WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 4, Issue 2, 524-548. Research Article ISSN 2277– 7105

EVALUATION OF NARINGENIN ROLE IN INHIBITON OF LUNG TUMOR PROGRESSION IN MICE

Vishnu Varthan Jagannathan Vaithiyalingam*[1], Sathish kumar Natarajan Muthureddy[1], and Srividya Ammayappan Rajam[2]

[1]Department of Pharmacology, JSS College of Pharmacy, Ooty, JSS University, Mysore.
[2]Department of Pharmaceutical biotechnology, JSS college of Pharmacy, Ooty, JSS University Mysore.

Article Received on
11 Nov 2014,

Revised on 06 Dec 2014,
Accepted on 31 Dec 2014

***Correspondence for Author**
Vishnu Varthan Jagannathan Vaithiyalingam
Department of Pharmacology, JSS College of Pharmacy, Ooty, JSS University, Mysore.

ABSTRACT

Naringenin, aglycone flavonoid possess certain activities like anti-oxidiant, anti-viral, anti- microbial, anti-allergic, anti-estrogenic, anti-diabetic, cardioprotective, anti-obesity, anti-inflammatory, hepatoprotective and also have anti-cancer characteristics like carcinogenic inactivation, cell cycle arrest, anti-proliferation, apoptosis, anti-angiogenesis and enhances anti-oxidant activity. The inhibitory effect of Naringenin in lung tumor progression estimated with adenocarcinoma (A549) cell lines *(in vitro)* and C57BL/6 mice *(in vivo)* in a tri-dose manner compared with standard chemotherapy drug cisplatin. The results of the present study revealed, a dose-dependent activity in Naringenin and combination with cisplatin at a higher dose which showed decreased tumor progression in mice. Combination of Naringenin with cispaltin demonstrate beneficiaryaction than individual action. In vitro studies carried out for estimation of cell survivial and Nitric Oxide (NO) level shows dose dependent action of nringenin with IC_{50} value of 42µg/ml. In vivo studies was carried out in C57BL/6 mice. Naringenin satisfied the condition of an anti-cancer molecule with its characteristics in fragmentation assay, Zymography assay, anti-oxidant and myeloperoxidase studies, than cispaltin which failed in anti-oxidant and myeloperoxidase effect. Both in vitro and in vivo establishes dose dependent decrease in NO levels. But whereas Naringenin showed adverse results in Matrix Metalloproteinase (MMP) enzymatic levels with increase in dose levels. In conclusion Naringenin could suppress the lung tumor

progression when given individually and also in combinatorial with standard chemotherapy drug.

KEYWORDS: lung cancer, naringenin, cisplatin, Matrix metalloproteinase, myeloperosidase, Nitric oxide.

INTRODUCTION

Lung cancer is one of the leading causes of cancer death in world. During carcinogenesis, DNA will get affected by multiple mechanisms, a general mechanism by which DNA gets affect by electrophilic substitution in base pairs, these effects reflects during DNA synthesis and DNA replication.[1] Lung cancer usually progressed through three steps (i) Exposure of carcinogens leads to cellular adaptation in lung tissue, i.e.)., in general healthy person are lined with *columnar epithelium cells*, whereas smokers are lined with *simple cuboidal epithelial cells*, and this irreversible cellular adaptation leads to altered cellular functions. (ii) Carcinogenic agents crusades DNA damage by altering base pair either of the following process like oxidation, reduction, mispairing, alkylation of pairs which inturns alters the genetic expression (initation) of the cells, this altered genetic expression changes clonal expansion of cells (promotion) promotes a cell into preneoplastic lesions. (iii) Formation of preneoplastic lesions develops into a tumor cells and acts in its own pathway like altering signal transduction pathway which directly link to proliferation and differentiation of cell. Due to altered signal transduction pathway tumor cells showed poor sensitivity towards growth inhibiting signals and break away the programmed cell death like apoptosis uncontrolled cellular replication.[2] It also enhances angiogenic activity & metastasis in the tumor progressing region. Outcome of the above process shows a genetic instability in tumor cells when compared with healthy cells.

Chemotherapy plays a significant role in treatment of cancer. It destroys tumor cells either by killing directly or indirectly, but also destroys the actively dividing cells like bone marrow, hair follicles.[3] Preferred route of chemotherapy drug is through intravenous route and no betterment was observed in addition of third agent to the double regimen. Many compounds which are in use today are derivatives of natural products. Flavonoids are a group of herbal constituents which consist of more than of 4000 polyphenolic compounds of plant origin, which plays a role in prevention of tumor progression like carcinogenic inactivation, cell cycle arrest, anti-proliferation, induces apoptosis, anti-angiogenesis enhances anti-oxidant activity.[4]

Naringenin is one of the naturally occurring flavonoid possess a wide range of pharmacological properties including anti-oxidant, anti-tumor, anti-atherogenic, anti-proliferative.[5] It promotes its action through protein kinase C (PKC) signaling pathway,[6] inhibits COX-2 pathway,[7] EGFR pathway (Ref) and also increases intracellular gap junction between communication of two cells.[8] Present study was aimed to evaluate the role of naringenin in lung tumor.

MATERIALS AND METHODS

Materials

Naringenin, Di-methyl sulphoxide (DMSO), 3(4,5-dimethylthiazol-2-yl) 2,5- diphenyl Tetrazolium (MTT), Griess reagent, Protease from Streptomyces griseus, Sigma Aldrich Bangalore, India; Fetal bovine serum (FBS), PAA laboratories Mumbai, India,Giem'sa stain, Ethidium bromide AR 98%, Methyl green, Xylene-Cyanol and Ehrlichs solution, Sodium dodecyl sulphate (SDS), Bromo phenol blue, Triton X-100, Brij-35,Loba Chemicals Mumbai, India; Acridine orange, Tris-base, HI-Media laboratory Mumbai, India; Coomasie blue G-250 (Brilliant blue G), SD Fine Chemicals Hyderabad, India.

Animal

8 weeks old adult C57BL/6 mice were obtained from Sri Venkateswara enterprises, Bangalore and maintained in Central Animal House, J.S.S.College of Pharmacy, Ootacamund. The animals were housed under the laboratory conditions (relative humidity 85 ± 2%, temperature 22 ± 1ºC and 12 h light and dark cycle). They were fed with standard rodent pellet diet (Gold Mohar, Lipton –India, Ltd.) and purified water *adlibitum*. The study was approved by the institutional animal ethics committee for animal care and use (JSSCP/IAEC/M.Pharm/ Ph.cology /03/2013-14).

Cell culture

Human lung carcinoma (A549) cell lines were purchased from vellore institute of technology department of virology (VIT; vellore). Cells were cultured with RPMI1640 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin. All cells were incubated at 37ºC with 5% CO_2 in an incubator chamber.

Cytotoxicity assay

Cytotoxicity was assessed by MTT method which was performed by 1 x 10^4 cells were plated in 96 well plates with Ham's F12 medium containing 10% FBS.[9] The cells were incubated

for 12 hours with 5% CO_2, 95% O_2 at 37ºC. FBS was removed from Ham's F12 medium and replaced with serum free medium (SFM) containing 1% BAS for 24 hours. After BSA was removed control plates received DMSO and treatment plates received with 10, 20, 30, 40 and 50µg of naringenin containing Ham's F12 medium. After 24 hours100µl of 0.5% MTT was added to the plates and then incubated at 37ºC for 24 hour. After incubation colour developed was read at 650nm in ELISA reader. The percentage of growth inhibition was calculated and then plotted on a graph.

Measurement of Nitric Oxide (NO) levels

A549 cell lines were cultured 1 x 10^4 cells plated in 96 well plates with Ham's F12 medium containing 10% FBS. The cells were incubated for 12 hour with 5% CO_2, 95% O_2 at 37ºC. FBS was removed from Ham's F12 medium and replaced with serum free medium (SFM) containing 1% BSA for 24 hour incubated for 37ºC for 24 hour.[10] After BSA was removed control plates received DMSO and treatment plates received with 100, 50, 25, 12.5, 6.25, 3.125 µg/ml of naringenin and cisplatin respectively. Combination of naringenin with cisplatin dose same as in *in vivo* doses (100:3.5, 50:1.75, 25:0.875, 12.5:0.437, 6.25:0.218, 3.1:0.109) containing Ham's F12 medium. After treatment culture medium was removed and mixed with equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylene diamine hydrochloride, 2% phosphoric acid) and incubated for 10min at room temperature. The absorbance was measured at 550nm in ELISA reader. Sodium nitrite ($NaNO_2$) was used for the standard curve of nitrite concentration. Where the combination of naringenin and cisplatin dose were selected to determine the *in vitro – in vivo* correlation (IVIVC).

***In vivo* anti-tumor activity**

Induction of lung tumor

Lung tumor was induced by subcutaneous injection with A549 cell lines (5 x 10^6 concentrations) at the right flank region.[11] Tumor growth was measured with digital vernier caliper once weekly and tumor growth volume is calculated every week by the formula given below. The animals which reach tumor size mean value above 100mm^3 were selected and grouped for the study; animals which did not reach required tumor size were excluded from the study.

$$\text{Tumor growth volume} = \left(\frac{[(\text{width})^2 \text{ x length}]}{2} \right)$$

After 30 days of tumor induction treatment progress were started. Naringenin was prepared in 0.5% Carboxyl Methyl Cellulose Sodium (CMC-Na) (0.5g of CMC-Na dissolved in 100ml of water) at a dose of 100mg/kg, 150mg/kg, and 200mg/kg of body weight ([5]), which was prepared by dissolving 100mg, 150mg, 200mg of naringenin in 10ml of 0.5% CMC-Na respectively. Cisplatin (7mg/kg) prepared by dissolving 7mg in 10ml of 0.5% CMC-Na and administrated. Drugs were freshly prepared and administrated in order to prevent degradation.

