Effect of Dieckol through Biochemical Markers during Hepatocarcinogenesis in Rats

Chapter - II
EFFECT OF DIECKOL THROUGH BIOCHEMICAL MARKERS DURING HEPATOCARCINOGENESIS IN RATS

INTRODUCTION

N-nitrosodiethylamine (NDEA), an environmental nitrosamine has been shown to be metabolized by cytochrome P-450-dependent monooxygenase system to its active ethyl radical metabolite (CH3 CH2\(^+\)) (Verna et al., 1996). The reactive product and the free radicals generated by P450 dependent enzymes interact with DNA producing mutation coupled with increased serum indices of liver function such as alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT); total bilirubin caused severe histopathological lesions in liver tissues followed by neoplastic transformation (Sivaramakrishnan et al., 2008; Singh et al., 2009). It is essential to have a specific and effective chemical and reactive oxygen species (ROS) scavenger to target xenobiotic metabolizing enzymes and multiple types of radicals in order to maintain cellular health. In particular, antioxidants are known to protect the cell from the deleterious effects of ROS and chemical carcinogens by preventing the metabolic activation of procarcinogens, inactivating carcinogens, blocking DNA binding sites and enhancing DNA repair mechanisms (Valko et al., 2006; Khan et al., 2008).

Recently, reported marine brown seaweed, showed a high content of polyphenols, known as phlorotannins, recognized to have defensive or protective functions against oxidative stress (Kang et al., 2012). The brown algae, E. cava, are plentifully produced on Jeju Island in Korea. It is popular in Korea and Japan as a food ingredient as a supplement for animal feed and fertilizer, and as a medicine. The total polyphenolic compounds (phlorotannins) are richer in E. cava than in other brown algae (Heo et al., 2005). The phlorotannin components of E. cava include such phenolic secondary metabolites as eckol (a closed – chain trimer of phloroglucinol), 6,6’-bieckol (a
hexmer), dieckol (a hexamer), phlorofucofuroeckol (a pentamer) and triphlorethol – A that are influential for biological activities (Ahn et al., 2007; Kang et al., 2005). Among these phlorotannins, dieckol is one of the the major active compounds (Lee et al., 2012). But there is no detailed studies hepatocarcinogenesis in rats and potential of dieckol through different experimental methods. Hence, the present study was aimed to validate the in-vivo antioxidant potential of dieckol in NDEA induced rats and chemical based was quantified.

**MATERIALS AND METHODS**

**Drug and chemicals**

Dieckol, NDEA, 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), reduced glutathione (GSH), 2, 20-dipyridyl, xylene orange, 2, 4-dinitrophenylhydrazine (DNPH), c - glutamyl -p- nitro anilide, and 5, 50- dithiobis - 2-nitrobenzoic acid were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). The rest of the chemicals utilized were obtained from a local firm (India) and were of analytical grade.

**Maintenance of animals**

Adult male albino Wistar rats (150–170g was used for the experiment. The animals were housed in plastic cages and maintained in a 12h light/12h dark cycle, 50% humidity and 25±3°C. The animals had free access to a standard pellet diet (Sai Enterprisei Pvt Ltd, Chennai, India) and water *ad libitum*. This study was approved (Vide.No.1038, 2013) by the Institutional Animal Ethics Committee of Annamalai University and was conducted in accordance with the “Guidelines for the Care and Use of Laboratory Animals”.

**Tumor induction**

Hepatocellular carcinoma was induced in male Wistar rats by providing 0.01% of *N*-nitrosodiethylamine (NDEA) through drinking water for 15 weeks (Ha et al., 2001; Arul and Subramanian 2012).
**Isolation of dieckol**

Protocol as same as that of first chapter in order to avoid the redundancy the methodology has not been repeated here.

**Dieckol preparation**

Dieckol was dissolved in saline and each rat received daily 1 ml at a dose of 40 mg/kg body weight orally by intragastric intubation throughout the experimental period (Kang et al., 2013).

**PROCESSING OF BLOOD AND TISSUE SAMPLES**

**Serum preparation**

Blood was collected in a dry test tube and allowed to coagulate at ambient temperature for 30 min. Serum was separated by centrifugation at 3000 rpm for 15 min.

**Plasma preparation**

The blood, collected in a heparinized centrifuge tube, was centrifuged at 2000 rpm for 10 min and the plasma was separated by aspiration, transferred into eppendorf tubes and stored at -4°C until analysis.

**Erythrocyte preparation**

After the separation of plasma, the buffy coat, enriched in white cells, was removed and the remaining erythrocytes were washed three times with physiological saline. A known volume of erythrocyte was lysed with hypotonic phosphate buffer at pH 7.4. The hemolysate was separated by centrifugation at 2500 rpm for 10 min and the supernatant was used for the estimation of enzymic antioxidants.

**Tissue homogenate preparation**

Liver and kidney tissues (250 mg) were sliced into pieces and homogenised in appropriate buffer in cold condition (pH 7.0) to give
20% homogenate (w/v). The homogenate were centrifuged at 1000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated and used for various biochemical estimations.

**Tissue sampling for histopathological study**

For histopathological study, three rats from each group were perfused with cold physiological saline, followed by formalin (10% formaldehyde). The liver and kidney were excised immediately and fixed in 10% formalin.

