CHAPTER V

CHEMOPREVENTIVE POTENTIAL OF *MATRICARIA CHAMOMILLA* THROUGH 7, 12- DIMETHYLBENZ (A) ANTHRACENE INDUCED ORAL CARCINOMA IN C57 BLACK MICE

5.1 INTRODUCTION

The imbalance in the antioxidant system creates oxidative stress [304]. Reactive Oxygen Species (ROS) and free radicals were formed in body as a result of normal respiration and it will be neutralized by natural antioxidant systems present in the body [305]. ROS also results from stress, radiation and toxins etc. Over production of ROS leads to the imbalance in natural antioxidant systems and leads to various disease conditions such as diabetes, cancer, atherosclerosis, neurodegenerative disorders and aging process [306]. DNA damage by ROS also leads to serious illness like cancer and other diseases. ROS induces DNA mutations causes G → T transversions [307], which ultimately leads to frequent mutations in p53 supressor genes [308]. Oral cavity is also susceptible to ROS due to the various habits such as tobacco smoking and inhalation of free radicals [309]. Oral cancer results from the increased oxidative and nitrosative stress along with the antioxidant system disturbances [310, 311]. Betel quid chewers maintain alkaline conditions in the oral cavity due to the presence of calcium hydroxide in the lime lead to the increase in ROS generation [312]. Continuous intake of pan masala and gutkha induces chronic inflammation and oxidative stress which further leads to cell proliferation, miscoding DNA adduct and inhibition of DNA repair [313].

The lungs and urine of cigarette smokers has found to contain 8-OHdG, a biomarker of oxidative stress, cancer and diabetes.
Antioxidants are the potential agents which prevent or postpone the degenerative disorders due to oxidative stress [314, 315].

Living systems were equipped with natural antioxidant systems, which may be enzymatic (CAT, SOD and GPx) or non-enzymatic (Vit C, Vit E, Carotenoids and Polyphenols) [316]. The oxidative stress was lowered by the following two mechanisms to protect the cell membrane from injury.

(a) Detoxification of ROS via CAT, GPx and SOD up regulation.

(b) Arresting the generation of free radicals [317].

Synthetic forms of antioxidants like butylated hydroxyl toluene, butylated hydroxyl anisole, tertiary butylated hydroxyl quinone and garlic acid esters have some negative side effects such as radio-sensitization, increased toxicity of other chemicals and mutagenic activity, etc and are too costly [318]. There is a necessity in the search of the natural antioxidants. Plants, spices and herbs are rich in phytochemicals such as flavonoids, phenols tend to possess antimicrobial, antiallergic, anticarcinogenic and antiaging properties [319, 320]. There is a promising research proceeds in search of natural antioxidant system in plants. It was recently demonstrated that turmeric down regulates COX-2 in oral premalignant and cancer cells in vitro conditions due to the presence of a yellow pigment, curcumin [321]. In vitro antioxidant analyses are the preliminary screening methods. Whereas for the preclinical studies, the native micro environment resides in the animals has more advantage than in vitro conditions. The phytochemicals effective in in vitro conditions may not give similar results in the in vivo conditions due to the interference in physiological interactions such as absorption, distribution, storage and excretion [322]. Matricaria chamomilla, belong to Asteraceae family are the most popular herb. The flowers of M. chamomilla contained several groups
of therapeutic compounds. It was used in the treatment of irritable bowel syndrome, sore stomach and cholesterol-lowering effects etc., [323]. This study was designed to analyze the *in vivo* anticancer and antioxidant activity of *M. chamomilla* in C57 black mice.

5.2 MATERIALS AND METHODS

5.2.1 ANIMALS

The C57 black mice, 4-6 weeks old and weighing about 25–30 g were purchased from SreeVenkateshwara Enterprises Pvt Ltd, Bangalore, and maintained in Central Animal House, at the KMCH College of Pharmacy, Coimbatore, India.

The C57 black mice were maintained in the cages made of polypropylene. The mice were given with the standard diet and water and 12 hrs light and dark cycle with the proper temperature and humidity. The study was approved by Institutional Animal Ethics Committee (Register number 685/Po/02/a/CPCSEA). The C57 black mice were maintained according to the guidelines of the Ethical Committee for Animal Care of KMCH College of Pharmacy and Indian National Law on animal care and use.

5.2.2 CHEMICALS

The carcinogen, 7,12-dimethylben[a]anthracene (DMBA) were purchased from Sigma Aldrich Chemical Pvt Limited, Bangalore, India. All the other chemicals obtained are of analytical grade.