Measurement of tumor growth volume

Tumor growth volume was measured with digital vernier calipers once in a week up to 30 days (for completion of tumor induction). The tumor growth volume was calculated by using the formula as given below. Treatment process begins according to the specified dose mentioned in table 2 and the tumor growth volume measured with digital vernier calipers once in a week till the completion of treatment period (31^{st} – 60^{th} day).

$$\text{Tumor growth volume} = \left(\frac{[(\text{width})^2 \text{ x length}]}{2} \right)$$

Lung tissue preparation

The animal protocol used in these studies is approved by Institutional Animal Ethics committees at J.S.S. College of Pharmacy, Ootacamund. C57BL/6 mice in which tumor has induced and after completion of treatment process the animals were sacrificed by an excess dose of ketamine (100mg/kg Body weight) and xylazine (10mg/kg body weight), were administrated intra-peritoneally. After scarification, the chest region was cut open, lungs were dissected and the tissue was stored at -80ºC for further estimation.

Zymography assay for MMP-2 and MMP-9

Gelatin zymography were performed by the following method: **Separating gel** 10.5% Sodium dodecyl sulphate (SDS) SDS-polyacrylamide with 2 mg/mL of gelatin (30% acrylamide & 0.8% bis-acrylamide (4.0 ml), 1.5 M Tris-HCL pH 8.8 (3.75 ml), 10% ammonium persulfate (0.05 ml), TEMED (0.01 ml), Gelatin 2mg/ml in 10% SDS (1.5 ml) and distilled water to reach final volume 15ml). **Stacking gel** 5% SDS-polyacrylamide (30% acrylamide & 0.8% bisacrylamide (0.65 ml), 1 M Tris-HCL pH 6.8 (1.25 ml), 10% ammonium persulfate (0.025 ml), TEMED (0.008 ml), 10% SDS (0.05ml) and distilled water (3ml) to reach final volume (5ml) were prepared. The addition of ammonium persulfate and

TEMED helps in polymerizing the gel. The gels were loaded with samples diluted in 1:1 non-reducing buffer (12.5% 0.5 M Tris–HCl pH 6.8, 10% glycerol, 4% SDS, and 0.05% bromophenol blue of final volume make up to 100ml) and the electrophoresis was carried out by applying a constant current of 35 mA at 90 V for approximately 5 h. After disassembling the apparatus, gels were washed 2 times in 2.5% triton- x 100 for 15 minutes and incubated on incubation buffer (Tris–HCl 50 mM, $CaCl_2$ 10 mM, NaCl 50 mM, pH 7.6) for 18 hours at 37^o C, then it is stained by 0.1% Coomassie brilliant blue R-250 solution (with 40% methanol and 10% acetic acid) for 4 h under gentle shaking and destained for 20 minutes with 25% ethanol and 8% acetic acid solution followed by 2.5% ethanol for 3 minutes and gels were visualized against white background for MMP bands which appears as blue colour bands.[12]

Fragmentation assay

DNA was isolated from the lung tissue by Phenol-Chloroform method. In a mortar and pestle 0.5gm of freshly excised tissue was blended until the tissue is ground to powder. Add approximately 10 volumes (w/v) of Lysis buffer and incubated for 1 hour at 37ºC. Proteinase K of final concentration 100µg/ml was prepared from 20 mg/ml which was added to the above solution and incubated in water bath for 3 hours at 50ºC. Equal volume of phenol-chloroform were added and two phases were gently mixed. Two phases were separated by centrifuging at 6000 rpm (rotations per minute) for 15 minutes. Aqueous phase was separated and centrifuge at 13000 rpm for 15 minutes. 0.2ml of 10M ammonium acetate and 2ml of ethanol were added to the pooled aqueous phase. DNA would immediately forms a precipitate. 1ml of TE buffer was added and store at 4ºC. The concentration of DNA was measured by the UV absorbance spectrophotometer. The amount of UV radiations absorbed by DNA stored in TE buffer is directly proportional to the quantity of DNA in the sample. By measuring the absorbance at 260 nm and 280 nm their ration A_{260}/A_{280} reflects the purity of DNA, (A260/A280) was 1.8. When the ratio is less than 1.8 it indicates that the preparation was contaminated either with proteins or with phenol. A standard of known DNA concentration was prepared and the readings were measured by UV spectrophotometer at 260 nm. As the DNA concentration increases, the Optical Density (OD) value also increases.[13]

Fragmentation assay was performed by gel electrophoresis method. The gel was prepared by agarose of 1% concentration in TAE buffer and 5µl of ethidium bromide were added. Pour the solution in gel plate and cool it, Load the DNA samples in the gel and submerge in TAE

buffer. Electrophoresis was carried out at 50mA for 2-3 hours and gel was viewed under UV transilluminator.[14]

Anti-oxidant assay

Assay of superoxide dismutase (SOD)

To 1 ml of tissue homogenate, 0.25 ml of chloroform and 0.5 ml of ethanol were added and mixed vigorously with vortex mixer. This mixture was centrifuged at 1800 rpm (rotations per minute) for 6 minutes. 100 µl of supernatant was taken and transferred into a test tube. The volume was made up to 2.25 ml with phosphate buffer (pH 7.8). Then 0.2 ml of EDTA, 0.1 ml NBT (Nitro Blue Tetrazolium), were added to the mixture and the readings were taken at 560 nm. After taking the initial readings the tubes were kept for illumination inside the chamber for 15 min. After 15 min the tubes were taken out and again taken the OD value at 560 nm. The difference between initial and final reading was recorded. The specific activity of the enzyme was expressed as Units/gram protein.[15]

Nitric Oxide (NO) scavenging activity

Nitric oxide in the lung tissue was determined by incubating 100 µl tissue homogenate with equal amount of Griess reagent (one part of 0.1% N (1-naphthyl)-diamine dihydrochloride in distilled water and 1 part 1% sulphanilamide in 5% concentrated H_3PO_4) for 10 minutes at room temperature. Absorbance was measured at 540 nm and the amount of nitric oxide was calculated from the $NaNO_2$standard curve. The amount of nitric oxide was expressed in Units/milligram protein for tissue.[15]

Assay of Catalase (CAT)

0.9 ml phosphate buffer with 0.1 ml tissue homogenate/serum and 0.4 ml H_2O_2 were added. The reaction was arrested after 60 sec by adding 2.0 ml dichromate-acetic acid mixture (ratio 1:4). The tubes were kept in a boiling water bath for 10 minutes, cooled and the colour developed was read at 590 nm. The specific activity of the enzyme was expressed as Units/milligram protein for tissue.[15]

Myeloperoxidase (MPO) assay

The enzyme activity was assayed by homogenizing the tissue sample in 20ml phosphate buffer (pH 7.4) and centrifuge at 10000 rpm for 10 min. The resulting pellet was suspended in 50ml of phosphate buffer (pH 6.0) containing 0.5%w/v hexadecyltrimethyl ammonium bromide. Sample was then centrifuged at 10000 rpm for 5 min and supernated was used for

MPO activity. MPO assay solution was prepared by mixing 107.6ml of distilled water, 12ml of 0.1M sodium phosphate buffer(pH 7.0), 0.192ml of guiacol, 0.4ml 0.1M H_2O_2 to makeup the final volume 120 ml. 0.02ml of lung homogenate was mixed with 0.980ml of MPO assay solution. The generation of tetra guiacol was measured spectrophotometrically at 470nm at 0 minute and 15 minute and the values are expressed as Units/Gram of tissue[16] (John chen *et al*., 2008). The MPO activity is calculated by the below mentioned formula.

$$\text{MPO activity} = \left(\frac{\Delta OD * Vt * 4}{E * \Delta t * Vs} \right)$$

Where,

ΔOD= change in absorbance;

Vt = total volume;

Vs = sample volume;

E (extinction coefficient) = 26.6 mM^{-1};

Δt = change in time.

Statistical analysis

The results were expressed as mean ± SEM, statistical analysis was performed using one-way ANOVA, followed by a post hoc test. The statistical analysis was done by using Graph pad prism version 5.01, Graph Pad Software Inc..USA. P value <0.05 is considered as significant.

RESULTS

Naringenin exhibits cytotoxicity action against lung cancer cell line

From the previous studies Naringenin possess cytotoxicity action tested against brain tumor cell lines[9] (Vanisree *et al*., 2011), hepatocellular carcinoma cell lines[17] Arul duraikannu *et al*., 2013). To observe whether Naringenin exerts same action against lung cancer cell line, Naringenin were added at various concentrations (10µg/ml-50µg/ml) to the A549 cell lines (doses were selected based on the brain tumor studies) for 24 hour observation (Table. 1). Naringenin established a dose dependent action, inhibits cell viability in lung cancer cell line. Its IC_{50} value was found to be 42µg/ml (Table 1,2 & Fig. 1).