**Experimental design**

The animals were randomized and divided into 4 groups (as shown in Table 1) of 8 animals each.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td>Control rats were given with corn oil orally for 16 weeks</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td>Rats received dieckol (40 mg/kg body weight, dissolved in saline) orally for 16 weeks.</td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td>Rats were induced with hepatocellular carcinoma by providing 0.01% NDEA through drinking water for 15 weeks.</td>
</tr>
<tr>
<td><strong>Group 4</strong></td>
<td>Rats treated with dieckol one week before the administration of 0.01% NDEA and continued along with carcinogen (NDEA) till the end of the experiment (i.e. 16 weeks).</td>
</tr>
</tbody>
</table>

After the last treatment, the rats were fasted over night, and euthanized by cervical dislocation. Blood samples were collected in tubes containing ethylenediamine tetra acetic acid (EDTA). The plasma was obtained after centrifugation (2000 x g for 20min at 4°C) and used for various biochemical measurements. The livers were excised immediately from the animals, washed with ice-chilled physiological saline, and stored at -80°C until analysis.
ESTIMATION OF LIVER MARKER ENZYMES

ACTIVITIES OF SERUM ASPARTATE AND ALANINE TRANSAMINASES

Oxaloacetate and pyruvate formed are coupled with 2, 4-DNPH to give the corresponding hydrazone, which gives brown colour in alkaline medium and this is measured colorimetrically by the method of Reitman and Frankel (1957).

Reagents

1. Buffered aspartate - \( \alpha \)-ketoglutarate substrate, pH 7.4.
2. Buffered alanine - \( \alpha \)-ketoglutarate substrate, pH 7.4.
3. DNPH colour reagent.
4. Sodium hydroxide: 0.4N
5. Working standard pyruvate: 2 mM

Procedure

0.5 ml of substrate was incubated for a few mins at 37\(^{\circ}\) C. Then added, 0.1 ml of serum and incubation was continued for an hour in the case of aspartate transaminase and 30 mins for alanine transaminase. Adding 0.5 ml of DNPH solution arrested the reaction and the tubes were kept at room temperature for 20 mins. 5.0 ml of 0.4 N sodium hydroxide was also added. A set of pyruvic acid standards was also treated in similar manner. The colour developed was read at 540 nm. Activity of AST and ALT were expressed in units/ml.

ASSAY OF ALKALINE PHOSPHATASE

Activity of alkaline phosphatase was assayed by the method of King and Armstrong (1951).
**Reagents**

2. Substrate – 0.01 M phenylphosphate disodium salt solution.
3. Folin-ciocalteau reagent
4. Sodium carbonate solution: 10%
5. Standard phenol solution: A solution of distilled crystalline phenol in water containing 5.0 µg in 1.0 ml was prepared.

**Procedure**

An incubation mixture containing 150 µM of bicarbonate buffer and 100 µM of substrate in 2.9 ml was preincubated at 37°C for 10 mins.

0.2 ml of serum was added to this and incubated at 37°C for 15 mins.

The reaction was arrested by the addition of 10 ml of Folin-ciocalteau reagent. The suspension was centrifuged and 2.0 ml of 10% sodium carbonate was added to the supernatant. The solution was incubated at 37°C for 10 mins.

Aliquots of standard phenol (2.5 - 10 µg) were also treated with Folin’s reagent and sodium carbonate. The blue colour developed was read at 640 nm. The enzyme activity was expressed in KA units.

**ASSAY OF LACTATE DEHYDROGENASE**

The activity of lactate dehydrogenase (LDH) was assayed by the method of Nieland (Varley et al., 1998) and was assayed spectrophotometrically according to the standard procedures using commercially available automated Roche/Hitachi 912 kit.

**Reagents**

1. Glycine buffer: 100 mM
2. Buffered substrate – 2.78 g of lithium lactate was dissolved in 125 ml of glycine buffer containing 75 ml of 0.1N NaOH solution. This was prepared just before use.
3. NAD⁺: 20 mM
4. DNPH: 0.2% of DNPH in 1 N HCl
5. NaOH: 0.4N

6. Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of buffer.

Procedure

To a set of tubes, 1 ml of the buffered substrate and 0.1 ml of serum was added and the tubes were incubated at 37° C for 15 min. After adding 0.2 ml of NAD solution, the incubation was continued for another 15 min. The reaction was then arrested by adding 1 ml of DNPH reagent and the tubes were incubated further period of 15 min at 37° C. 0.1 ml of serum was added to blank tubes after arresting the reaction with DNPH. 7 ml of NaOH solution was added and the colour developed was measured at 420 nm. Stable aliquots of the standard were also analyzed by the same procedure.

The enzyme activity was expressed in IU/L.

ACTIVITY OF SERUM γ-Glutamyl Transferase

The activity of γ-glutamyl transferase (GGT; E.C.2.3.2.1) was estimated by the method of Rosalki et al. (1970). The p-nitroaniline liberated by the enzyme in the presence of substrate (L-γ-glutamyl-p-nitroanilide) produces a yellow colour, which was estimated spectrophotometrically at 410nm.