5.2.3 EXPERIMENTAL DESIGN

The black mice in the first group were served as control. It was rubbed with liquid paraffin for 14 weeks, three times per week on left buccal pouches.

The animals in group II was served as toxic control and were rubbed with DMBA (0.5%) mixed in liquid paraffin for 14 weeks, three times per week.
The animals in group III C57 served as standard group and were given orally 5 fluorouracil 20 mg/kg daily and rubbed with DMBA (0.5%) mixed in liquid paraffin for 14 weeks, three times per week.

Group IV and group V animals were treated with the doses of *M. chamomilla* extracts orally, 250 and 500 mg per kilogram body weight per day respectively. The treatment was started before one week of tumor induction and continued on alternative days to 0.5% DMBA in liquid paraffin painting, until the sacrifice of the C57 black mice.

The experiment was ended after 14 weeks. All mice were sacrificed by cervical dislocation after anesthesia given between 7-9 AM, after overnight fasting. The blood and buccal cavity of the treated and control groups were selected for the various morphological, biochemical and antioxidant parameters.

**Table 5.1: Experimental animal groupings**

<table>
<thead>
<tr>
<th>Group I</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>Tumor control</td>
</tr>
<tr>
<td>Group III</td>
<td>Treatment with standard drug (5-Fluorouracil) 20 mg/kg body weight</td>
</tr>
<tr>
<td>Group IV</td>
<td><em>M. chamomilla</em> extract (250 mg/kg body weight)</td>
</tr>
<tr>
<td>Group V</td>
<td><em>M. chamomilla</em> extract (300 mg/kg body weight)</td>
</tr>
</tbody>
</table>

5.2.4 Assessment Of Morphological Parameters

**5.2.4.1 Body Weight**

The body weight of C57 black mice were recorded every week from the day of tumor induction and compared with the untreated mice and the results were recorded [324].

**5.2.4.2 TUMOR AND LIVER WEIGHT**
The animals in all the groups were dissected and the weight of the tumor and liver were calculated. Also other parameters such as tumor incidence, burden and latency were also noted [325].

### 5.2.5 ASSESSMENT OF BIOCHEMICAL PARAMETERS

#### 5.2.5.1 DETERMINATION OF ASPARTATE AMINOTRANSFERASE (AST)

Aspartate aminotransferase, also called Glutamate Oxaloacetate Transaminase (GOT) converts L-aspartate and \( \alpha \) ketoglutarate and form the products, oxaloacetate and L-glutamate by transamination reaction. The compound 2, 4- Dinitrophenyl hydrazine forms hydrazone in coupling reaction with the formed oxaloacetate. The resulting brown colored complex can be measured colorimetrically.

#### 5.2.5.1.1 REAGENTS

Buffered aspartate (pH 7.4); 2,4- DNPH reagent; 4N sodium hydroxide; working pyruvate standard; solution I (prepared by diluting one part of reagent 3 - 10 parts of purified water).

#### 5.2.5.1.2 PROCEDURE

The analysis included for this study was the blank, standard, test (for each serum sample) and control (for each serum sample). The 0.25 ml of buffered aspartate was added in all the test tubes. The test serum was added to test group tubes and 0.05 ml of working pyruvate standard into the standard tubes. After proper mixing, all the tubes were kept for incubation for 60 minutes at 37\(^\circ\)C, after which 0.25 ml each of 2, 4- DNPH was added into all the test tubes. Then, 0.05 ml of distilled water and 0.05 ml of each serum sample was added to the blank and the serum control tubes respectively. The mixture was then incubated at room temperature for 20 mins and 2.5 ml from the solution I was added to all test tubes. It was mixed and optical density was measured in a spectrophotometer at 505 nm within 15 minutes [318].

The enzyme activity was calculated as
\[
AST \left( \frac{IU}{L} \right) = \frac{Ab(T) - Ab(C)}{Ab(S)} - Ab(B) \times \text{concentration of standard}
\]

5.2.5.2 DETERMINATION OF ALANINE AMINOTRANSFERASE (ALT)

Alanine amino transferase, also known as Glutathione Peroxidase (GPT) converts L-alanine and α ketoglutarate and form pyruvate and L-Glutamate by transamination reaction. The formed pyruvate reacts with 2, 4–Dinitrophenyl hydrazine resulting in the formation of the hydrazone complex. The resulting brown colored complex can be measured colorimetrically.

5.2.5.2.1 REAGENTS

Buffered alanine (pH 7.4), 2,4–DNPH, 4N sodium hydroxide, working pyruvate standard, solution I (prepared by diluting 1 part of reagent 3 to 10 parts of purified water).

