed, centrifuged for 10 min at 10,000g and the supernatant was collected. Protein concentration of supernatant was determined by following the method of Lowry et al [O.H. Lowry, N.J. Rosebrough, A.E. Farr, R.J. Randall, Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275]. Scramble and NF-kBp65

siRNA were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following manufacturer's instruction.

4. Western blots

5 Western blot was performed according to our previous description [S. Dasgupta, 5. Bhattacharya, A. Biswas, S. S. Majumdar, S. Mukhopadhyay, S. Ray and S. Bhattacharya (2010) NF- κ B mediates lipid-induced fetuin-A expression in hepatocytes that impairs adipocyte function effecting insulin resistance. *Biochem. J.* 429: 451-462]. Briefly, 40-50 μ g of protein from control or treated cell lysates were resolved on 10% or 12.5% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA) with the help of Semi-Dry trans blot Apparatus (Bio-Rad Trans-Blot® SD Cell, USA). The membranes were first incubated with different primary antibodies at 1:1000 dilutions followed by respective alkaline phosphatase conjugated secondary antibodies at same dilutions using SNAP i.d. apparatus (Millipore, Bedford, MA). The protein bands were detected by developing with 5-bromo 4-chloro 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

20 5. RT-PCR and Real Time PCR

Total RNA was extracted from different incubations using TRI-reagent according to the manufacturer's protocol. RT-PCR was performed with First strand cDNA synthesis kit, Fermentas Life Sciences, Revert Aid™, Hanover, MD, USA. Relative gene expression was further confirmed by Real-time PCR (Applied Biosystems Inc. CA, USA). PCR was performed using gene specific primers in the following reaction conditions: initial activation step at 95°C for 15mins, denaturation at 95°C for 30s, annealing at 50-55°C for 30s and final extension at 72°C for 30s x 40 cycles, *gapdh* was simultaneously amplified in separate reactions. C_T value was corrected using corresponding *gapdh* controls. Primer sequences used were as follows; NF-kBp65 (forward: 5'-ccatcagggcagatctcaaacc-3' reverse: 5'-gctgctgaaactctgagttgtc-3'; Gene bank accession no. NM_021975.3), Bcl-2 (forward: 5'-TGGGATGCCTTTGTGGAAC-3', reverse: 5'-GAGACAGCCAGGAGAAATCAAAC-3'; Gene bank accession no. NM_000657.1), Cyclin D1 (forward: 5'-GGATGCTGGAGGTCTGCGAGGAAC-3', reverse: 5'-GAGA-GGAAGCGTGTGAGGCGGTAG-3'; Gene bank accession no.

NM_053056.2). *gapdh* (forward: 5'-gccatcaacgaccccttc-3', reverse: 5'-agccccagccttctcca-3'; Gene bank accession no. J04038.1).

6. Gel Filtration and Immunoaffinity Chromatography to purify NF-kB

- 5 To prepare NF-kB rich fraction, gel filtration chromatography was performed on a Sephadex G-75 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) column (2x50 cm) equilibrated with Tris-Cl buffer (10 mM Tris-Cl, pH-7.4) by following an earlier description from this laboratory [D. Basu, S. Bhattacharya, Purification of two types of gonadotropin receptors from carp ovarian follicles: overlapping recognition by two
- 10 different ligands, Gen. Comp. Endo. 129 (2002) 152-162]. The flow rate was maintained at 1 ml/min and 1.0 ml fractions were collected just after loading the skeletal muscle cell lysate. A standard gel filtration molecular weight marker from Sigma Chemical Co., St. Louis, USA, was run to determine the approximate molecular weight of the eluted proteins. Fractions around 66 kDa were pooled, lyophilized to
- 15 reduce the volume and subjected to 10% SDS-PAGE. To identify NF-kB p65 there, the gel was transferred to PVDF membrane for Western blot analysis. It was further purified by immunoaffinity chromatography where CNBr activated Sepharose 4B beads were conjugated with the anti-NF-kBp65 antibody. Coupling of the antibody was carried out by following the procedure previously described from this laboratory.
- 20 Briefly, NF-kB p65 antibody coupled Sepharose 4B beads were loaded on a 5 ml column. Unbound antibody was removed by washing with coupling buffer (pH-8.5) followed by acetate buffer (0.1 M, pH-4.0). The remaining active groups on the beads were blocked with glycine buffer (0.2 M, pH-8.0). The sample was loaded on the column and kept overnight at 4°C. The unbound proteins were eluted with 10 mM Tris-
- 25 HCl buffer, pH-7.4 while NF-kBp65 bound to the ligand was eluted with elution buffer (10 mM Tris-HCl, pH -7.4) containing 2.0 M KI as a chaotropic agent. The volume of each eluted fraction was 1.0 ml. The fractions were collected until the O.D. at 280 nm reached the baseline, pooled and dialyzed against the buffer (10 mM Tris-HCl, pH -7.4) and lyophilized. The purity of NF-kB p65 was examined in a 10% SDS-PAGE
- 30 where it gave a single protein band which also crossreacted with anti-NF-kB p65 antibody.

7. Electrophoretic Mobility Shift Assay (EMSA)

- EMSA was performed by using nuclear extracts prepared from different
- 35 incubations in cancer cells using oligonucleotide probes specific for NF-kB binding site

(Wild: 5'- AGTTGAGGGGACTTTCCCAGGC -3', mutants: 5'- AGTTGAGGGGAACTTTCCCAGGC -3', AGTTGAGGGAAAACTTTCCCAGGC -3'). The probes were end-labelled with [$\gamma^{32}\text{P}$]-ATP using T4 polynucleotide kinase and then incubated with 10 μg of nuclear extracts of control and Dn treated MCF-7 and PC3 cells in a 20 μl of binding reaction for 45 min. Reaction mixtures were resolved on 5% polyacrylamide gel and visualized by PhosphorImager (GE Healthcare, USA).

8. [^3H]-leucine incorporation study

MCF-7 and PC3 cells were incubated in Kreb's Ringer Phosphate (KRP) buffer supplemented with 0.2% bovine serum albumin. To determine the effect of Dn on NF-kBp65 protein synthesis, cells were incubated with 10 $\mu\text{Ci/ml}$ of [^3H]-leucine in the presence or absence of Dn. Cells were also transfected with NF-kBp65 siRNA or scramble siRNA. On termination of incubation cells were lysed and NF-kBp65 was pulled down by anti-NF-kBp65 antibody. Radioactive count incorporation to NF-kBp65 was measured in Liquid Scintillation counter (Perkin Elmer, Tri-Carb 2800TR).