Reagents

1. Tris-HCl (0.1 M) - Glycylglycine (0.05 M) buffer (pH 9): 1.57 gm of Tris-HCl and 660 mg of glycylglycine was dissolved in distilled water and the pH was adjusted to 9 with 1 N NaOH and made up to 100 ml.

2. Tris-HCl Buffer (0.1 M; pH 8.5): 1.57 gm of Tris-HCl was dissolved in 100 ml of distilled water and the pH was adjusted to 8.5 with 1 N NaOH.
3. L-γ-glutamyl-p-nitroanilide substrate (6.25 mM): 190 mg of L-γ-glutamyl-p-nitroanilide was dissolved in 100 ml of prewarmed Tris-HCl-glycylglycine buffer.

4. Acetic acid - 10%

5. Standard p-nitroanilide (1 μmole/ml): 13.8 mg of p-nitroanilide was dissolved in 100 ml of Tris-HCl buffer.

**Procedure**

0.05 ml of serum was made up to 0.5 ml by the addition of L-γ-glutamyl-p-nitroanilide substrate and was incubated at 37°C for 30 min. The standard tubes taken at a concentration ranging from 0.1 to 0.4μmole were also incubated as above. The reaction was arrested by the addition of 2.5 ml of 10% acetic acid. Simultaneously, a control without serum was also subjected to the above treatment and incubation excepting that serum was added after arresting the reaction. The yellow colour developed after the addition of acetic acid was measured at 410nm against the blank using spectrophotometer.

The activity of GGT was expressed as IU/L of serum.

**ESTIMATION OF SERUM BILIRUBIN**

The level of serum bilirubin was estimated by the method of Malloy and Evelyn (1937). Serum bilirubin was estimated by Van den Bergh reaction. It was based on the formation of purple coloured azobilirubin when bilirubin reacts with diazotised sulphanilic acid.

**Reagents**

1. Absolute methanol

2. HCl - 1.5%

3. Diazo reagent
   Solution A: 1g of sulphanilic acid was dissolved in 15ml of conc. HCl and made up to 1 litre with water.
Solution B: 0.5g of sodium nitrate was dissolved in water and made up to 100ml. Freshly prepared before use by adding 0.3ml of solution B to 10ml of solution A.

4. Standard bilirubin solution: 10mg/100ml chloroform.

**Procedure**

0.2 ml of serum was diluted to 2 ml with distilled water in two tubes marked as test and blank. To the test, 0.5 ml of the diazo reagent and to the blank, 0.5 ml of 1.5% HCl was added. Finally to both tubes, 2.5 ml of methanol was added and the tubes were kept at room temperature for 30 min. The colour developed was read at 540nm. For a standard curve, one in five dilutions of stock standard in methanol was made to obtain a solution containing 2 mg/100 ml.

The level of serum bilirubin was expressed as mg/dl.

**ESTIMATION OF ALPHA-FETOPROTEIN (AFP)**

**Principle**

The solid phase is coated with monoclonal AFP-specific antibody labelled with a ruthenium complex. An aliquot of sample containing AFP gets conjugated with biotinylated monoclonal AFP-specific antibody and forms a sandwich complex when incubated. After incubation, streptavidin-coated microparticles are allowed to react with biotin of the sandwich complex. The reaction mixture is aspirated into the measuring cell where the microparticles are captured onto the surface of the electrode. Unbound substances are removed with ProCell. Application of a voltage to the electrode induces chemiluminescent emission which is measured by a photomultiplier. The results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent bar code.

The values of AFP are expressed as ng/mL.
PHASE I ENZYMES: ASSAY OF CYTOCHROME P450

The activity of cytochrome P450 was assayed by the method of Omura and Sato, (1964). This method is based on the formation of a pigment with an absorbance between 450-490 nm.

**Reagents**

1. Phosphate buffer, 0.2 M, pH 7.4
2. Sodium dithionite
3. Carbon monoxide (CO) gas: CO can be generated freshly by the action of concentrated sulphuric acid on formic acid.

**Procedure**

To 1 mL of buffer, 0.1 mL of sample was added followed by few mg of sodium dithionite. The CO gas was gently bubbled for approximately 1 min and the absorbance was read at 450 and 490 nm. The difference in absorption spectrum was used to calculate cytochrome P450 content using the extinction coefficient 91mM⁻¹cm⁻¹.

Values are expressed as nmoles of cytochrome/mg protein.

PHASE II ENZYMES: ASSAY OF GLUTATHIONE-S-TRANSFERASE (E.C.2.5.1.18)

Activity of glutathione-S-transferase (GST) was measured in tissue homogenate by following the increase in absorbance at 340nm using CDNB as substrate by the method of Habig *et al.* (1974).

\[
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{Non-Se-GPx}} \text{GSSG} + 2\text{H}_2\text{O}
\]

**Reagents**

1. Phosphate buffer - 0.3 M, pH 6.5
2. Reduced glutathione - 30 mM
3. CDNB - 30 mM was prepared in 95% ethanol.
Procedure

The reaction mixture contained 1 ml of phosphate buffer, 0.1 ml of CDNB, 0.1 ml of tissue homogenate and 0.7 ml of distilled water. The reaction mixture was incubated at 37°C for 5 min and then the reaction was started by the addition of 0.1 ml of 30 mM glutathione. The absorbance change was read at 340nm for 5 min. Reaction mixture without the enzyme was used as the blank. 9.6 was the difference in the micromolar extinction coefficient between CDNB-GSH conjugate formed/min/mg proteins.