5.2.5.2.2 PROCEDURE

The systems used in this study included blank, standard, test (for each serum sample) and control (for each serum sample). The 0.25 ml of buffered alanine was added into all test tubes. This was followed by 0.05 ml of serum in the test group tubes and 0.05 ml of working pyruvate standard into the standard tubes. After proper mixing, all the tubes were incubated for 60 minutes at room temperature, after which 0.25 ml each of 2,4-DNPH reagent was added into all test tubes. Then, 0.05 ml of distilled water and 0.05 ml of each serum sample was added to the blank and the serum control tubes respectively. The solution was incubated at room temperature for 20 mins and 2.5 ml from the solution I was added and mixed properly. The optical density was read at 505 nm within 15 minutes in a spectrophotometer with purified water as a standard [326].
\[ ALT \left( \frac{IU}{L} \right) = Ab(T) - \frac{Ab(C)}{Ab(S)} - Ab(B) \times \text{Concentration of standard} \]

5.2.5.3 DETERMINATION OF ALKALINE PHOSPHATASE (ALP)

The enzyme alkaline phoshatase present in the serum forms inorganic phosphate and phenol from phenyl phosphate at pH 10. The phenol combines with 4-aminoantipyrine and an oxidizing agent potassium ferri cyanide to form an orange-red complex in alkaline conditions. The intensity of the color is proportional to the enzyme activity which can be measured spectrometrically.

5.2.5.3.1 REAGENTS

Buffered substrate, chromogen reagent and phenol standard

5.2.5.3.2 PROCEDURE

The working solution was prepared by reconstituting a single vial of buffered substrate with 2.2 ml of water. It was followed by adding 0.5 ml of working buffered substrate and 1.5 ml of purified water in to the test tubes. The test tubes were mixed properly and then incubated at 37°C for about 3 mins. Then 0.05 ml of serum and phenol standard were added to test and standard tubes respectively. It was again mixed well and then incubated for 15 minutes at 37°C. Thereafter, one ml of chromogen reagent and 0.05 ml of serum was added to the control tube. It was mixed and the optical density of all the tubes was read against purified water at 510 nm [327].
\[ ALP (KA) = OD(\text{Test}) - OD(\text{Control})/OD(\text{Standard}) - OD(\text{Blank}) \times 10 \]

5.2.5.4 DETERMINATION OF PROTEINS

This method is a combination of both Folin-ciocalteau and Biuret reaction which involves two step reactions. Protein binds with the copper in alkaline medium and reduces it to Cu++ in first step reaction. The formed Cu++ catalyses the oxidation reaction of the aromatic amino acids by the reducing reaction of Phosphomolybdotungstate to heteropolymolybdanum, which leads to the formation of blue color which is measured at 640 nm in the second step reaction.

5.2.5.4.1 REAGENTS

Alkaline copper reagent
Solution A: 2% w/v of sodium carbonate in 0.1 N NaOH.
Solution B: 0.5% w/v copper sulphate in 1% sodium potassium tartarate
50 ml of solution A was mixed with 1 ml of solution B just before use.

Folin’s phenol reagent commercial reagent, 1:2 dilutions bovine serum albumin (BSA).

5.2.5.4.2 PROCEDURE

The 0.1 ml of liver homogenate was mixed with 0.9 ml of water and 4.5 ml of alkaline CuSO₄. The solutions were maintained at 37°C for about 10 minutes after which 0.5 ml of Folin’s reagent was added and incubated for 20 mins. The intensity of the blue color formation represents the concentration of the protein measured at 640 nm and expressed in mg/g of tissue [328].

5.2.5.5 ESTIMATION OF UREA
Urea is the product of the catabolism of protein containing nitrogen. The increased level of blood urea is called hyper uremia. The urea estimation was analyzed by the method of Urease-GLDH: enzymatic UV test.

**5.2.5.5.1 REAGENTS**

Reagent 1: 120 mmol/L TRIS (pH 7.8), 7 mmol/L 2-oxoglutarate, 0.6 mmol/L ADP, 6 KU/L Urease and 1 KU/L GLDH
Reagent 2: 0.25 mmol NADH
Reagent 3: 40 mg/dl standard

**5.2.5.5.2 PROCEDURE**

To the one ml of reagent 1, 250 µl of reagent 2 were added. To this 10 µl of serum of test animal were added, mixed well and the reading were represented in mg/dl [329].

**5.2.5.6 ESTIMATION OF URIC ACID**

The uric acid formed during the purine metabolism as the end product. The increased uric acid levels in blood serum leads to the mono sodium urate crystal formation, accumulated around the joints is the most common complication of gout. Uric acid estimation was done by the method of TOOS enzymatic photometric test.