9. [$\gamma^{32}\text{P}$]-ATP incorporation study

MCF-7 and PC3 cells were incubated without or with daidzein (Dn) or transfected with NF-kBp65 siRNA or scramble siRNA in the presence of 80 μM [$\gamma^{32}\text{P}$]-ATP and on termination of incubation, cell lysates were subjected to immunoprecipitation with anti-pNF-kBp65 antibody. Radioactive count incorporation to pNF-kBp65 was measured in Liquid Scintillation counter (Perkin Elmer, Tri-Carb 2800TR).

Example 13

MCF-7 and PC3 cells were treated without (Con) or with Dn (3 and 5 $\mu\text{g/ml}$ respectively) or transfected with NF- κB p65 siRNA (p65KO) or scramble siRNA (Mock). Control cells contained DMSO as vehicle. Total RNA extracted from these cells was subjected to qPCR analysis using NF- κB p65 gene specific primers. *gapdh* was used as internal control. MCF-7 and PC3 cells were incubated without or with Dn or transfected with NF- κB p65 (p65KO) or scramble (Mock) siRNA followed by the incubation of [^3H]-leucine or [$\gamma^{32}\text{P}$]-ATP. NF- κB p65 or pNF- κB p65 was pull-down from the cell lysates and % incorporation of radiolabelled leucine or phosphate into NF- κB p65 or pNF- κB p65 was determined by radioactive counting in liquid scintillation counter. Figures are one of the representatives of five individual experiments. Data represented are means $\pm$ SEM, * $p < 0.01$ vs Con, # $p < 0.001$ vs Con.

Our results demonstrate that suppression of NF- κ B p65 gene expression by p65siRNA and a flavone compound; daidzein (Dn) reduced both inactive and phosphorylated form of NF- κ B in cancer cells. The reason for selecting both p65siRNA and Dn to suppress NF- κ B is that NF- κ B p65 siRNA specifically inhibits its mRNA whereas Dn occupies promoter κ B segment where NF- κ B binds to express its gene. We therefore had two modes of NF- κ B gene suppression to study the importance of equilibrium between inactive and active NF- κ B in NF- κ B dependent cancer cells. Inhibition of NF- κ B gene expression in MCF-7 and PC3 cells consequently reduced its protein expression and this coincided with the significant decrease of its phospho-form. To examine whether such reduction is due to inhibition of NF- κ B protein synthesis and that effected subdued phosphorylation, we incubated MCF-7 and PC3 cells with [3 H]-leucine or [γ 32p]-ATP followed by immunoprecipitation of active and inactive NF- κ B with their respective antibodies. NF- κ B protein synthesis was markedly reduced because of suppressed NF- κ B gene expression by NF- κ Bp65 siRNA or Dn. This corresponded with the significant decline of phosphorylated NF- κ B indicating that active NF- κ B pool in these cells is dependent on its de novo synthesis.

Advantages of present invention-

1. Till date an inhibitor of NF- κ B synthesis has not been reported, hence, novelty of our research investigation lies on this achievement as daidzein has been found to be an effective inhibitor of NF- κ B gene expression. Since it blocks transcription of NF- κ B p65.
2. Daidzein inhibits NF- κ B synthesis by reducing its gene expression.
3. For last few years there are several reports that in some cancers such as lung cancer, breast cancer, pancreatic cancer, colon cancer and in brain cancer NF- κ B remains overexpressed. The reason behind this robust expression of NF- κ B in these cancers has been reported to be their dependence on NF- κ B. Such an excess expression of NF- κ B is required for their survivability of these cancer cells Saitoh et al. (2010) Cancer 70: 263-270, Yu et al. (2003) Oncology 65: 37-45, Yu et al. (2004) Int J Colorectal Dis 19: 18-22).

Therefore in these NF- κ B dependent cancer cells, an inhibitor of NF- κ B expression would be a suitable intervention for their growth and survivability. Daidzein will serve this purpose as this is the only inhibitor of NF- κ B expression known till date through our investigation. This is indeed a great advantage for daidzein.

Claims:

- 5 1. A method for inhibiting NF- kappaB gene expression which comprises administering to a vertebrate in need of treatment, a therapeutically effective amount of atleast one compound selected from the group consisting of isoflavone obtainable from a soyabean plant belonging to the genus Glycine, and pharmaceutically acceptable salts thereof.
- 10 2. A method as claimed in claim 1, wherein isoflavone selected are daidzein and daidzin extracted from soybean.
3. A method as claimed in claim 1, wherein the cell lines used were selected from the group consisting of PC-3 Prostrate Cancer cell line, and MCF-7 Breast Cancer cell line.
- 15 4. A method as claimed in claim land 2, wherein the NF-KappaB inhibition is used to treat diseases selected from the group comprising of Breast Cancer, Prostate Cancer and Diabetes.
5. A method as claimed in claim 1-4, wherein the effective amount of Daidzein used
is about 3-5µg /ml.
- 20 6. A method of treating human Prostrate cancer, Breast cancer, and Diabetes comprising administering orally to a subject in need thereof isoflavone selected from the group consisting of Diadzein and Diadzinin the range of 3-5pg /ml.
7. Use of Diadzein and Diadzin as an inhibitor of NF-kb gene expression.
- 25 8. Use of Diadzein and Diadzin for the treatment of human Prostrate cancer, Breast cancer, and Diabetes.