Table 1 Cytotoxicity effect of naringenin in human adenocarcinoma (A549) cell lines by MTT assay.

S.NO	Negative control	Naringenin treatment 10µg/ml	Naringenin treatment 20µg/ml	Naringenin treatment 30µg/ml	Naringenin treatment 40µg/ml	Naringenin treatment 50µg/ml
1.	1.140	0.9502	0.917	0.889	0.854	0.774
2.	1.143	0.944	0.913	0.893	0.857	0.776
3.	1.147	0.947	0.918	0.893	0.855	0.784
4.	1.139	0.952	0.920	0.891	0.849	0.781
5.	1.142	0.956	0.918	0.897	0.852	0.789
6.	1.143	0.945	0.915	0.895	0.847	0.783
7.	1.145	0.955	0.911	0.894	0.852	0.784
8.	1.143	0.952	0.918	0.892	0.823	0.786
Mean ± SEM	1.142 ± 0.011	0.950 ± 0.015***	0.916 ± 0.010***	0.893 ± 0.010***	0.848 ± 0.013***	0.782 ± 0.017***

Values expressed as Mean ± SEM; n = 8, statistical significance. ***P<0.001 vs. negative control; one way ANOVA followed by Dennett's multiple comparison test.

Table 2 Percentage inhibition of cell growth with treatment of naringenin in human adenocarcinoma (A549) cell lines.

S.NO	Naringenin Concentration (µg/ml)	percentage inhibition of cell growth
1.	Negative control	0
2.	10	16.82
3.	20	28.31
4.	30	36.01
5.	40	48.89
6.	50	72.02

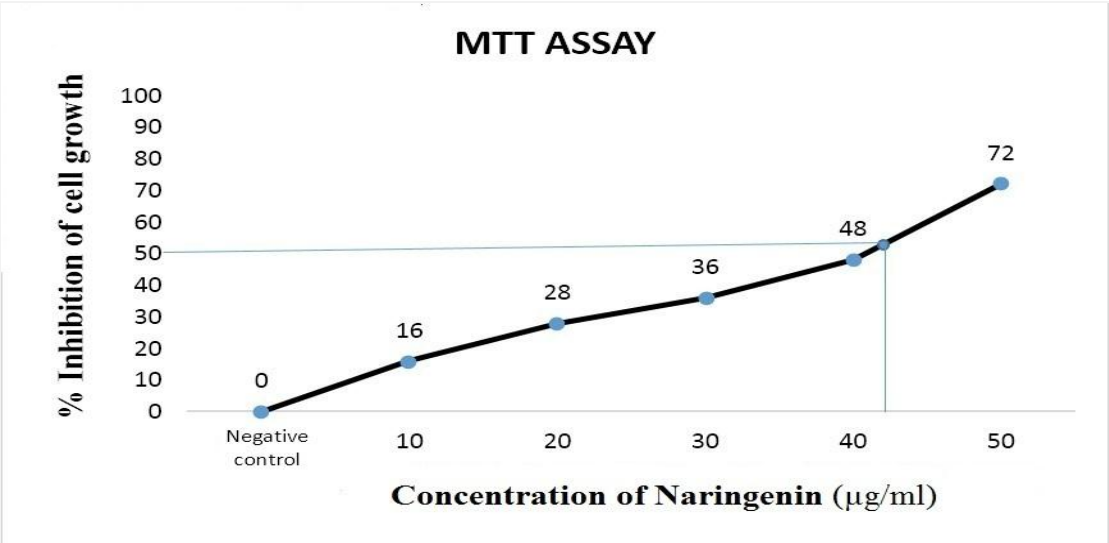


Figure 1- Percentage inhibition of cell growth with treatment of naringenin in human adenocarcinoma (A549) cell lines.

Effect of Naringenin in decreasing Nitric Oxide (NO) levels in A549 cell lines

As shown in Fig. 2 Naringenin demonstrated decreased NO levels in A549 cell lines. Dose dependent response was observed in Naringenin and along with that combination of cisplatin showed marked reduction in NO levels.

Naringenin exhibits therapeutic activity against lung tumor *in vivo*

To observe whether Naringenin exerts a therapeutic response against lung tumor in *in vivo* as in *in vitro*, solid tumor model was implanted in C57BL/6 mice by using A549 cell lines. Tumor growth volume was measured regularly and treatment process was initiated after the desired tumor growth volume attained. From the observations as shown in table no 3 & 4 and figure no 3 , Naringenin treated mice showed reduced tumor volume with respect to increase in dose concentration, whereas the combination of Naringenin 200mg/kg and cisplatin 7mg/kg results in significant decrease in tumor growth volume.

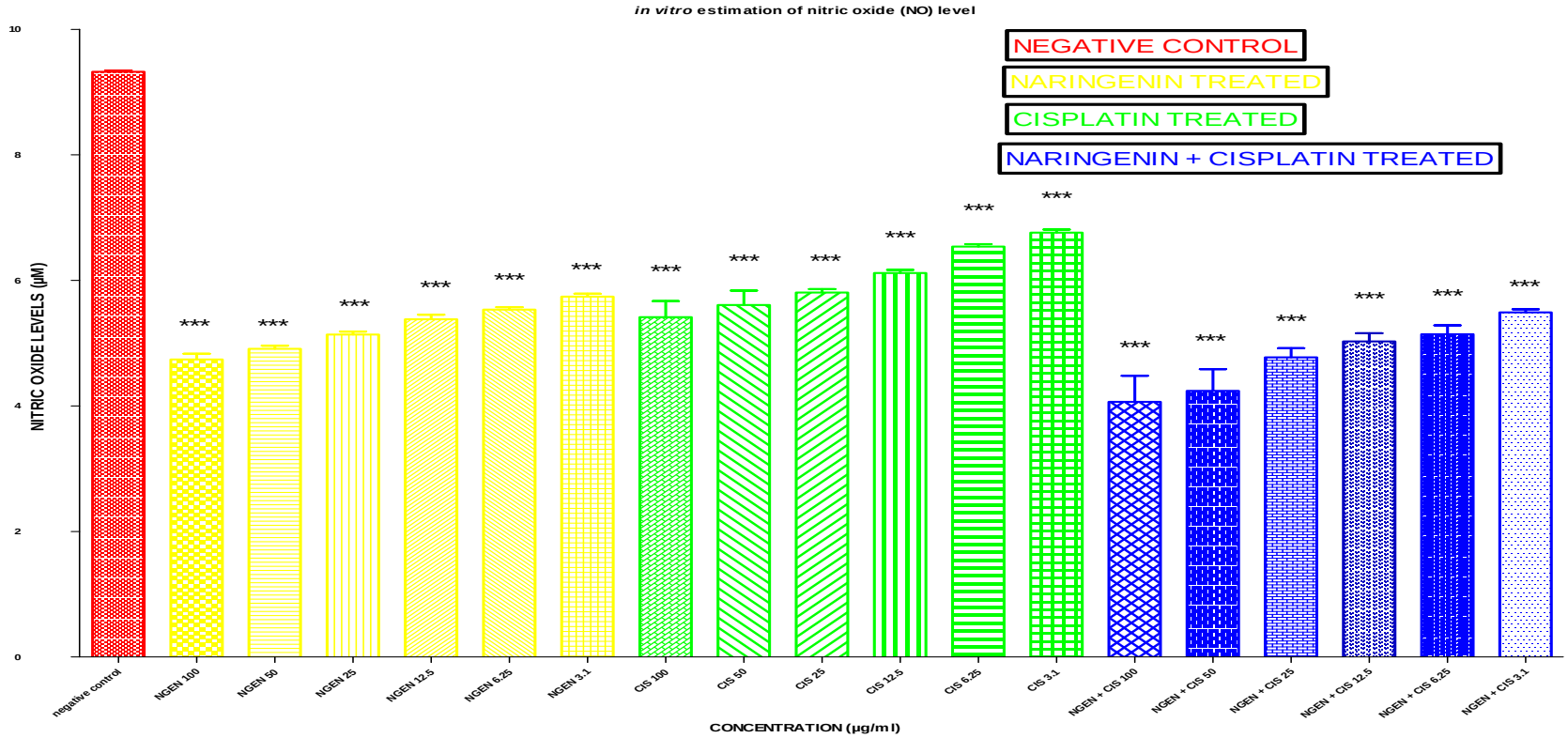


Figure- 2 Estimation of Nitric Oxide (NO) concentration on A549 cell lines with naringenin, cisplatin and combination of naringenin and cisplatin treatment.

Value expressed as mean ± SEM; n = 19, statistical significance. *P<0.05 vs. negative control; one way ANOVA followed by Dunnett's multiple comparison test.