Calculation

\[
\text{Activity} = \frac{\text{OD} \times 3 \times 100}{9.6 \times 5 \times \text{mg protein}}
\]

The activity of GST was expressed as μmoles of CDNB-GSH conjugate formed/min/mg protein.

DETERMINATION OF LIPID PEROXIDATION

ESTIMATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS)

The level of thiobarbituric acid reactive substances (TBARS) in plasma and tissues were estimated by the method of Niehuis and Samuelsson (1968). In this method, malondialdehyde and other TBARS were measured by their reactivity with TBA in acidic condition to generate a pink coloured chromophore, which was read at 535nm.

Reagents

1. Tris-HCl buffer - 0.025 M, pH 7.5
2. TCA - 15%
3. HCl - 0.25 N
4. TBA - 0.38%
5. TBA-TCA-HCl reagent - (1 : 1 : 1 v/v)
6. Standard (1, 1', 3, 3' tetramethoxy propane) - 0.16 ml of 3 M solution of standard tetramethoxy propane was made up to 100 ml with double distilled water. From that to 1 ml of was taken and made up to 100 ml with double distilled water, which served as working standard.

**Procedure**

The tissue homogenate was prepared in Tris-HCl buffer (pH 7.5). 1 ml of the tissue homogenate/0.5 ml of plasma was treated with 2 ml of TBA-TCA-HCl reagent and mixed thoroughly. The mixture was kept in a boiling water bath for 15 min. After cooling, the tubes were centrifuged for 10 min and the supernatant was taken for measurement. A series of standard solution were also treated in a similar manner. The absorbance of chromophore was read at 535nm against the reagent blank.

The values were expressed as mM/g – tissue or mM/dl - plasma

**ESTIMATION OF LIPID HYDROPEROXIDES**

The level of lipid hydroperoxides (LOOH) in plasma and tissues were estimated by the method of Jiang et al. (1992). In this method, oxidation of ferrous ions (Fe^{2+}) under acidic conditions in the presence
of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560nm.

**Reagents**

1. Tris-HCl buffer - 0.025 M, pH 7.5
2. Pure methanol
3. Sulphuric acid (H$_2$SO$_4$) - 250 mM
4. BHT
5. Xylenol orange
6. Ammonium ion sulphate
7. Fox reagent - 88 mg of BHT, 7.6 mg of xylenol orange and 9.8 mg of ammonium ion sulphate were added to 90 ml of methanol and 10 ml of 250 mM H$_2$SO$_4$.
8. Standard - 0.2 M H$_2$O$_2$.

**Procedure**

1.8 ml of the Fox reagent was mixed with 0.2 ml of the tissue homogenate/0.2 ml of plasma. Then incubated for 30 min at room temperature and read at 560nm.

The values were expressed as mM/g – tissue, 10$^{-5}$ mM/dl – plasma

**DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS**

**ESTIMATION OF ASCORBIC ACID (VITAMIN C)**

The level of ascorbic acid was estimated by the method of Omaye *et al.* (1979). The ascorbic acid was oxidized to copper to form dehydro ascorbic acid and diketoglutaric acid. These products when treated with DNPH form the derivative, bis-2, 4-dinitrophenyl hydrazone that undergoes rearrangement to form a product with absorption maxima
at 520nm. Thiourea provides a mild reducing medium, which helps to prevent the interference from ascorbic acid chromogens.

**Reagents**

1. Tris-HCl buffer - 0.025 M, pH 7.5
2. DNPH reagent - 2 g of DNPH was dissolved in 100 ml of 9 N H₂SO₄.
   To this 4 g of thiourea was added and mixed.
3. TCA - 6%
4. TCA - 4%
5. H₂SO₄ - 85%
6. Standard ascorbic acid - 10 mg of L-ascorbic acid was dissolved in 100 ml of 4% TCA. This was diluted to prepare a working standard of concentration 100 µg/ml.
7. Activated charcoal.

**Procedure**

0.5 ml of plasma/tissue homogenate was mixed thoroughly with 1.5 ml of 6% TCA and centrifuged for 20 min at 3500 × g. To 0.5 ml of the supernatant, 0.5 ml of DNPH reagent was added and mixed well. The tubes were allowed to stand at room temperature for an additional 3h. Removed, placed in ice-cold water and added 2.5 ml of 85% H₂SO₄ and allowed to stand for 30 min. A set of standards containing 10-50 µg of ascorbic acid were taken and processed similarly along with a blank, containing 0.5 ml of 4% TCA. The colour developed was read at 530nm.

Ascorbic acid values were expressed as µM/mg - tissue, mg/dl - plasma.
ESTIMATION OF $\alpha$-TOCOPHEROL (VITAMIN E)

$\alpha$-tocopherol was estimated by the method of Desai (1984). This method involves the reduction of ferric ion to ferrous ion by $\alpha$-tocopherol and the formation of a red coloured complex with 2, 2$'$-dipyridyl. Absorbance of the chromophore was measured at 520nm.