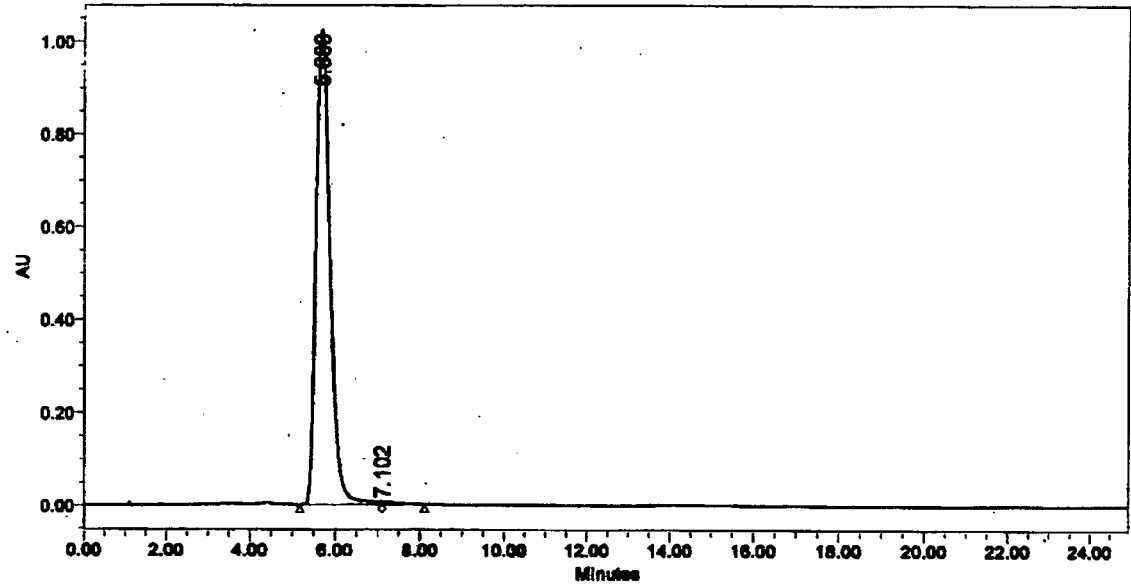


Figure 1

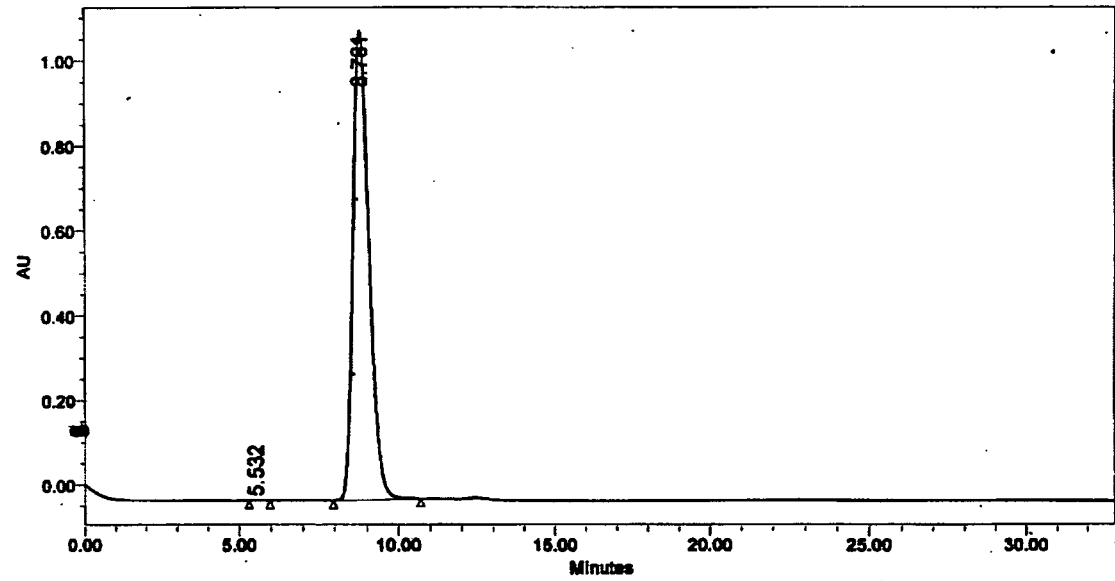


Figure 2

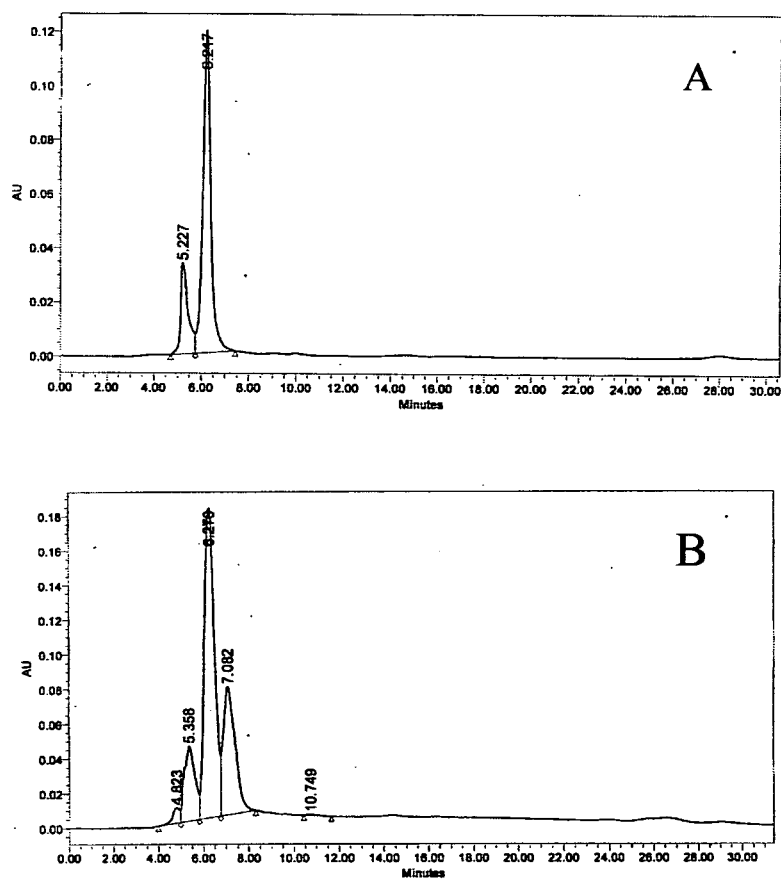
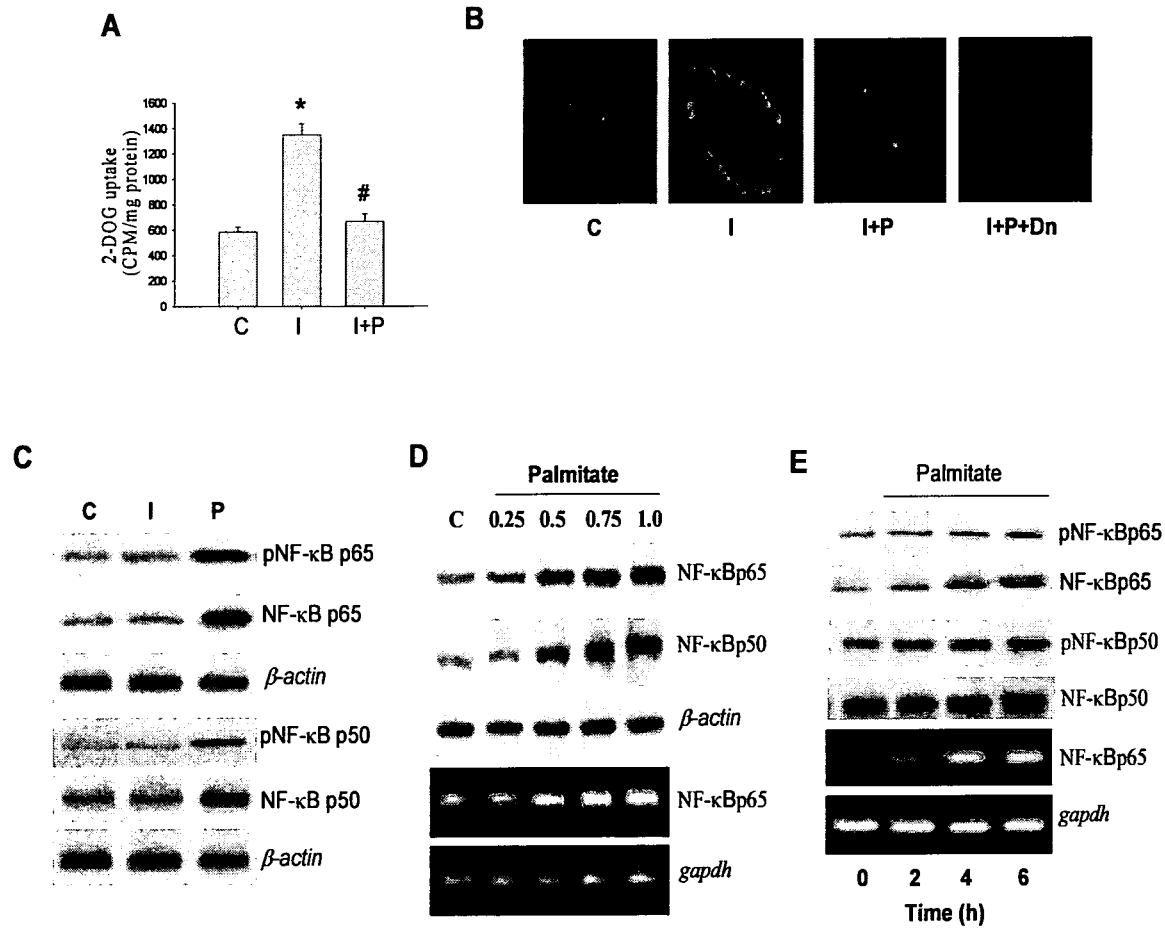