**5.2.5.6.1 REAGENTS**

Reagent 1: 100 mmol/L Phosphate buffer (pH 7), 1 mmol/L TOOS, 1 KU/L Ascorbate oxidase

Reagent 2: 100 mmol/L Phosphate buffer (pH 7), 0.3 mmol/L 4-amino antipyrine, 10 µmol/L K₄(Fe(CN)₆), 1 KU/L Peroxidase, 50 U/L Uricase

**5.2.5.6.2 PROCEDURE**
To the 800 µl of reagent 1, 20 µl of test serum were added. It was then incubated at 30°C for 5 mins. To this 200 µl of reagent 2 was added and again incubated at 37°C for 5 minutes and the readings were recorded in mg/dl [329].

5.2.6 ASSESSMENT OF ANTIOXIDANT ENZYMES

5.2.6.1 PREPARATION OF TISSUE HOMOGENATE

After treatment with methanol extracts of plants, the rats were sacrificed, liver of mice were isolated and washed with normal saline and stored for 12hrs for \textit{in vivo} antioxidant studies. The separated liver was homogenized with Teflon coated homogenizer with 0.1M Tris-HCl buffer of pH 7.4 to obtain 10% homogenate. The resulting mixture was centrifuged at 10000 rpm for 10 minutes at 5°C. The supernatant was collected and used for \textit{in vivo} studies.

Antioxidant enzymes such as SOD, CAT, GPx, GSH, Vitamin C and LPO were determined in all the liver tissues of all the groups of mice.

5.2.6.2 ESTIMATION OF SOD

Estimation of SOD was done based on the inhibition of oxidation of adrenaline to adrenochrome by the enzyme SOD. Liver homogenate (0.5 ml) was blended with 0.5 ml of water, 0.25 ml of ethanol and 0.15 ml of chloroform. The solution was shaken for one minute and then centrifuged at 2000 rpm and the supernatant was analyzed to estimate the concentration of enzyme. To the 0.5 ml of supernatant, 1.5 ml of buffer and 0.4 ml of epinephrine were added to initiate the reaction and OD per minute was measured at 480 nm in a UV-VIS spectrophotometer (UV 1700, Szhimadzhu).

“The change in optical density per minute at 50% inhibition to adrenochrome transition by the enzyme is taken as one enzyme unit and SOD activity was expressed in U/mg” [330].

5.2.6.3 ESTIMATION OF CATALASE
Estimation of catalase was based on the rapid decomposition of hydrogen peroxide generated during the β-oxidation of fatty acids by flavoprotein dehydrogenase. To the 0.1 ml of liver homogenate, one ml of phosphate buffer and H$_2$O$_2$ were added. The reaction was inhibited by adding 0.2 ml dichromate acetic acid reagent. The standard solution was H$_2$O$_2$ and it was taken in the range of 4 to 20 µl. The above solutions were kept in the water bath for about 10 mins and the OD value was measured spectrophotometrically at 570 nm (UV 1700, Szhimadzhu).

“Catalase activity in liver homogenate is expressed as nmoles of H$_2$O$_2$ consumed per minute per mg of protein at 37°C” [331].

### 5.2.6.4 LIPID PEROXIDATION

In this method, an indicator of oxidative stress, malondialdehyde and other TBARS were estimated by their reaction with thiobarbituric acid (TBA) in an acidic condition and form a pink coloured chromophore.

To the one ml of liver homogenate, 0.2 ml of 4% SDS, 1.5 ml of acetic acid in HCl of pH 3.5 and 15 ml of 0.8% TBA of pH 7.4 were added. The above mixtures were kept in a boiling water bath for about 1 hr. The resulting tube was centrifuged at 1200 g for about 10 minutes. The pink colored complex was read against a blank at 532 nm.

“The LPO was expressed as $n$ moles of MDA per mg of protein with 1,1,3,3,-tetra-ethoxypropane as the standard”[332].

### 5.2.6.5 GPx

The glutathione was calculated by the reaction with 5,5- dithio-bis-(2-nitro benzoic acid) [DTNB] which develops a product that absorbs at 412 nm. To the 0.1 ml of liver homogenate, EDTA, sodium azide, reduced glutathione and H$_2$O$_2$ were added. To this 0.4 ml of buffer were mixed and maintained at 37°C for 10
mins. To this, 0.5 ml of TCA was added to arrest reaction and then the tubes were centrifuged. The supernatant was collected and 3ml of sodium hydrogen phosphate, 1 ml of DTNB were added to the above mixture and the optical density was read at 412 nm.

“Glutathione peroxidase activity, in serum is expressed as μg/mg” [333].