Reagents

1. Petroleum ether 60-80°C
2. Double distilled ethanol
3. 2, 2$'$-dipyridyl in ethanol - 0.2%
4. Ferric chloride in ethanol - 0.5%
5. Stock standard - 100 mg of $\alpha$-tocopherol in 100 ml of distilled ethanol.
6. Working standard - Stock solution was diluted to a concentration of 10 $\mu$g/ml in distilled ethanol.

Procedure

To 0.1 ml of plasma/lipid extract, 1.5 ml of ethanol and 2 ml of petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80°C. To this 0.2 ml of 2, 2$'$-dipyridyl solution and 0.2 ml of ferric chloride solution was added. Mixed well and kept in dark for 5 min and added 2 ml of butanol. The intense red colour developed was read at 520nm. Standard $\alpha$-tocopherol in the range of 10-100 $\mu$g were taken and treated similarly along with blank containing only the reagent.

The values were expressed as $\mu$M/mg – tissue, mg/dl - plasma
ASSAY OF ENZYMIC ANTIOXIDANTS

ESTIMATION OF SUPEROXIDE DISMUTASE (E.C.1.15.1.1)

The activity of superoxide dismutase (SOD) was determined by the method of Kakkar et al. (1984). Superoxide radicals react with NBT in the presence of NADH and produce formazan blue. SOD removes the superoxide radicals and inhibits the formation of formazan blue. The intensity of colour is inversely proportional to the activity of the enzyme.

Reagents

1. Sodium pyrophosphate buffer - 0.025 M, pH 8.3
2. PMS - 186 µM
3. NBT - 300 µM
4. NADH - 780 µM
5. Glacial acetic acid
6. n-butanol
7. Chloroform (CHCl₃)
8. Ethanol

Procedure

0.5 ml of tissue homogenate was diluted to 1 ml with water. Then added 2.5 ml of ethanol and 1.5 ml of CHCl₃ (all the reagents were chilled). This mixture was shaken for 1 min at 4°C and then centrifuged. The enzyme activity in the supernatant was determined. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 µM PMS, 0.3 ml of 30 µM NBT, 0.2 ml of 780 µM NADH, appropriately diluted enzyme preparation and water in a total volume of 3 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90 sec the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The intensity of
the chromogen in the butanol layer was measured at 560nm against butanol blank. A system devoid of enzyme served as control.

One unit of the enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one minute under the assay conditions and expressed as specific activity in units/mg protein.

**ESTIMATION OF CATALASE (E.C.1.11.1.6)**

The activity of catalase (CAT) was determined by the method of Sinha (1972). Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H$_2$O$_2$. The chromic acetate formed was measured at 620nm.

**Reagents**

1. Phosphate buffer - 0.01M, pH 7.0
2. H$_2$O$_2$ - 0.2 M
3. Potassium dichromate - 5%
4. Dichromate-acetic acid reagent - 1:3 ratio of potassium dichromate was mixed with glacial acetic acid. From this 1 ml was diluted again with 4 ml acetic acid
5. Standard H$_2$O$_2$ - 0.1 ml of 0.2 M H$_2$O$_2$ was diluted to 100 ml using distilled water.

**Procedure**

To 0.9 ml of phosphate buffer, 0.1 ml of tissue homogenate and 0.4 ml of H$_2$O$_2$ were added. After 60 sec, 2 ml of dichromate acetic acid reagent was added. The tubes were kept in boiling water bath for 10 min and the colour developed was read at 620nm. Standards in the range of 2-10 µmol were taken and proceeded as test with blank containing reagent alone.

The activities were expressed as µmoles of H$_2$O$_2$ consumed/min/mg protein.
ESTIMATION OF GLUTATHIONE PEROXIDASE (E.C.1.11.1.9)

The activity of glutathione peroxidase (GPx) was estimated by the method of Rotruck et al. (1973). A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of GSH for a specified time period. Then the remaining GSH was measured by the method of Ellman.

\[
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{Se-GPx}} \text{GSSG} + 2\text{H}_2\text{O}
\]

Reagents

1. Tris-HCl buffer - 0.4 M, pH 7.0
2. Sodium azide - 10 mM
3. TCA - 10%
4. Ethylenediaminetetraacetic acid (EDTA) - 0.4 mM
5. H₂O₂ - 20 mM
6. Ellman’s reagent - 19.8 mg of DTNB in 100 ml of 1% sodium citrate solution.
7. Reduced glutathione - 2 mM

Procedure

To 0.2 ml of Tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of tissue homogenate were added. To the mixture, 0.2 ml of glutathione followed by 0.1 ml of H₂O₂ was added. The contents were mixed well and incubated at 37°C for 10 min along with a tube containing all the reagents except sample. After 10 min the reaction was arrested by the addition of 0.5 ml of 10% TCA, centrifuged and the supernatant was assayed for glutathione by the method of Ellman (1959).
The activities were expressed as µg of GSH consumed/min/mg protein.

**Statistical analysis**

Data were analysed by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using a statistically software package (SPSS for Windows, V. 13.0, Chicago, USA). Results were presented as mean ± S.D. p-values < 0.05 were considered as statistically significant.