Figure 3



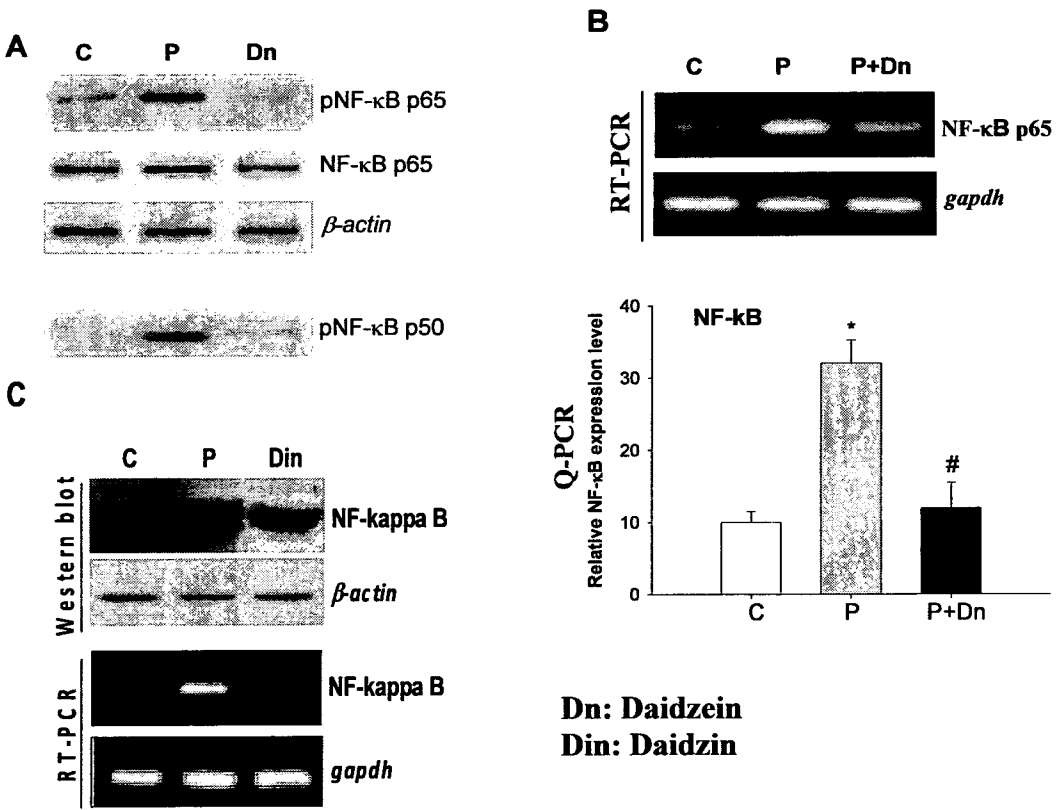


Figure 5

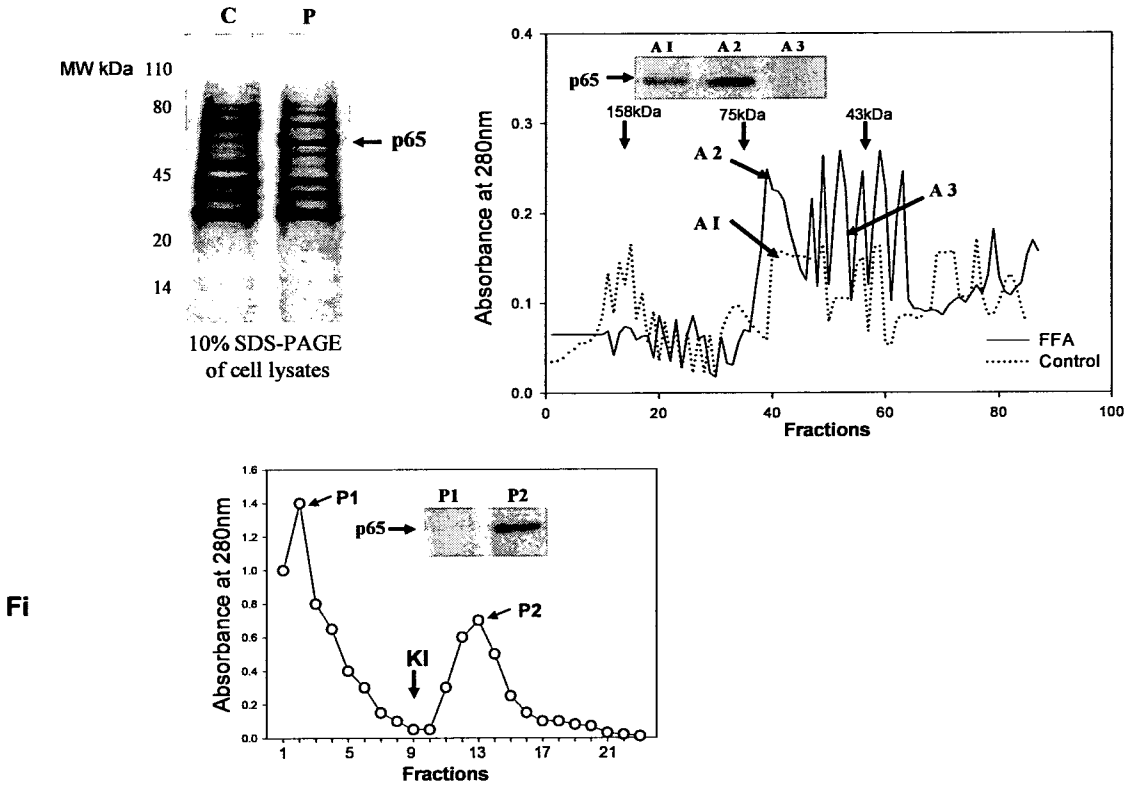