5.2.6.6 GSH

The reaction between DTNB and GSH forms a compound 2-nitro-5-thiobenzoic acid and GSSG, where 2-nitro-5-thiobenzoic acid yield a stable yellow colored complex, which is proportional to GSH concentration.

To the 250 μL of tissue homogenate, 1 ml of 5% TCA was added and the above solution was centrifuged at 3000 g for about 10 minutes at room temperature. To 250 μL of the above supernatant, 1.5 ml of 0.2 M phosphate buffer was added and mixed well. The 250 μL of 0.6 mM of Ellman’s reagent (DTNB solution) was added to the above mixture and the optical density was taken at 412 nm within 10 minutes.

“The amount of glutathione expressed as μg/mg protein” [334].

5.2.6.7 VITAMIN C

To the 0.5 ml of homogenate, 1.5ml of 6% TCA2 was added. The mixture was centrifuged at 6000 rpm for 20 mins. The supernatant6 was collected and 0.5 ml of DNPH reagent was added and maintained for 3 hrs at 37°C. To this solution, 2.5 ml of H₂SO₄ was added and the readings were taken at 530 nm.

5.2.7 HISTOPATHOLOGY OF LIVER, KIDNEY AND TUMOR

The isolated liver, kidney and tumor tissues were immediately fixed with 10% formalin, dehydrated in increasing concentration of ethanol, cleared in the xylene solution
and paraffin embedded. The prepared tissue sections were cut into 2-3 µm pieces by using rotary microtome. The prepared sections were stained by using hematoxylin-eosin dye for photomicroscopic observations [335].

5.2.8 IHC MARKERS
5.2.8.1 COX-2

Tumor sections were immuno stained with the COX-2 primary monoclonal antibody of rabbit obtained from BioGenex Life Sciences Pvt. Ltd., India. The prepared tissues were added with the primary antibody and it was incubated in a humid chamber for overnight at 4º C. After incubation, the tissues were stained by labeled HRP as a secondary antibody. The bounded enzyme was revealed using DAB in Tris buffered saline. The sections were then processed by dehydration, clearing and mounting. The cells showed the positive staining for COX-2 was counted manually. The percentage of stained cells in each group was counted in the selected hot spots.

“The COX-2 expression was indicated in the terms of percentage” [336].

<table>
<thead>
<tr>
<th>STAINING</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative staining</td>
<td>-</td>
</tr>
<tr>
<td>Positive staining (Grade I)</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Positive staining (Grade II)</td>
<td>5-30%</td>
</tr>
<tr>
<td>Positive staining (Grade III)</td>
<td>&gt;30%</td>
</tr>
</tbody>
</table>

5.2.8.2 p53 and Ki-67
The expression of p53 and Ki 67 were analyzed by the following method. The selected tissue sections were deparaffinized with xylene. The rehydration of the tissue sections were done with the increasing concentrations of ethanol. The rehydrated tissues were then kept in 3% H$_2$O$_2$ dissolved in methanol for about 10 minutes to cut off the activity of the endogenous peroxidase. The sections were blocked with nonspecific antibody by mixing in 1% BSA diluted with PBS for about 20 minutes. The processed tissues were incubated with the PAb 240, an anti-p53 monoclonal antibody of mouse at 1:50 dilution at room temperature for 2 hrs. The tissues were washed with PBS and treated with antimouseIgG conjugated with HRP for 30 mins at room temperature. The diaminobenzidine tablet was used to develop the color (Sigma). The development of brown precipitate formed by nucleus shows the presence of p53 protein. The slides were counter stained with Mayer's haematoxylin, DPX mounted and observed by light microscopy [337].

The percentage of positively stained cells were determined and expressed in the percentage.

### Table 5.3: Grading of oral cancer by p53

<table>
<thead>
<tr>
<th>Positive staining for p53</th>
<th>Oral carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;50%</td>
<td>+++</td>
</tr>
<tr>
<td>26-50%</td>
<td>++</td>
</tr>
<tr>
<td>5-25%</td>
<td>+</td>
</tr>
<tr>
<td>&lt;5%</td>
<td>-</td>
</tr>
</tbody>
</table>

In oral dysplasia and oral cancer, tissue sections less than 5% positive were considered as negative.

### 5.2.9 STATISTICAL ANALYSIS

All the data expressed are mean ± standard deviation of six mice in five groups. All the grouped data are statistically evaluated with SPSS/19 software.

### 5.3 RESULT AND DISCUSSION
Figure 17: The control, standard, toxic control and M. chamomilla treated animal groupings
5.3.1 ASSESSMENT OF MORPHOLOGICAL PARAMETERS

5.3.1.1 BODY AND LIVER WEIGHT

The body and liver weight of the control and extract treated were observed after 18 weeks of cancer induction to analyze the toxicological effect of the plant extracts.