**RESULTS**

Table 1 shows the total number and size of nodules in experimental animals. The dieckol treated group: 4 showed a significant decrease in the number of nodules and nodule size when compared with group 3 animals. NDEA treatment increased the relative liver weight to body weight compared to the control (group I). However, administration of dieckol to group 4 significantly reduced the relative liver weight compared to group 3 animals.

Table 2 shows the body and liver weights of control and experimental animals. The body weights were significantly decreased in NDEA-treated animals as compared with control. Treatment of dieckol to NDEA-treated rats significantly improved the body weight as compared to group 3 animals. Figure 10 shows the schematic representation of rat liver of control and experimental animals.
Table 2: Effect of dieckol on number and size of nodules and tumour incidence in control and experimental animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dieckol</th>
<th>NDEA</th>
<th>NDEA+Dieckol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumour incidence</strong></td>
<td>-</td>
<td>-</td>
<td>6/6</td>
<td>2/6</td>
</tr>
<tr>
<td><strong>Total number no of nodules</strong></td>
<td>-</td>
<td>-</td>
<td>107*</td>
<td>56**</td>
</tr>
<tr>
<td><strong>Average number of nodules/bearing liver</strong></td>
<td>-</td>
<td>-</td>
<td>17.83 ± 1.5*</td>
<td>9.33 ± 0.5**</td>
</tr>
</tbody>
</table>

**Relative size (% of total numbers)**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dieckol</th>
<th>NDEA</th>
<th>NDEA+Dieckol</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1mm</td>
<td>-</td>
<td>-</td>
<td>54 (50.5)</td>
<td>31 (55.3)</td>
</tr>
<tr>
<td>&gt;1 mm&lt;3 mm</td>
<td>-</td>
<td>-</td>
<td>33 (30.8)</td>
<td>17(30.3)</td>
</tr>
<tr>
<td>&gt;3mm</td>
<td>-</td>
<td>-</td>
<td>20 (18.7)</td>
<td>8 (14.3)</td>
</tr>
</tbody>
</table>

*Significantly different from control p < 0.001 ANOVA followed by DMRT.

**Significantly different from group 3 p < 0.001 ANOVA followed by DMRT.
Table 3: Body weight, organ weight, food and water intake in control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Food and Water intake</th>
<th>Organ weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>150 ± 20.04</td>
<td>295 ± 19.09</td>
<td>6.6 ± 0.8</td>
</tr>
<tr>
<td>Dieckol</td>
<td>155 ± 19.65</td>
<td>301 ± 20.04</td>
<td>6.4 ± 0.7</td>
</tr>
<tr>
<td>NDEA</td>
<td>160 ±16.25*</td>
<td>195 ± 12.58*</td>
<td>3.2 ± 0.5*</td>
</tr>
<tr>
<td>NDEA+Dieckol</td>
<td>156 ±17.61**</td>
<td>234 ± 17.83**</td>
<td>6.2±0.5**</td>
</tr>
</tbody>
</table>

*Significantly different from control p < 0.001 ANOVA followed by DMRT.
**Significantly different from group 3 p < 0.001 ANOVA followed by DMRT.
Figure 9: Schematic representation of rat livers in control and experimental animals

**HISTOPATHOLOGICAL CHANGES**

The histological examinations in figure 10 basically support the results obtained from serum enzyme and tumor marker assays. Figure 10 shows the normal architecture (Control) and cells cytoplasm of hepatic cells with granulated cytoplasm, central vein, small uniform nuclei and nucleolus. Dieckol treated animals showed normal architecture depicting the non-toxic nature of dieckol (Figure 10b). NDEA alone (Figure 10c) showed loss of architecture and tumor cells which were smaller than normal cells with granular cytoplasm and large hyperchromatic nuclei, whereas group 4 animals pretreated with dieckol showed few neoplastically transformed cells and hepatocytes maintaining near normal architecture (Figure 10d),
Figure 10: Histopathological changes of liver in control and experimental rats

![Image of liver tissue showing normal architecture and neoplastic changes](image)

(A) Liver tissue of the normal group (control) showed hepatic lobule having normal architecture (40x, HE). (B) Liver tissue of the Dieckol treated group showed hepatic lobule having normal architecture (40x, HE). (C) NDEA alone showing loss of architecture, mitotic, granular cytoplasm and neoplastic cells. (D) Few neoplastically transformed cells and hepatocytes maintaining near normal architecture observed in Dieckol (40x, HE) animals.

Biochemical assays

Figure 11 shows the status of phase I (cytochrome P450) and phase II detoxication enzymes (GST), in the liver of control and experimental animals in each group. The activity of GST was significantly decreased, whereas the status of cytochrome P450 was increased in liver of tumor-bearing animals (NDEA) as compared to control animals. Administration of dieckol to NDEA treated animals significantly decreased phase I and elevated phase II enzyme activities compared to NDEA. Although treatment with dieckol alone (Dieckol) showed no significant difference in the activities of phase 1 enzymes, the activities of phase II enzymes in the liver were significantly increased compared to control.
The changes in the levels of molecular markers of alpha feto protein (AFP) and carcinoembryonic antigen (CEA) in control and experiment animals are depicted in Figure 12. In NDEA treated rats the levels of AFP and CEA were significantly increased compared to control. Administration of dieckol significantly decreased the levels of AFP and CEA compared to control. Administration of dieckol alone significantly decreased the levels of AFP and CEA compared to control.