Figure 6

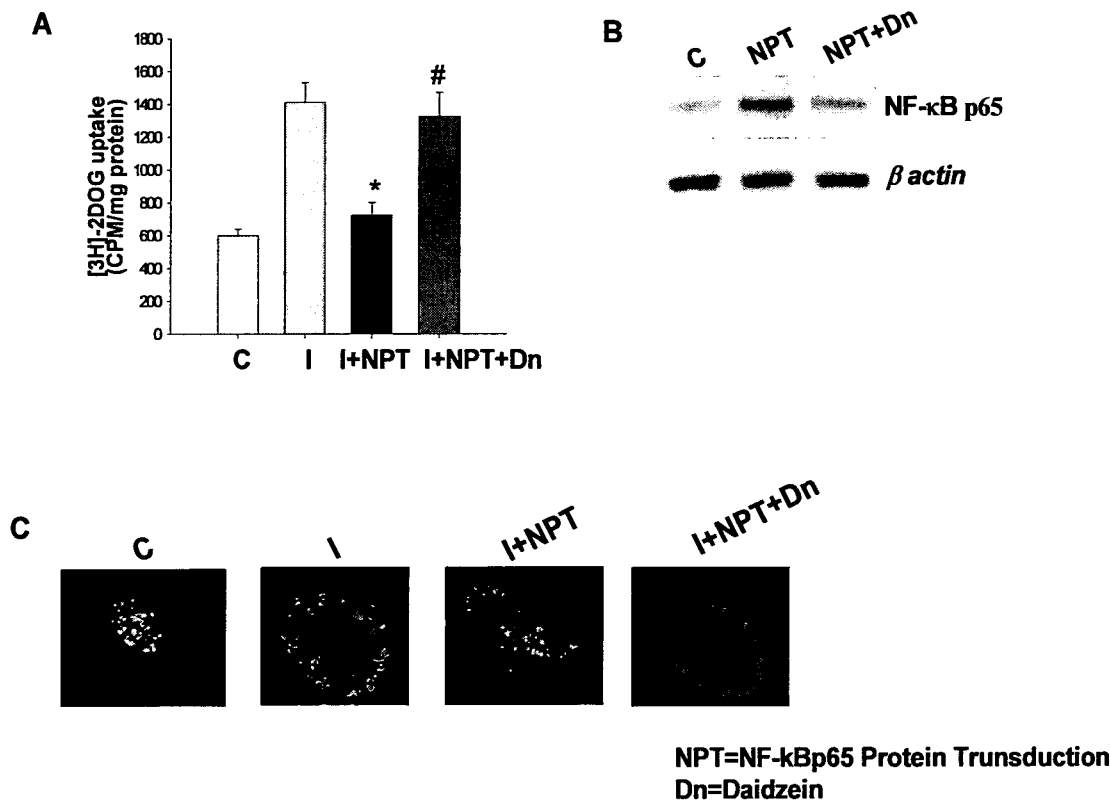
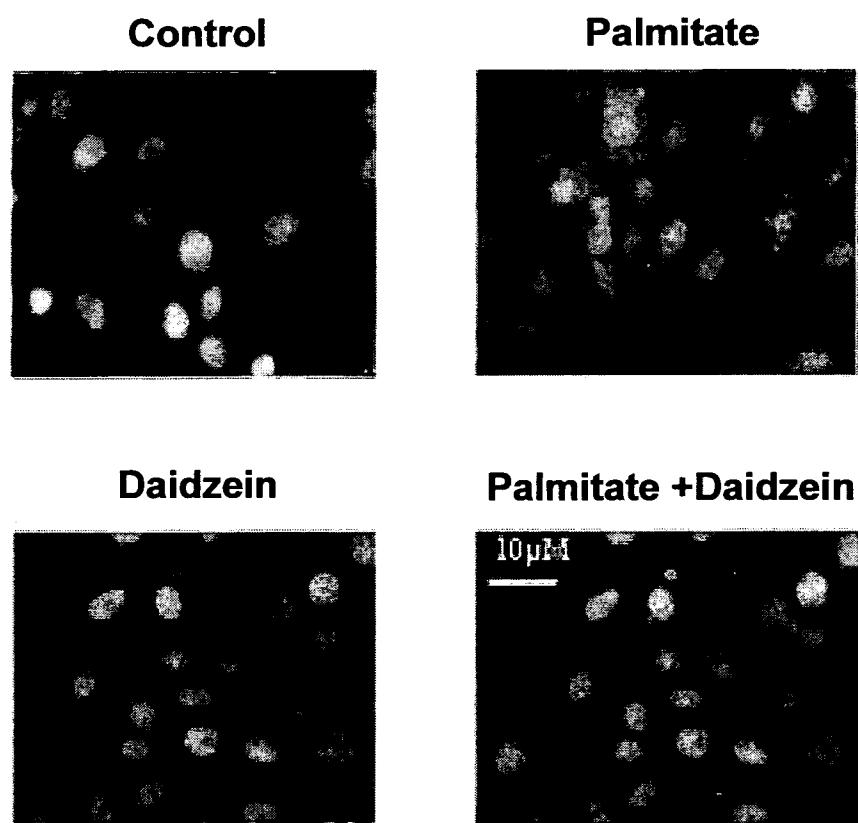