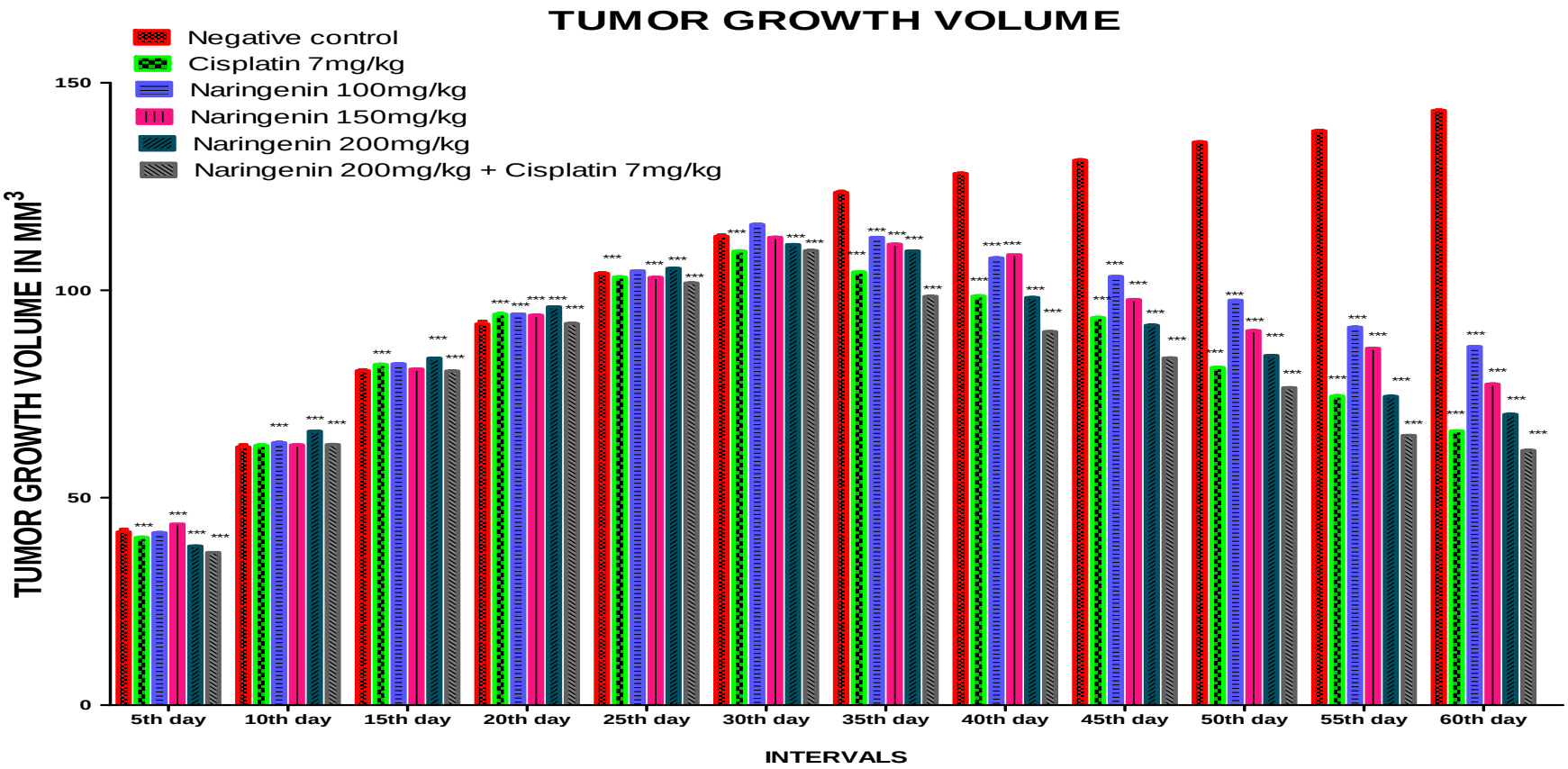


Figure 3 Evaluation of tumor growth volume in A549 cell lines induced lung tumor induced in C57BL/6 mice/

Value expressed as mean ± SEM; n = 6, statistical significance: ***P<0.001 vs. negative control; two way ANOVA followed by Bonferroni post comparison test.

Table- 3 Tumor growth volume measured in A549 cell lines induced lung tumor in C57BL/6 mice before treatment.

S.NO	Duration of tumor volume measure-ment	negative	Cisplatin 7mg/kg	Naringenin 100mg/kg	Naringenin 150mg/kg	Naringenin 200mg/kg	Naringenin 200mg/kg + cisplatin 7mg/kg
Before treatment							
1.	5th day	41.76 ± 0.61 ###	40.42 ± 0.09	41.55 ± 0.06	43.66 ± 0.01	38.33 ± 0.01***	36.80 ± 0.01***
2.	10th day	62.25 ± 0.52###	62.58 ± 0.19***	63.26 ± 0.09***	62.74 ± 0.01	66.05 ± 0.02***	62.82 ± 0.01
3.	15th day	80.61 ± 0.21###	82.09 ± 0.08***	82.26 ± 0.07***	81.02 ± 0.03***	83.63 ± 0.02***	80.52 ± 0.02
4.	20th day	91.87 ± 0.68###	94.30 ± 0.11***	94.27 ± 0.03***	94.03 ± 0.03***	96.01 ± 0.02***	92.01 ± 0.02
5.	25th day	104.08 ± 0.10###	103.19 ± 0.09***	104.68 ± 0.04***	103.10 ± 0.03***	105.33 ± 0.02***	101.74 ± 0.01***
6.	30th day	113.03 ± 0.32 ###	109.43 ± 0.10***	115.89 ± 0.11***	112.76 ± 0.01	111.02 ± 0.02***	109.58 ± 0.01***

Value expressed as mean ± SEM; n = 6, statistical significance: # P<0.05 vs. control. *P<0.05 vs. negative control; two way ANOVA followed by Bonferroni post comparison test.

Table- 4 Tumor growth volume measured in A549 cell lines induced lung tumor in C57BL/6 mice after treatment.

S.NO	Duration of tumor volume measure-ment	negative	Cisplatin 7mg/kg	Naringenin 100mg/kg	Naringenin 150mg/kg	Naringenin 200mg/kg	Naringenin 200mg/kg + cisplatin 7mg/kg
After treatment							
7.	35th day	123.58 ± 0.28 ###	104.40 ± 0.08***	112.74 ± 0.02***	111.09 ± 0.03***	109.47 ± 0.01***	98.53 ± 0.020***
8.	40th day	128.14 ± 0.09 ###	98.62 ± 0.08***	107.80 ± 0.02***	108.58 ± 0.02***	98.25 ± 0.02***	90.01 ± 0.02***
9.	45th day	131.34 ± 0.14 ###	93.40 ± 0.09***	103.35 ± 0.02***	97.75 ± 0.01***	91.57 ± 0.01***	83.67 ± 0.01***
10.	50th day	135.72 ± 0.07 ###	81.42 ± 0.05***	97.57 ± 0.03***	90.25 ± 0.01***	84.26 ± 0.01***	76.50 ± 0.01***
11.	55th day	138.45 ± 0.01 ###	74.52 ± 0.11***	91.07 ± 0.06***	86.03 ± 0.02***	74.45 ± 0.02***	65.00 ± 0.03***
12.	60th day	143.35 ± 0.09###	66.11 ± 0.02***	86.45 ± 0.05***	77.36 ± 0.04***	70.09 ± 0.03***	61.39± 0.03***

Value expressed as mean ± SEM; n = 6, statistical significance: # P<0.05 vs. control. *P<0.05 vs. negative control; two way ANOVA followed by Bonferroni post comparison test.

Naringenin action in expression of matrix metalloprotinease (MMP-2 & MMP-9) in lung tissue

As an investigative study, Naringenin action was evaluated in the expression of matrix metalloproteinase, which was visualized by Zymography technique Fig 4. Matrix metalloproteinase was highly expressed in untreated mice than with Naringenin treated mice and also cisplatin with a combination of Naringenin showed marked reduction in MMP expression than vehicle control mice.

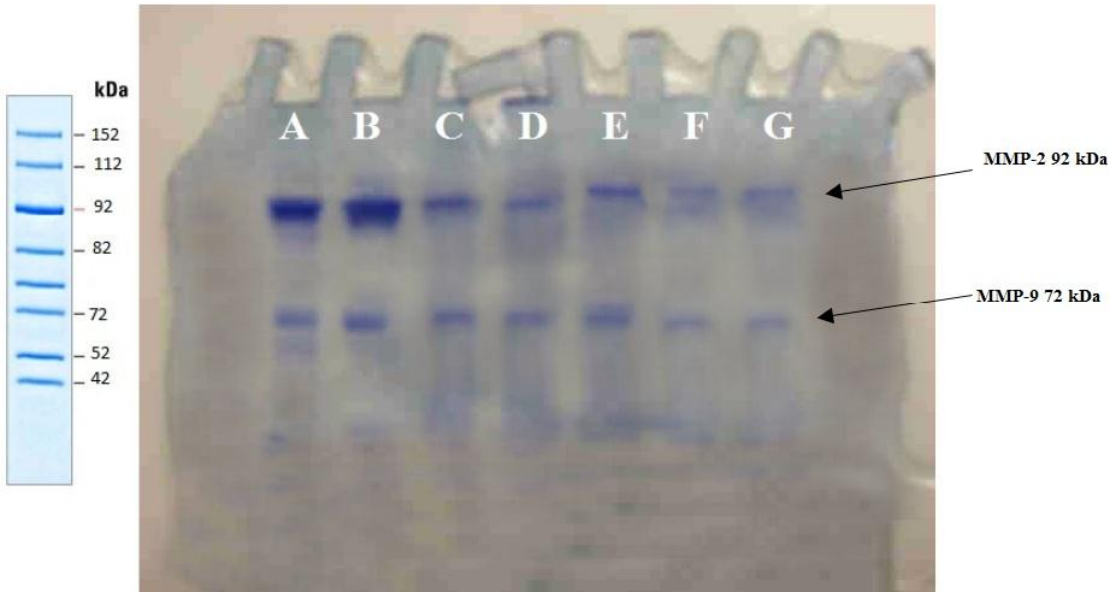


Figure 4. Estimation of Matrix Metalloproteinase expression (MMP – 2 and MMP - 9) in A549 cell lines induced lung tumor in C57BL/6 mice treated with naringenin and cisplatin.