The changes in the levels of lipid peroxidation products in control and experiment animals are depicted in Figure 13a and 13b. In NDEA treated rats the levels of TBARS and lipid hydroperoxides were significantly increased compared to control. Administration of dieckol significantly decreased the levels of TBARS and LPO compared to control. Administration of dieckol alone significantly decreased the levels of TBARS and LPO compared to control.

Figures 14a and 14b illustrate the levels of enzymatic and non-enzymatic antioxidants namely SOD, CAT, GPx, vitamin C, vitamin E and GSH in liver and plasma of control and experimental rats. A significant decrease in the activities of enzymatic antioxidants and non-enzymatic antioxidants in NDEA treated rats was observed. Treatment with dieckol on groups 4 significantly increased the levels of enzymatic and non-enzymatic antioxidants in liver compared to NDEA treated rats. Administration of dieckol alone significantly increased the enzymatic and non-enzymatic antioxidants compared to control.

Figure 15 shows the levels of serum hepatic marker enzymes in control and experimental rats. NDEA induced hepatocarcinogenesis caused abnormal liver function in all rats. Activities of serum hepatospecific enzymes such as AST, ALT, ALP, LDH, GGT, and the level of bilirubin were significantly increased (p < 0.05) in NDEA treated animals when compared to control animals. However, dieckol treatment to (NDEA+Dieckol) leads to a reversal in the values towards
Figure 11: Activities of phase I and II enzymes in the liver of experimental and control animals

NDEA - N-Nitrosodiethylamine; * Significant different from control p<0.01, ** Significant different from control p<0.001, *** Significant different from NDEA p<0.001.

ANOVA followed by DMRT, a) μ moles of cytochrome P450, b) μ moles of CDNB conjugated with GSH per minute.

Figure 12: Changes in the levels of AFP and CEA in serum of experimental and control rats

Values NDEA - N-Nitrosodiethylamine; * Significant different from control p<0.001, ** Significant different from NDEA p<0.001, ANOVA followed by DMRT.
Figure 13a: Changes in the levels of lipid peroxidation in liver of experimental and control rats

TBARS

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dicékol</th>
<th>NDEA</th>
<th>NDEA+Dicékol</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM/g tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

Lipid Hydperoxides

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dicékol</th>
<th>NDEA</th>
<th>NDEA+Dicékol</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM/g tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

NDEA - N-Nitrosodiethylamine; *Significant different from control p<0.01, **Significant different from NDEA p < 0.01, ***Significant different from NDEA p<0.01, 
●Significant different from NDEA p<0.001 ANOVA followed by DMRT.

Figure 13b: Changes in the levels of lipid peroxidation in Plasma of experimental and control rats

TBARS

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dicékol</th>
<th>NDEA</th>
<th>NDEA+Dicékol</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

Lipid Hydperoxides

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dicékol</th>
<th>NDEA</th>
<th>NDEA+Dicékol</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-5 mmol/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

NDEA - N-Nitrosodiethylamine; *Significant different from control p<0.01, **Significant different from NDEA p < 0.01, ***Significant different from NDEA p<0.01, 
●Significant different from NDEA p<0.001 ANOVA followed by DMRT.
Figure 14a: Changes in the levels of enzymatic and non-enzymatic antioxidant status of liver in control and experimental rats

NDEA - N-Nitrosodiethylamine; *Significant different from control p <0.05, **Significant different from control p <0.001, ***Significant different form control p <0.05, †Significant different from NDEA p <0.01, †† Significant different from NDEA p <0.001

ANOVA followed by DMRT. #Units of enzyme activities are expressed as: SOD – one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 min. CAT – 1 mol of hydrogen peroxide consumed per min. GPx – lg of glutathione consumed per min.

Chapter II
Figure 14b: Changes in the levels of enzymatic and non-enzymatic antioxidant status of plasma in control and experimental rats

NDEA - N-Nitrosodiethylamine; *Significant different from control p <0.05, **Significant different from control p <0.001, ***Significant different from control p <0.01, ♣♣ Significant different from NDEA p <0.001 ANOVA followed by DMRT.

Units of enzyme activities are expressed as: SOD – one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1min. CAT – 1 mol of hydrogen peroxide consumed per min. GPx – 1g of glutathione consumed per min.
Figure 15: Effect of dieckol on the activities of liver marker enzymes in the serum of control and experimental groups of rats

* Significant different from group 1 \( p < 0.05 \), ✡ Significant different from group 3 \( p < 0.01 \). ✡✡ Significant different from group 3 \( p < 0.001 \) ANOVA followed by DMRT.
normal control. No significant changes were observed in dieckol alone treated rats compared to control.

**Discussion**

Administration of dieckol prevented the development of NDEA-induced hepatic carcinoma by modulating xenobiotic metabolizing enzymes, oxidant and antioxidant status as well as decreasing the liver marker enzymes. Our results are in line with other chemopreventive agents (Ramakrishnan *et al.*, 2007; Sivaramakrishnan *et al.*, 2008; Singh *et al.*, 2009, Arul and Subramanian 2013).