Figure7

**Figure 8**

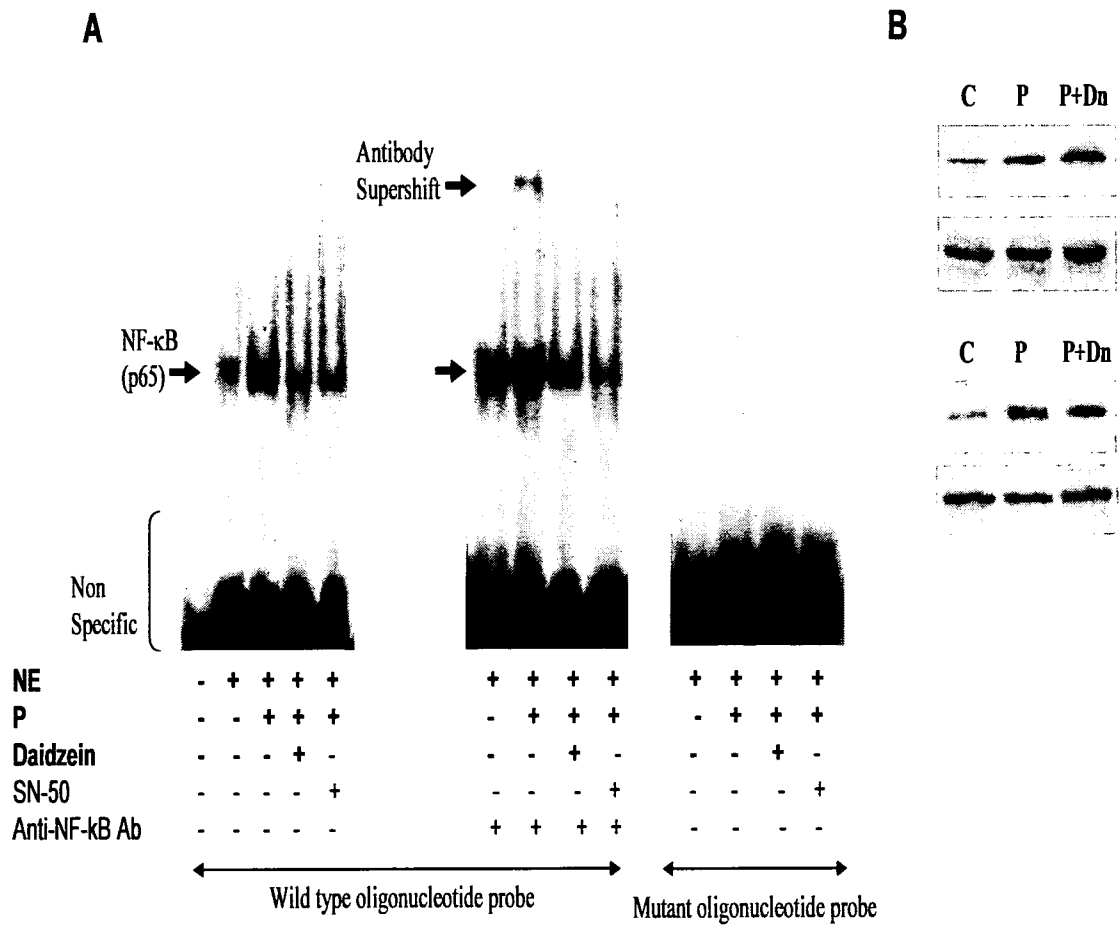


Figure 9

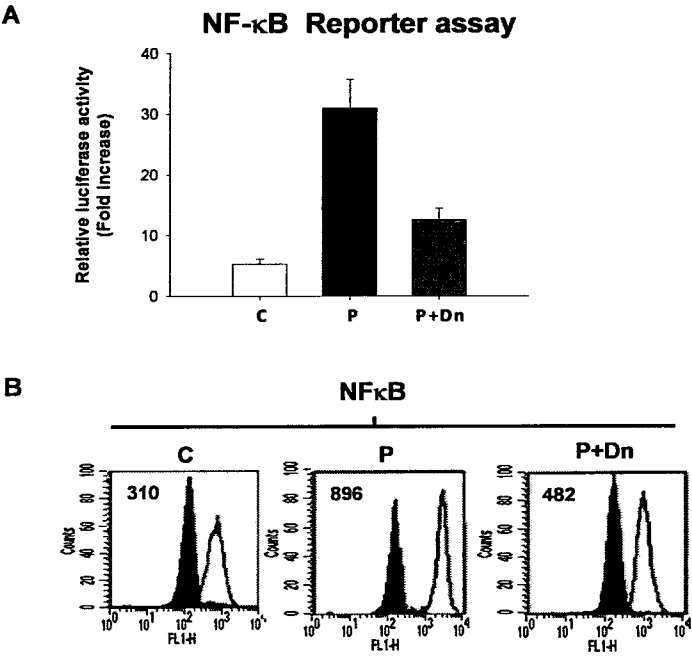


Figure 10

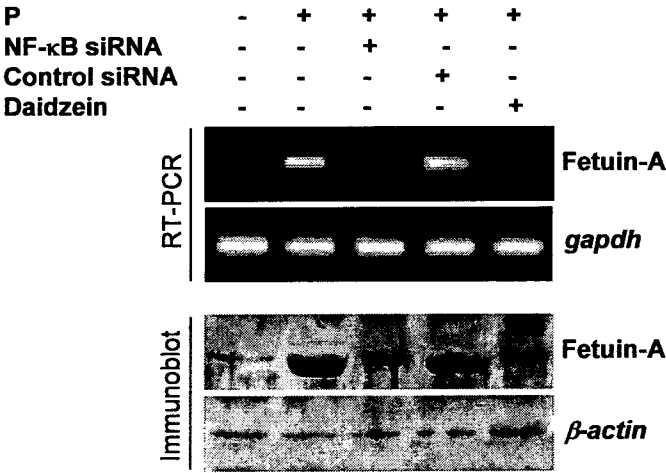


Figure 11

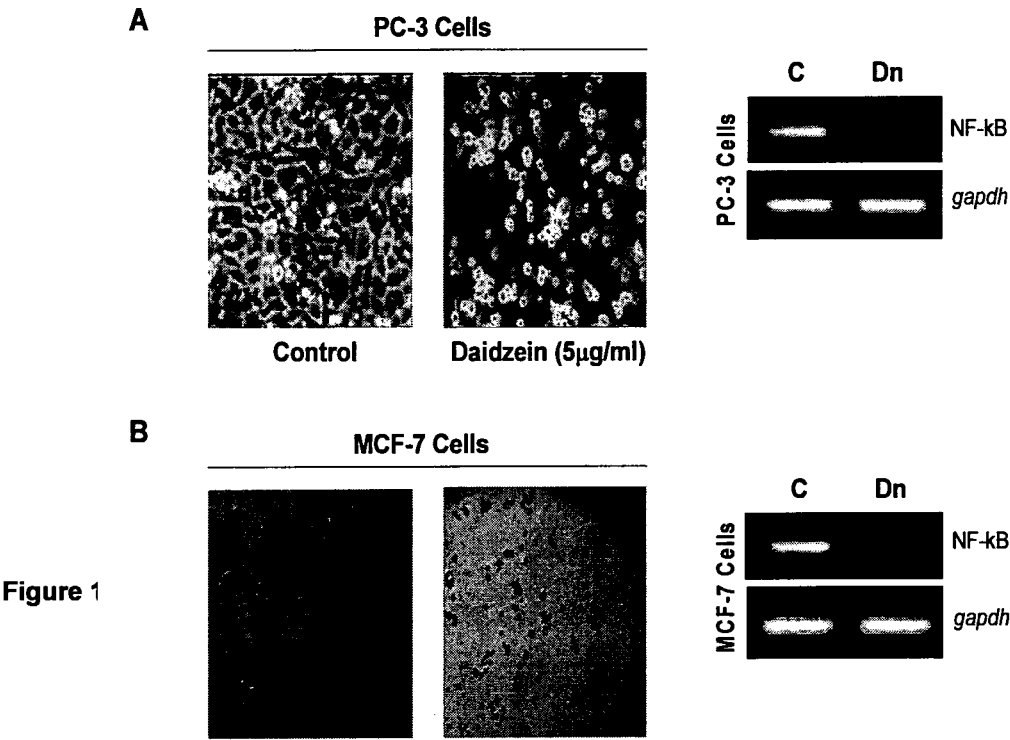