Where A = Vehicle control, D = Naringenin 100mg/kg
B = Negative control E = Naringenin 150mg/kg
C = Cisplatin 7mg/kg F = Naringenin 200mg/kg
G = Naringenin 200mg/kg + Cisplatin 7mg/kg

Apoptosis action and DNA fragmentation assay by Naringenin

Fragmentation of DNA is key to assess for apoptosis, which was evaluated by gel electrophoresis. From the observations, Naringenin reflects similar effects of apoptosis which was observed in brain tumors Fig5. The combination of cisplatin with Naringenin evidences that combined action raised more chances than of fragmentation than when Naringenin was alone administrated.

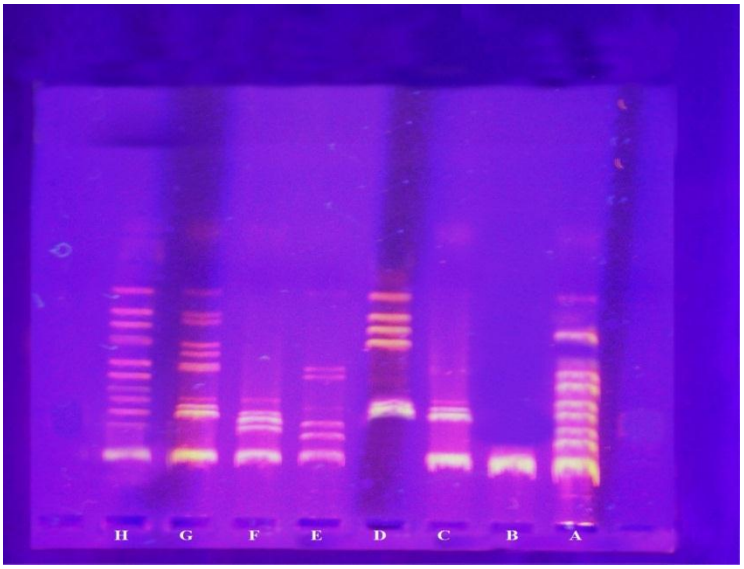


Figure 5 Detection of apoptosis by DNA fragmentation assay.

A = DNA Ladder (molecular weight 150 kDa) E = Naringenin 100mg/kg

B = Vehicle control F = Naringenin 150mg/kg

C = Negative control G = Naringenin 200mg/kg

D = Cisplatin 7mg/kg H = Naringenin 200mg/kg + Cisplatin 7mg/kg

Naringenin as antioxidant

From the previous studies, it was confirmed that Naringenin possess anti oxidant properties. Based on the observation's antioxidant properties in lung tumor were performed, Naringenin alone when administrated exhibits a significant increase in antioxidant levels of SOD in Fig 6, Catalase in Fig 7 and decrease in NO Fig 8. As depicted in picture Naringenin established a dose dependent action. Where as mechanism of action of cisplatin in combination of Naringenin demonstrated controversy result, that these molecule combinations won't succeed in enhancing anti oxidant levels.

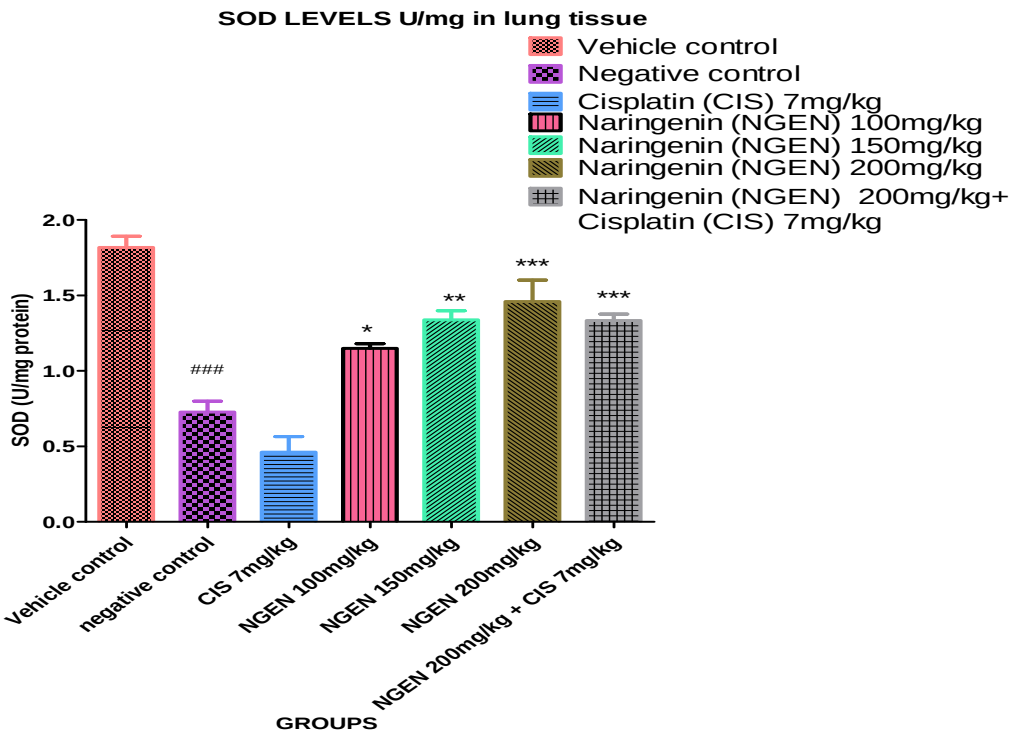


Figure 6 Estimation of Super Oxide Dismutase (SOD) levels in lung tissue homogenate.

Values expressed as mean ± SEM, n=6, statistical significance: ### P<0.001 vs. control. * P<0.05, ** P<0.01, *** P<0.001 vs. negative control; one way ANOVA followed by Dunnett's multiple comparison test. Route of administration: Oral.

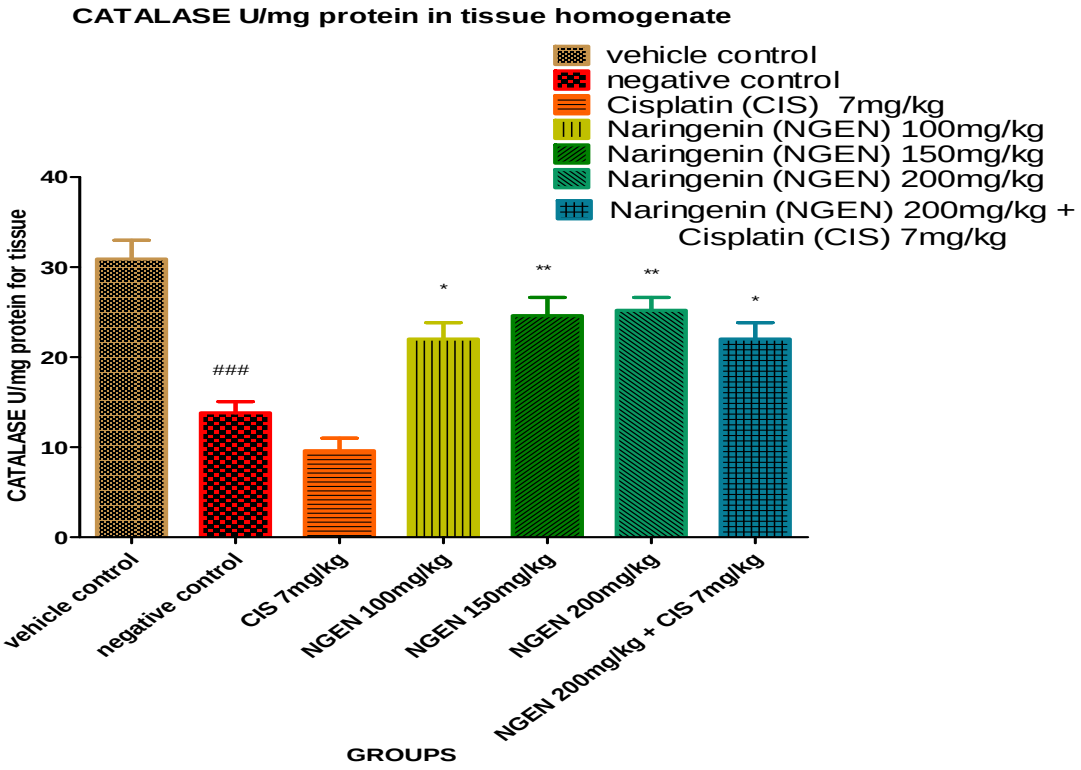


Figure 7 Estimation of Catalase level in lung tissue homogenate.

Values expressed as mean ± SEM, n=6, statistical significance: ### P<0.001 vs. control. * P<0.05, ** P<0.01 vs. negative control; one way ANOVA followed by Dunnett's multiple comparison test. Route of administration: Oral.