The body weight and liver weight of mice were observed to be almost equal to the treated (Group IV and V) mice, when compared to the Group I control. This concludes the non-toxicological effect of the plant extract. Increase or decrease in the weight concludes the disfunctioning of the organ.

Table 5.4: Effect of *M. chamomilla* on liver weight of C57 black mice

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CONTROL</th>
<th>ONLY DMBA</th>
<th>DMBA + STD</th>
<th>DMBA + EXT 250mg/kg</th>
<th>DMBA + EXT 500mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUMOR WEIGHT (gm)</td>
<td>2.302±0.346</td>
<td>3.198±0.105</td>
<td>3.221±0.126</td>
<td>3.031±0.126</td>
<td>2.302±0.346</td>
</tr>
</tbody>
</table>

Figure 5.2: Body weight analysis of treated and untreated C57 black mice
5.3.1.2 TUMOR LATENCY, INCIDENCE, BURDEN AND WEIGHT

Tumor incidence was decreased (68.67%) in the *M. chamomilla* treated animals (Group V) when compared to other groups.

Tumor burden were significantly decreased in low dose MC treated group (Group IV) and tumor latency were extended for groups III, IV and V when compared to group II. These results clearly indicate the chemopreventive activity of MC against oral cancer.

According to the literature, the life prolongation of an animal, decrease in the volume of the tumor and viable cell count are some of the important characteristics of the anticancer agent [338-341].
5.3.2 ASSESSMENT OF BIOCHEMICAL PARAMETERS

The biochemical enzymes (SGOT, SGPT, and ALP) and total protein were analyzed to confirm the normal functioning of liver. The biochemical liver marker enzymes and total protein were reduced in the *M. chamomilla* treated groups when compared to the DMBA induced mice and the level of enzymes were maintained almost equal to the healthy mice (Group I).

The methanol extract of *M. chamomilla* restored the enzyme levels SGOT (42.67 U/L), SGPT (145.33 U/L), ALP (162.67 U/L) and total protein (0.161 ± 0.0056) in the high dose treated groups (Group V).
The change in the serum enzymes indicates liver damage and the disturbances of cell metabolism [342]. The restorations of liver enzymes in the *M. chamomilla* pre-treated groups maintain the enzyme level almost equal to the healthy mice indicates the anticancer activity of *M. chamomilla*. The elevation in the DMBA treated group may proceed to secondary carcinoma and metastasis [343].

The level of liver protein were significantly decreased in 5-FU treated mice (0.199), *M. chamomilla* treated groups (Group IV- 0.232± 0.0204 and Group V 0.161 ± 0.0056) when compared to the DMBA induced tumor groups (Group II 0.318± 0.011). The increase in protein level is an indicator of inflammation of liver. The comparison of results of protein estimation for the control and treated groups, it is confirmed that *M. chamomilla* can prevent the liver damage caused during the cancer.

### 5.3.2.1 EFFECT OF MC EXTRACT ON SGOT, SGPT AND ALP IN C57 BLACK MICE
5.3.2.2 UREA AND URIC ACID

The reduction in the urea and uric acid levels in *M. chamomilla* treated groups were observed when compared to the DMBA tumor induced group indicates the normal functioning of kidneys. Group II animals showed the higher level of urea (57.1± 2.802) and uric acid (4.33 ± 0.165) indicates the nephrotoxicity. Groups III, IV and V restored the levels of urea and uric acid represents the prevention of kidney damage by the 5-FU and *M. chamomilla*.

**TABLE 5.7: EFFECT OF M. CHAMOMILLA EXTRACT ON UREA AND URIC ACID IN TUMOR INDUCED C57 BLACK MICE**
5.3.3 ASSESSMENT OF ANTIOXIDANT ENZYMES

The present analysis was evaluating the *in vivo* antioxidant activity of methanolic extract of *M. chamomilla* in DMBA induced oral carcinoma in C57 black mice.

5.3.3.1 LIPID PEROXIDATION

The levels of basal lipid per oxidation were significantly increased in toxic control (Group II) might be due to the increase in the free radicals generation during cancer induction. The lipid peroxidation level decreased in the pretreated groups (Groups III, IV & V) and represents the scavenging activity of standard drug and the extract treated groups towards the free radicals.