Figure 1

Figure :12

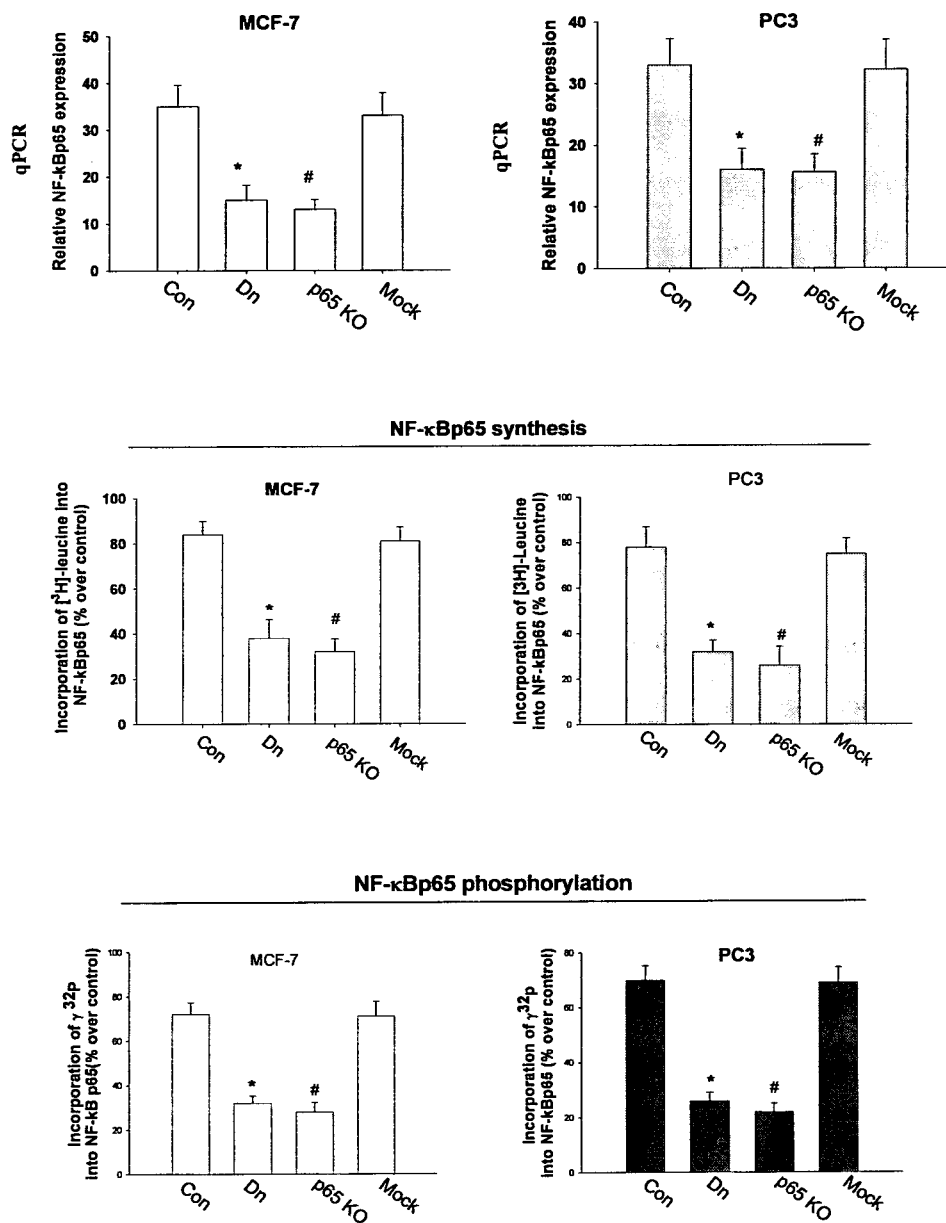
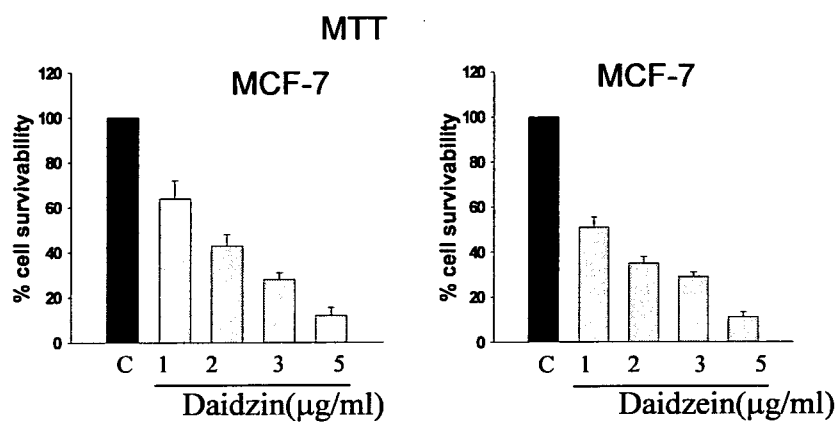
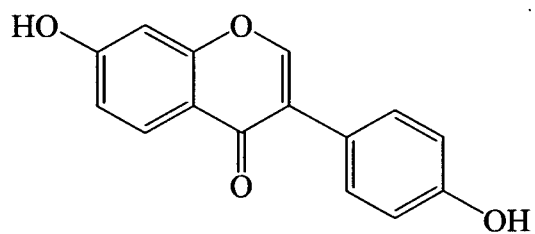
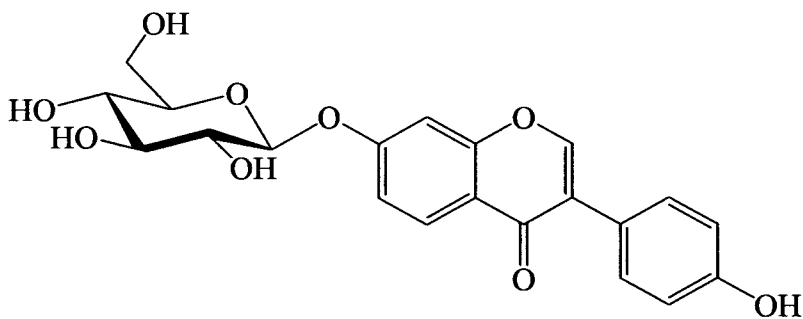