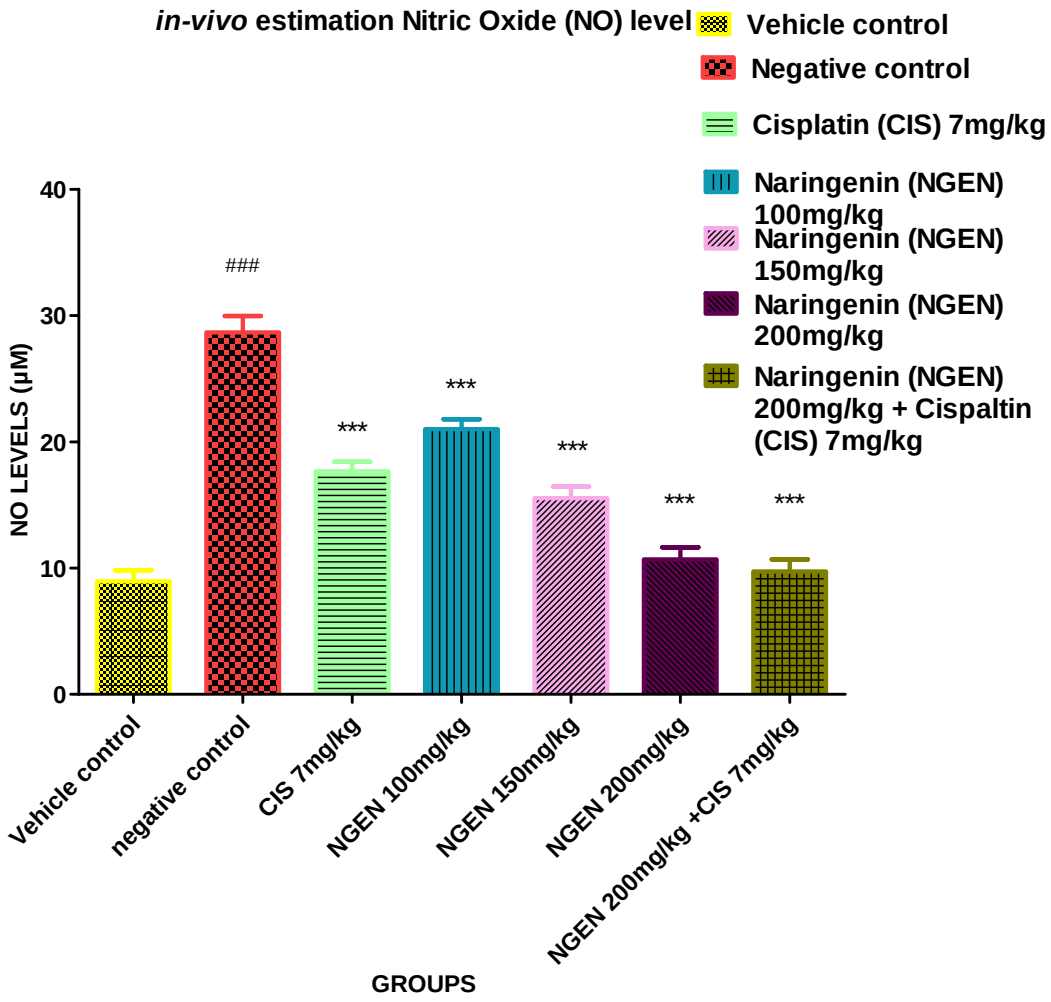


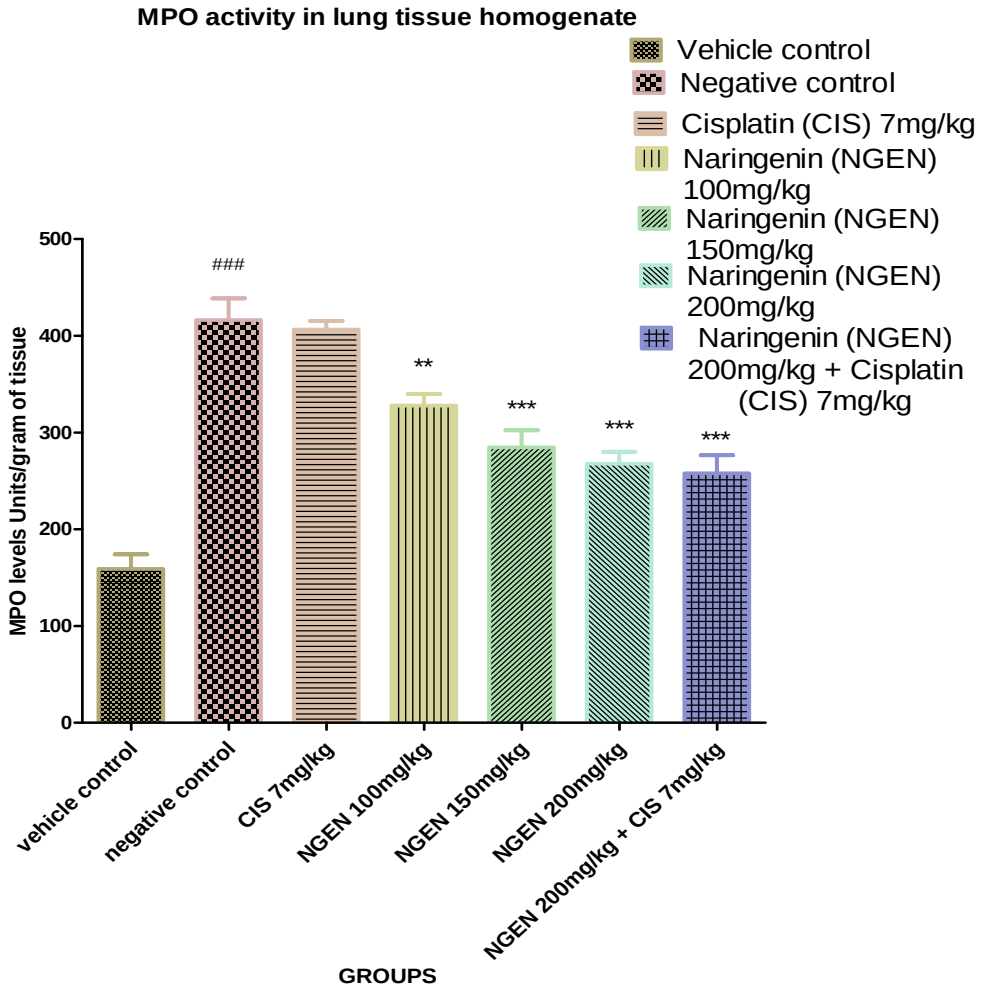
Figure 8 Estimation of Nitric Oxide levels in lung tissue homogenate.

Values expressed as mean ± SEM, n=6, statistical significance: ### P<0.001 vs. control. ***P<0.001 vs. negative control; one way ANOVA followed by Dunnett's multiple comparison test. Route of administration: Oral.

Naringenin action on myeloperoxidase in lung tissue

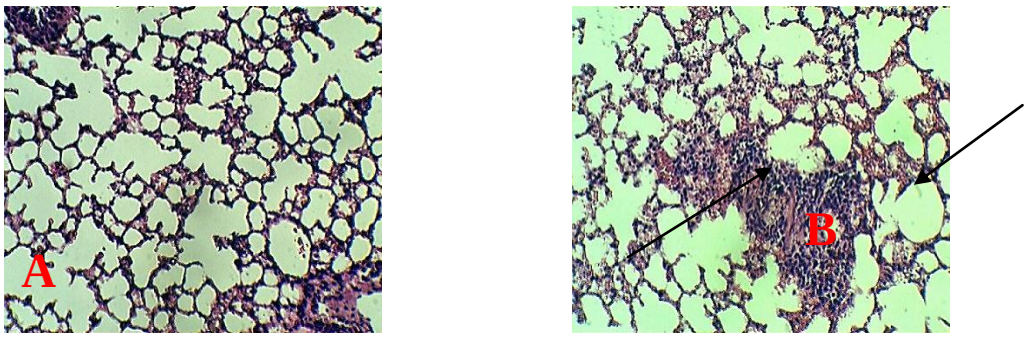
Based upon observation in Fig 9, Naringenin when administrated alone was found to capable in reduction of MPO levels with respect to that of dose, whereas cisplatin showed very less or no activity against MPO levels, also a combination of Naringenin with cisplaitn fails in reducing MPO levels.

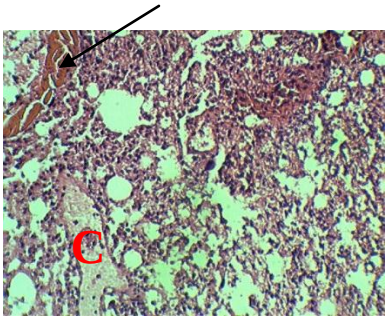
Figure 9 Estimation of Myeloperoxidase (MPO) levels in A549 cell lines induced lung tumor in C57BL/6 mice with naringenin and cisplatin treatment

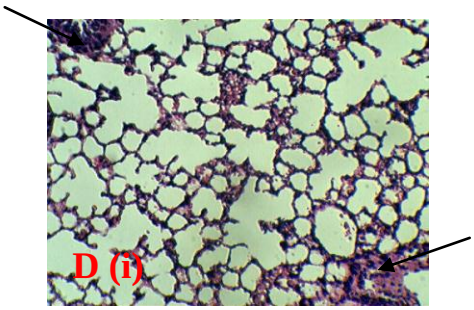


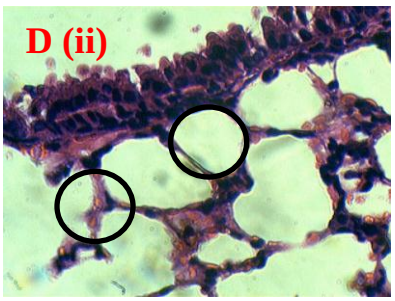
Values expressed as mean ± SEM, n=6, statistical significance: ### P<0.001 vs. control. ** P<0.01, *** P<0.001 vs. negative control; one way ANOVA followed by Dunnett's multiple comparison test. Route of administration: Oral.