ROS production increases when there is an imbalance in the natural antioxidant defense mechanisms. LPO oxidizes the polyunsaturated fatty acids which causes long term damage of cells [335, 336]. MDA, a lipid peroxidation product indicates the level of ROS generation in tissue [337]. Increased LPO decrease the cell membrane fluidity and membrane bound enzymes and receptors were disturbed [338]. In our study, the LPO decreased in the pre-treated group indicate the antioxidant activity of *M. chamomilla*. 

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>only DMBA</th>
<th>DMBA + STD</th>
<th>DMBA+EXT 250 mg/kg</th>
<th>DMBA+EXT 500 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea(mg/dl)</td>
<td>20.03±2.793</td>
<td>57.1±2.802</td>
<td>17.77±1.109</td>
<td>28.53±0.659</td>
<td>23.17±1.514</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>3.5±0.2033</td>
<td>4.33±0.165</td>
<td>2.267±0.111</td>
<td>2.53±0.1115</td>
<td>2.033±0.0558</td>
</tr>
</tbody>
</table>
Table 5.8: Effect of *M. chamomilla* extracts on LPO in C57 black mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>LPO (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.131 ±0.002</td>
</tr>
<tr>
<td>Toxic control</td>
<td>0.147 ±0.004</td>
</tr>
<tr>
<td>Standard drug</td>
<td>0.110±0.004</td>
</tr>
<tr>
<td><em>M. chamomilla</em> (250 mg/kg body weight)</td>
<td>0.132±0.001</td>
</tr>
<tr>
<td><em>M. chamomilla</em> (500 mg/kg body weight)</td>
<td>0.096±0.008</td>
</tr>
</tbody>
</table>

### 5.3.3.2 SOD, CAT and GPx

The levels of SOD and CAT were increased in toxic control (Group II) when compared to the normal mice shows the increase in the free radical formation. The levels in the standard drug (Group III) and extract treated (Groups IV and V) shows significant reduction in the enzyme levels represents the free radical scavenging activity of the plant extracts.

The levels of GPx increased in the group II, nearing to normal in groups III and IV and decreased in group V shows the markedly prevented DMBA induced alteration and maintained enzyme levels of the *M. chamomilla* extract.

SOD is a metallo enzyme requires zinc and copper for its activity. CAT enzymes are heme containing proteins [344]. Both enzymes protect the cells from ROS. GPx also decreases lipid hydroperoxides by attacking polyunsaturated fatty acids in cell membrane [348]. The enzymatic antioxidants (SOD, CAT and GPx) decreases in pre-treated group of *M. chamomilla* high dose (500 mg/kg body weight) indicate the preventive antioxidant activity.
5.3.3.3 GSH AND VIT C

The level of GSH in standard drug (Group III) and *M. chamomilla* pre-treated groups (Groups IV and V) showed the lower GSH levels than the toxic control animals (Group II) indicate the antioxidant activity of *M. chamomilla*.

The lower content of Vitamin C in pre-treated group (Group IV) showed the hydroxyl scavenging activity of *M. chamomilla*.
GSH, an intracellular non protein sulphydryl compound is the hydrophilic antioxidant [349]. In our study, a decreased GSH level in the pretreated group shows the preventive formation of ROS. Also Vit C is an antioxidant prevents cataracts and the cancers of throat, oral, stomach and pancreas [350]. Our results also confirmed the above statement.

### 5.3.4 HISTOPATHOLOGY

#### 5.3.4.1 LIVER

Histopathological studies of the liver sections from control groups (Group I) shows normal lobular architecture. Individual hepatocytes show binucleation and cystoplasmic vacuolation. Portal triad appears no significant pathology. The central vein and sinusoids exhibits mild dilatation. Liver sections from the toxic control (Group II) hepatocytes appear binucleation and cytoplasmic vacuolation. Hepatic parenchyma shows interface hepatitis. Portal triad exhibits mild periportal inflammation. The central vein and sinusoids have mild dilatation. Standard group (Group III) appears no significant pathology. Liver section from the extract treated at low dose shows normal lobular architecture. Individual hepatocytes have binucleation and cytoplasmic vacuolation. Portal triad exhibits portal triaditis. The central vein and sinusoids shows mild dilatation. High dose of *M. chamomilla* extract treated sections of the liver appears normal lobular architecture.
Figure 5.6: Histopathology of liver cells (A) Control (B) Toxic control (C) Standard drug (D) *M. chamomilla* extract 250 mg/kg (E) *M. chamomilla* 500 mg/kg in DMBA induced oral carcinoma in C57 black mice
5.3.4.2 KIDNEY

The histopathological studies of kidney section from control group (Group I) shows normal cortex and medulla. The glomeruli appear normal morphology. Both the tubes show no significant pathology. The interstitium exhibits within normal limit. Blood vessels observed as mild congestion.