Metabolic biotransformations of NDEA by cytochrome P450 enzymes produce O6-ethyldeoxyguanosine and O4 and O6-ethyldeoxythymidine, active ethyl radical metabolite (CH3 CH2+) which is responsible for initiation of carcinogenesis (Pegg, 1983; Aiub *et al.*, 2003). A subsequently reactive product of NDEA can be detoxified by phase II enzymes such as GST. Increased activity of cytochrome P450 and decreased activity of GST observed in the present study provide evidence for the development of HCC in NDEA treated animals. Our results provide evidence that dieckol could act as a dual acting agent by decreasing phase I cytochrome P450 and increasing phase II enzyme GST and block initiation of NDEA-induced hepatocarcinogenesis. Several studies have documented that dual acting agent are ideal chemopreventive agents with high efficacy (Moon *et al.*, 2006; Ganapathy *et al.*, 2008; Arul and Subramanian 2013).

Modulation of XMEs by dieckol is correlated with equilibrium between oxidant and antioxidant balance, which is tilted towards the antioxidant side. The reactive metabolite of NDEA and the free radicals generated by P450-dependent enzymes disturb antioxidant status and facilitate lipid peroxidation (LPO) and decreased antioxidant status thereby producing several toxic products, such as malondialdehyde (MDE) and 4-hydroxynonenal. These products can attack cellular
targets including DNA, thereby inducing mutagenecity and carcinogenicity. Decreased activities of enzymatic and non-enzymatic antioxidants in NDEA–induced HCC may be due to over-utilization of these antioxidants to scavenge the products of lipid peroxidation and indicate the complete disruption of the antioxidant defense mechanism of the liver. Our results are in line with other workers reported in literature (Jeyabal et al., 2005; Sivaramakrishnan et al., 2008; Singh et al., 2009; Arul and Subramanian 2013).

Animals treated with dieckol could protect the cell through inhibition of lipid peroxidation as evidenced from the decreased levels of tissue TBARS and lipid hydroperoxides. Our results suggest that anti-lipid peroxidative role of dieckol is probably mediated by its ability to scavenge free radicals due to the presence of 4-hydroxyl group in the b-ring possessing electron donating properties (Ahn et al., 2007). In addition, dieckol could prevent the membrane from free radical attack and thus protect the membrane and inhibit lipid peroxidation. Furthermore, dieckol inhibits lipid peroxidation and generation of free radicals by enhancing antioxidant status as evidenced by increasing the levels of SOD, catalase and GPx and non-enzymatic antioxidants, vitamin E and D, and GSH. Our findings demonstrate that the modulation of the delicate balance between oxidant and antioxidants by various natural phytochemicals such as dieckol is a rational approach to block tumour progression. Antioxidant potential of dieckol have also documented in CCL4 induced hepatotoxicity (Kang et al., 2013b).

Generally liver damage induced by NDEA reflects instability of liver cell metabolism and membrane instability subsequently causes distinctive changes in the serum enzyme activities. Upon liver injury, liver marker enzymes (AST, ALT, and ALP) enter into the circulatory system due to altered permeability of membrane (Sivaramakrishnan et al., 2008; Singh et al., 2009). It correlates with our results, which showed increased activities of the enzymes in the serum of HCC.
induced animals. Serum LDH, a cytoplasmic marker enzyme and GGT, a membrane-bound enzyme are other well known indicators of liver damage during various physiological and pathological conditions such as hepatocarcinogenesis (Bulle et al., 1990; Ramakrishnan et al., 2007). The increased activities of AST, ALT, ALP and LDH observed in the present study are indicators for NDEA-induced liver damage and HCC. A significant increase in the concentration of serum bilirubin in NDEA treated rats is also consistent with the presence of hepatic damage (Bulle et al., 1990; Singh et al., 2009). Alpha-fetoprotein is a tumour marker produced by regenerating hepatic tumours. Several studies have documented that exposure of rats to certain carcinogens such as NDEA causes an elevation of circulating AFP levels (Sivaramakrishnan et al., 2008; Singh et al., 2009; Taha et al., 2010).

Administration of dieckol attenuated NDEA induced hepatocarcinogenesis, as shown by the reverted activities of AST, ALT, ALP, LDH, and decreased the concentration of bilirubin and α-fetoprotein. Our results suggest that dieckol aids in parenchymal cell regeneration in liver, thus protecting liver cell and membrane integrity by scavenging free radicals and enhancing antioxidant status, thereby decreasing enzyme leakage and hinder the process of carcinogenesis. Several studies provide evidence that could decrease the liver marker enzymes in serum during chemically-induced hepatotoxicity by inhibiting free radicals and lipid peroxidation and enhancing antioxidants (Lee et al., 2004; Renugadevi et al., 2010).

In conclusion, administration of dieckol effectively suppressed the NDEA-initiated hepatocarcinomas and its preneoplastic lesions by modulating xenobiotic-metabolizing enzymes (XMEs) and attenuated lipid peroxidation through scavenging of free radicals and enhancing antioxidant status and reverting liver marker enzymes and this could be the initial step in the prevention of HCC in Wistar rats.
Further studies are underway to elucidate the chemopreventive effects of dieckol on molecular mechanisms such as cell proliferation, apoptosis, invasion, metastasis and angiogenesis during NDEA-induced hepatocellular carcinoma.