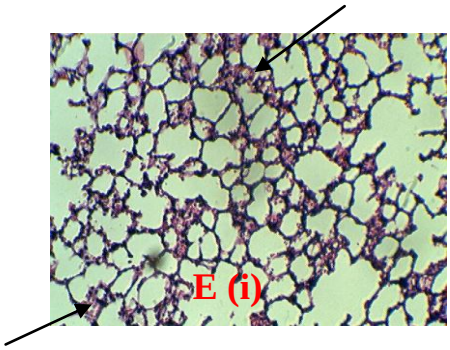
Histopathology

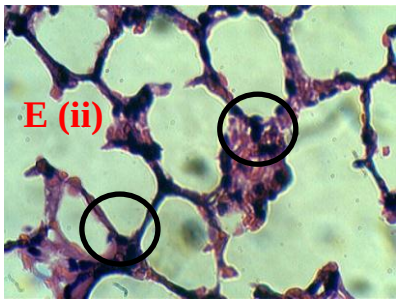


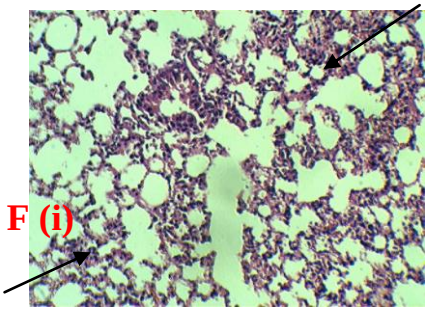


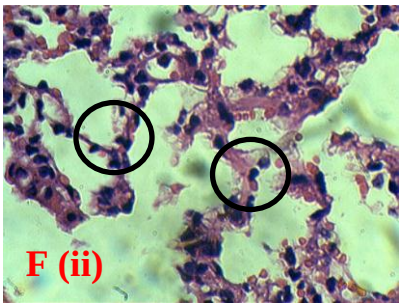


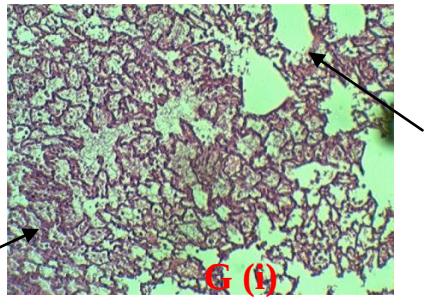


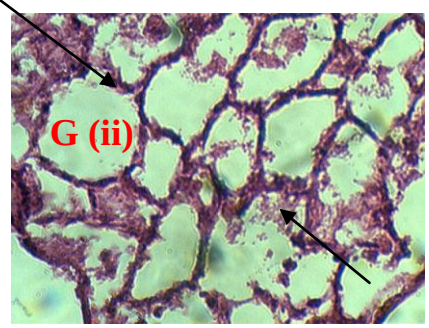












Based on the present study as depicted in the picture, A – 10 X represent a healthy lung tissue of mice. picture B – 10 X confirms the occurrence of tumor formation, whereas picture C –

40 X (cisplatin 7mg/kg), D (naringenin 100mg/kg) (i) 10 X& D (ii) 40 X, E(naringenin 150mg/kg) (i) 10 X& E (ii) 40 X,F(naringenin 200mg/kg) (i) 10 X& F(ii) 40 X, G (naringenin 200mg/kg + cisplatin 7mg/kg)(i) 10 X& G (ii) 40 X, showed decreased tumor presence with reduced nodule formation corresponds with dose of cisplatin and Naringenin. Where as in the combination of cisplatin with Naringenin showed marked reduction of the tumor, which was represented in picture G.

DISCUSSION

Naringenin a flavanone present in many citrus fruits belonging to the class of flavonoids exhibits a diverse pharmacological action including anti-inflammatory, antioxidant and anti-proliferative effect.[5] Naringenin promotes antitumor effects by several mechanism such as inhibition of COX-2 mediated cell proliferation,[7] cell cycle arrest at the G1phase, down regulation of the connexin expression[8] and upregulation of protein kinase C (PKC) signaling pathways associated in reduction of tumor progression.[6]

The present study was designed to evaluate the role of naringenin in inhibition of lung tumor progression. *In vitro* estimation was carried out to determine the cytotoxicity and nitric oxide level. The cytotoxicity effect of naringenin in A549 cell lines by MTT assay showed a dose dependent decrease in cell survival. TheIC_{50}value of naringenin was found to be 42µg/ml and percentage inhibition of cell growth was proportional to the concentration of naringenin. The cytotoxicity induced by naringenin may be due to cell cycle arrest at the G1 phase which was reported by Vanisree *et al*., (2011) for naringenin in C6 glioma cell lines for brain tumor study.

Nitric oxide (NO) plays an important role in tumor progression via inflammatory pathway. Among the treated groups combination of naringenin with cisplatin showed the marked reduction in NO levels. Reduction of NO levels may be due to antioxidant effect of naringenin by scavenging $NO^{\bullet}$radical[18] and also decreased NO levels by inhibiting nitrite production and iNOS expression which was reported by[19] for naringenin action in macrophages. In the *in vivo* studies, the tumor growth volume of the mice was recorded. The tumor volume of the tumor induced mice reached maximum size of 115 ± 0.11 within 30 days of the initiation time. After the treatment, the tumor growth volume was found to be decreased with respect to the dose of Naringenin. In the combination of Naringenin with cisplatin showed marked reduction in tumor volume 61.39± 0.03, than given in individual doses of Naringenin even at higher dose levels 70.09 ± 0.03. In the present findings, the

expression of Matrix Metalloproteinase (MMP – 2 and MMP - 9) was qualitatively estimated by gelatin Zymography method. Dose dependent decrease in expression of MMP - 2 and MMP - 9 was observed in all treated groups. Naringenin might promoted its action by down regulation of Bcl-2.[20] Interestingly, we have observed that MMP's expression was below normal level in naringenin treated animals.

Since MMP's also play important role as targets for other therapies, the reason behind the same has to address in future studies. DNA damage will occur as single and double strand breaks and polycyclic aromatic hydrocarbon adducts.[21] Similarly DNA mutation is a change that occur in the base sequence by hydrolysis, oxidation, alkylation which causes alterations in protein function and regulation.[22] Naringenin on HepG-2 cells (hepatocellular carcinoma) cleaved DNA at various sites to produced fragments thus lead to nuclear morphological changes through endonucleases enzymes (Bal 31 endonuclease). Nuclear changes such as chromatin condensation, blebbing of cells are the characteristics of a cell which undergoes apoptosis.[17] In the present findings fragmentation of DNA was qualitatively estimated in gel electrophoresis for determination of apoptosis. Increase in fragmentation was observed with respect to the concentration of naringenin. Better fragmentation was observed in combination of naringenin 200mg/kg with cisplatin 7 mg/kg. It indicates naringenin induces apoptosis through fragmentation of DNA.

In the present study anti-oxidant enzymes such as SOD, catalase were estimated in lung tissue homogenates. Increased levels of SOD and catalase were found to be dose dependent in the groups treated with naringenin. However, cisplatin doesn't have action over antioxidant enzyme level. Antioxidant activity of naringenin is due to interference in mitochondrial electron chain which inhibits the oxidation of cellular components in tumor cells.[19]

Cisplatin induce ROS mediated cell damage and cell death by destabilizing the intercellular redox homeostasis results in further generation of thiol radicals by damaging the thiol (R-SH) groups in biological system[20] It leads to generation free radicals.[21] Nitric Oxide (NO) plays a central role as endothelium-relaxing factor within the central nervous system.[22] In tumor progression, inflammatory pathway enhances the cytokinase, which increases the level of NO by up regulation of iNOS. Increased levels of NO directly damage DNA, inhibition of DNA synthesis and inhibition of rate limiting enzyme ribonucleotide reductase[23] NO level was found to be reduced in naringenin treated groups and the combination of naringenin 200mg/kg and cisplatin 7mg/kg. Cisplatin involves in reduction of Nitric oxide level by down

regulation of NOS (Nitric Oxide synthase) enzyme and interfering with the iNOS, eNOS, nNOS which depends upon Ca^{2+} / calmoludin. Cisplatin blocks the signals of cytokines which are necessary for the activation of iNOS and Ca^{2+}dependent nitric oxide production pathway.[24]

Naringenin would also promoted its action through interfering with the COX-2 pathway and inhibition of nitrite production by reduced expression of iNOS.[25] In the inflammatory condition, higher infiltration of neutrophils and monocytes accumulation occurs in tumor site which in turn activates of K-ras induced IL-8expression through NF-kB pathway, results in enhancement of tumor growth, metastasis and endothelial cell recruitment to tumors. MPO is released due to oxidative burst of polymorphonuclear neutrophile (PMN) occurs during inflammatory process, thereby enzymatically active MPO catalyze the formation of certain reactive species and also act with hydrogen peroxide and chloride to produces the powerful oxidant hypochlorous acid (HOCl). In addition, patients with increased levels of PMN were observed in broncho-alveolar space of bronchio alveolar carcinoma showed reduced survival rates.[26]