Figure 13

**Figure 14.**Structure of daidzein 1Structure of daidzin 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/001580

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/352 A61K31/7048 A61P35/G0 A61P3/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal , BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 127 572 A2 (BASF AG [DE]) 29 August 2001 (2001-08-29) abstract page 2, paragraph 4 - paragraph 5 table 4 page 6, line 29; example 3 page 8, paragraph 23; claims 1-3,5 -----	1,2,4-8
X	DATABASE WPI Week 200449 Thomson Scientific, London, GB; AN 2004-513205 XP002664508, & JP 2004 196720 A (NICHIMO KK) 15 July 2004 (2004-07-15) abstract ----- -/--	1,2,4-8



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

29 November 2011

Date of mailing of the international search report

06/12/2011

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Hoff, Philippe

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/001580

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 200382 Thomson Scientific, London, GB; AN 2003-885516 XP0Q2664509, 6 JP 2003 286166 A (NICHIMO KK) 7 October 2003 (2003-10-07) abstract -----	1,2,4-8
X	wo 93/23069 AI (KELLY GRAHAM EDMUND [AU]) 25 November 1993 (1993-11-25) abstract page 9, paragraph 2 page 15, last paragraph - page 16, paragraph 3; claims 1,4,6,10, 12,14, 18 -----	1,2,4-8
X	VALACHOVICOVA TATIANA ET AL: "Soy isoflavones suppress invasiveness of breast cancer cells by the inhibition of NF-κB/AP-1-dependent and -independent pathways", INTERNATIONAL JOURNAL OF ONCOLOGY, LYCHNIA, GR, vol. 25, no. 5, 1 January 2004 (2004-01-01), pages 1389-1395, XP008145541, ISSN: 1019-6439 abstract page 1390, left-hand column, paragraph 2; figure 5; table I -----	1,2,4-8
X	ALONSO V ET AL: "Phytoestrogen modulation of bone-related cytokines and its impact on cell viability in human prostate cancer cells", LIFE SCIENCES, PERGAMON PRESS, OXFORD, GB, vol. 85, no. 11-12, 9 September 2009 (2009-09-09), pages 421-430, XP026564270, ISSN: 0024-3205, DOI: 10.1016/j.lfs.2009.07.005 [retrieved on 2009-07-23] abstract figure 3 page 426, left-hand column, paragraph 1 ----- -/--	1-8

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/001580

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JIN S ET AL: "Daidzein induces MCF-7 breast cancer cell apoptosis via the mitochondrial pathway. ", ANNALS OF ONCOLOGY : OFFICIAL JOURNAL OF THE EUROPEAN SOCIETY FOR MEDICAL ONCOLOGY / ESMO FEB 2010 LNKD- PUBMED: 19889614, vol . 21, no. 2, February 2010 (2010-02) , pages 263-268, XP002664510, ISSN: 1569-8041 the whole document -----	1-8
X	CHOI EUN JEONG ET AL: "Daidzein causes cell cycle arrest at the G1 and G2/M phases in human breast cancer MCF-7 and MDA-MB-453 cells", PHYTOMEDICINE (JENA) , vol . 15, no. 9, September 2008 (2008-09) , pages 683-690, XP002664511, ISSN: 0944-7113 the whole document -----	1-8
X	SINGH AJITA V ET AL: "Soy phytochemicals prevent orthotopic growth and metastasis of bladder cancer in mice by alterations of cancer cell proliferation and apoptosis and tumor angiogenesis", CANCER RESEARCH, vol . 66, no. 3, February 2006 (2006-02) , pages 1851-1858, XPQ02664512 , ISSN: 0008-5472 abstract page 1852, left-hand column, paragraph 3 page 1855, right-hand column, paragraph 3; figure 5 page 1857, right-hand column, paragraph 2 - paragraph 3 -----	1,2,7
X, P	KARIEB SAHAR ET AL: "Phytoestrogens Directly Inhibit INF-alpha-Induced Bone Resorption in RAW264.7 Cells by Suppressing c-fos-Induced NFATc1 Expression" , JOURNAL OF CELLULAR BIOCHEMISTRY, vol . 112, no. 2, February 2011 (2011-02) , pages 476-487 , XP002664513, abstract; figure 6 -----	1,2,7

INTERNATIONAL SEARCH REPORT

Information on patent Family members

International application No

PCT/IB2011/001580

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 1127572	A2	29-08-2001	CN	13 1837 1 A	24- 10-2001
			EP	1127572 A2	29-08-2001
			JP	2001233768 A	28-08-2001

JP 2G04196720	A	15-07 -2004	NON E		

JP 2003286166	A	07- 10-2003	NON E		

wo 9323069	AI	25- 11- 1993	AT	275959 T	15- 10-2004
			AU	683838 B2	27- 11- 1997
			AU	4052593 A	13- 12- 1993
			BR	9306393 A	15-09- 1998
			CA	2 136233 AI	25- 11- 1993
			DE	69333624 DI	21- 10-2004
			DE	69333624 T2	15-09-2005
			EP	0656786 AI	14-06- 1995
			EP	1477 178 A2	17- 11-2004
			ES	2225822 T3	16-03-2005
			JP	H07506822 A	27-07- 1995
			JP	2004002304 A	08-0 1-2004
			NO	944435 A	18- 11- 1994
			NZ	25205 1 A	28- 10- 1996
			PT	656786 E	30- 11-2004
			US	RE40792 EI	23-06-2009
			US	5830887 A	03- 11- 1998
			US	6497906 BI	24- 12-2002
			US	6562380 BI	13-05-2003
			US	66422 12 BI	04- 11-2003
			US	20030782 14 AI	24-04-2003
			US	20040488 12 AI	11-03-2004
			US	2004 10656 1 AI	03-06-2004
			US	2004 1707 13 AI	02-09-2004
			US	2007 179099 AI	02-08-2007
			wo	9323069 AI	25- 11- 1993