CONCLUSIONS

In present findings, MPO levels were found to be decreased with naringenin treatment. Cisplatin doesn't have any action over MPO reduction levels. This may be due to cisplatin activates caspase-1 present in neutrophils and macrophages which enhance certain inflammatory mediators such as IL-1ß and IL-18, TNF-a (tumor necrotic factor – alpha) at the tumor site.[27]

Histopathological findings, cisplatin treated group has mononuclear diving lining cells indicates the activation of mononuclear phagocyte system (MPS) which is a part of immune system. Naringenin treated groups showed dose dependent decrease in pneumonia and thickening of septa and the obtained results was found to be correlated in the reduced NO and MPO levels by inactivation of inflammatory process mediated by COX-2 pathway and NF-kB pathway respectively[28] Combination of Naringenin with cisplatin showed the reduced tumor progression by reducing pneumonia and thickening of septa which was observed in histological sections. Severe infiltration was observed which may due to cisplatin action through enhanced inflammatory pathway, but reduced MPO levels confirms the action of naringenin, which counter act the action of cisplatin in reducing the inflammatory pathway (COX-2 and NF-kB). In conclusion, we suggest based upon the results Naringenin possesses

anti-cancer effect against lung tumor and in combinatorial therapy with cisplatin, it satisfies in many criteria except few situations. Further studies to be carried, to find out molecular level actions of Naringenin against lung tumor.

ACKNOWLEDGEMENT

The corresponding author would like to thank Mr. Viral Laxmanbai Patel, Mr. shashank Mulakutla, Mr. Kaldihindiramasatyanrayana Raju, Mr. Nilesh Ambore, Mr.Shashank tummala, Ms. Asha Jose for their kindly help while carrying out this project. He also would like to thank JSS University for providing all the facilities to carry out this research.

REFERENCES

1. Pietanza M, Sandra P, Angelo Catherine. 'The molecular pathogenesis of small cell lung cancer', Cancer Bio & Therapy, 2011*;* 10(1): 1-10.
2. Hitoshi Kitamura, Takuya Yazawa, Koji Okudela, Hiroaki shimoyamada and Hanako Sato. 'Molecular and genetic pathogenesis of lung Cancer: Differences Between small-Cell and non-Small-Cell Carcinomas', The Open Pathology Journal, 2008*;* 2(1): 106-114.
3. Giuseppe Giaccone, Carlos Ferreira G, Cynthia Huisman. 'Novel approaches to the treatment of non-small cell lung cancer, Critical Reviews in Oncology/Hematology, 2002, 41(1): 57-77.
4. Edy Meiyanto, Adam Hermawan, Anindyajati. 'Natural Products for Cancer-targeted therapy: Citrus flavonoids as Potent Chemopreventive agents', Citrus Flavonoids as Potent Chemo preventive Agents, 2012; 13(1): 427-437.
5. Devan sabarinathan, Arambakkam Janardhanam Vanisree. 'Naringenin, a flavanone alters the tumorigenic features of C6 glioma cells', Biomedicine & Preventive Nutrition, 2011*;* 1(1): 19-24.
6. Wang X. 'The expanding role of mitochondria in apoptosis', *journal of Genes Development;* 2001; 15: 2922–2933.
7. Bernard Weinstein (1988)' Molecular mechanism of carcinogenesis', Cancer research, 1998*;* 48(1): 4135-4143.
8. Qing-Yi, Yusheng Jin, Jenny T.Mao, Zuo-Feng zhang, David Heber. 'Green tea inhibits cycolooxygenase-2 in non-small cell lung cancer cells through the induction of Annexin-1'. Biochemical and Biphysical research Communication, 2012; 427: 725-730

9. Vanisree, Devan sabarinathan, Arambakkam Janardhanam. 'Naringenin, a flavanone alters the tumerogenic features of C6 glioma cells', Biomedicine & Preventive Nutrition, 2011; 1(1): 19-24.
10. Jing Hsien Chen, Hui Hsuan Lin, Tai An Chiang. 'Gaseous Nitrogen oxide promotes human lung cancer cell Line A549 Migration, Invasion, and Metastasis via iNOS-Mediated MMP-2 Production', Toxicological sciences, 2008; 106(2): 364-75.
11. Gangjun Du , Guang Han, Shuo Zhang, Haihong Lin, Xianchuang Wu, Mei wang, Linlin Lu, Lijuan Yu, Wei liang, Liyan ji. 'Baicalin suppresses lung carcinoma and lung metastasis by SOD mimic and HIF-1α inhibition', European Journal of Pharmacology, 2010; 630(1): 121-30.
12. Phoon MC, Narasaraju T, Sim MK, Seet JE, Vincent T. Chow (2012) 'Doxycycline treatment attenuates acute lung injury in mice infected with virulent influenza H3N2 virus: Involvement of matrix metalloproteinases', Experimental and Molecular Pathology, 2012; 92(1): 287-93.
13. Ammayappan rajam srividya, Hardik Taakore, Devkant Tyagi, Pritam Majumdar, James, Vishnu Varthan VJ, Patel Jenish H and Lad Krunal V. 'Isolation, quantification and purity estimation of DNA from various sources', Bulletin of Pharmaceutical Research, 2011; 1(3): 4-9.
14. Sanyal SN, Poonam Thakur. 'Chemo preventive role of preferential COX-2 Inhibitor Diclofenac in 9, 10-Dimethybenz(a)anthracene Induced experimental lung carcinogenesis ', American Journal of Biomedical Sciences, 2009; 2(3): 275-88.
15. Grace Berlin VM, Siddikuzzaman (2012) 'Inhibition of metastatic lung cancer in C57BL/6 mice by liposome encapsulated all trans retinoic acid (ATRA)', International Immunopharmacology, 2012; 14(1): 570-79.
16. John Chen W, Micheal Breckwoldt (2008) 'Myeloperoxidase-targeted imaging of active inflammatory lesions in murine experimental autoimmune encephalomyelitis', Oxford journals, 2008; 131(004): 1123-33.
17. Arul Duraikannu, Perumal Subramanian. 'Inhibitory effect of naringenin (Citrus flavonone) on N-nitroso diethylamine induced hepato carcinogenesis in rats', Biochemical and Biophysical Research Communications, 2013; 434(1): 203-09.
18. Ganesh Chandra Jagetia, Tiyyagurakoti Reddy. 'Modulation of radiation-induced alteration in the anti-oxidant status of mice by naringenin', Cancer Research, 2005; 77: 780-94.

19. Chia lun Chao, Ching Sung Weng, Nen Chung Chang, Jih Shyong Lin, Shung Te Kao, Feng Ming Ho. 'Naringenin more effectively inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in macrophages than in microglia', Nutrition Research, 2010*;* 30(1): 858-64.
20. Narendran Krishnakumar, Nechikkad Subburayan Karthikeyan. 'Enhanced anticancer activity of naringenin-loaded nanoparticles in human cervical (HeLa) cancer cells', Biomedicine & Preventive Nutrition, 2011*;* 1(1): 223-31.
21. Asif Ali, Saba Abdi. 'Role of ROS modified human DNA in the pathogenesis and etiology of cancer', Cancer Letters, 1999; 142(1): 1-9.
22. Georgakilas, Thomas Kryston B, Anastassiya Georgiev B, Polycarpos Pissis. 'Role of oxidative stress and DNA damage in human carcinogenesis', Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2011; 711(1): 193-201.
23. Brozovic A, Ambriovic-Ristov A, Osmak, M. 'The relationship between cisplatin-induced reactive oxygen species, glutathione, and BCL-2 and resistance to cisplatin', Critical Review of Toxicology, 2010; 40: 347–59.
24. Han Bing Wang, Hong Zhen Liu, Xian Jie Wen, Qiao ling Zhou, Cheng Xiang Yang. 'The effects of combined treatment with sevoflurane and cisplatin on growth and invasion of human adenocarcinoma cell line A549', Biomedicine and Pharmacotherapy, 2013; 67(1): 503-19.
25. Lei Qin, Lingtao jin, Linlin Lu. 'Naringenin reduces lung metastasis in a breast cancer resection model', Protein & Cell, 2011*;* 2(6): 207-216.
26. Wislez M, Rabbe N, Marchal J, Milleron B, Crestani B, Mayaud C. Hepatocyte growth factor production by neutrophils infiltrating bronchio alveolar subtype pulmonary adenocarcinoma: role in tumor progression and death.. Cancer Research, 2003; 63(1): 1405-12.
27. Sarah Faubel, Danical Jubanovic, leonidreznikov, ,Hilarysomerset C, Harles A. Dinarello, and Charles l. Edelstein. 'Caspase-1-deficient mice are protected against cisplatin induced apoptosis and acute tubular necrosis'. Kidney International, 2004; 66(1): 2202-13.
28. Mary Fruh, Timothy Zacharewski, Kevin Connor, James Howell, Ichen Chen and stephen safe. 'Naringenin: a weakly estrogenic bioflavonoid that exhibits antiestrogenic activity', Biochemical Pharmacology, *1995;* 50(9): 1485-93.