The section from Group II shows normal cortex and medulla. The glomeruli show global mesangeal hypercellularity. Both the tubules appear no significant pathology. The interstitium exhibits within limit. Blood vessels show mild congestion.

The sections from the standard drug treatment (Group III) normal cortex and medulla. The glomeruli appear normal morphology. Both the tubules show no significant pathology. The interstitium exhibits within normal limit. Blood vessels show mild congestion.

The sections from the M. chamomilla treated groups (Groups IV and V) appear normal cortex and medulla. The glomeruli show global mesangeal hypercellularity. Both the tubules observed as no significant pathology. The interstitium appears within normal limit. Blood vessels exhibit mild congestion.

There was no necrosis observed in the kidney and liver of the animals treated with apaziquone and pyrithione zinc respectively. This shows the anticancer activity of the compounds against oral cancer [101,351].
Figure 5.7: Histopathology of kidney cells (A) Control (B) Toxic control (C) Standard drug (D) *M. chamomilla* extract 250 mg/kg (E) *M. chamomilla* 500 mg/kg in DMBA induced oral carcinoma in C57 black mice
5.3.4.3 TUMOR

The normal and tumor cells can be distinguished morphologically by microscopic observations [343].

The section from group II shows lymphoid tissue with areas appearing seromucinous glands. The observations concluded the circumcised lesion composed of cells arranged in closed crowded glands in back to back fashion change in the shape and number of cells. Surrounding stroma appears scattered lymphocytic infiltrates.

The section from group III exhibits fragment of lymphoid tissues with areas showing normal seromucinous glands. No evidence of malignancy/ granuloma.

The section from group IV appears fragment of lymphoid tissues with areas showing normal seromucinous glands. No evidence of malignancy/ granuloma.

The section from group V had lymphoid tissues with areas appearing normal seromucinous glands. Individual cells are having clear cytoplasm with uniform vesicular nuclei, powdery chromatin and inconspicuous nucleoli. No mitosis/necrosis is identified. Surrounding stroma appears scattered lymphocytic infiltrates.
Figure 5.8: Histopathology of tumor cells (B) Toxic control (C) Standard drug (D) *M. chamomilla* extract 250 mg/kg (E) *M. chamomilla* 500 mg/kg in DMBA induced oral carcinoma in C57 black mice

5.3.5 IHC MARKERS

5.3.5.1 COX 2

The expressions of COX 2 were analyzed in all the five groups and the results were represented in table 5.8 and figure 5.9.
Table 5.11: Expression of COX 2, p 53 and Ki 67 in treated and untreated C57 black mice

<table>
<thead>
<tr>
<th>IHC MARKERS</th>
<th>COX 2</th>
<th>p53</th>
<th>Ki 67</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP I</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GROUP II</td>
<td>3+</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>GROUP III</td>
<td>2+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GROUP IV</td>
<td>1+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GROUP V</td>
<td>3+</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The higher expressions of COX 2 were observed in the group II (Grade 3) followed by groups III and V (Grade 2) and the least expression was observed in group IV (Grade 1). COX 2, an early response gene which is induced only in certain conditions like oncogenes, UV radiation and carcinogens, etc. [353, 354]. It is usually under-expressed in normal conditions and up regulated in pathological conditions [355] due to the inactivation of tumor suppressor genes (p53), proto-oncogene activation (Ras) and EGF receptor activation [356, 357]. There are many researches available to report the upregulation of COX 2 in oral cancers [358, 359].

COX 2 possesses an important role in the progression of cancer. It act directly on cancer cells, increase mitosis and thereby cell division and indirectly by nurturing blood vessels which leads to immune system modulation [360]. COX 2 regulates the Vascular Endothelial Growth Factor (VEGF) expression in head and neck cancer [361].
5.3.5.2 p53

p53, a tumor suppressor gene, regulate the growth and proliferation of cells [362]. It is very hard to detect p53 in normal cells and can be activated only during the malignant conditions [363]. According to our results, p53 was undetectable in groups I, III, IV, V and highly expressed in group II (Grade 2). This result was correlated with the observations of Xie et al.,[364] stated that 61% of tongue carcinoma people expressed p53 and mutations of p53 were observed in OSCC patients [365].

Figure 5.9: Immunohistochemical staining of oral cancer tissues for the expression of COX 2 in C57 black mice
p53 arrests cell cycle when there is an DNA damage [366]. There is an close association between the p53 expression and grading of cancer. The expression of p53 increases with the stages of cancer [367-369].

Figure 5.10: Immunohistochemical staining of oral cancer tissues for the expression of p53 in C 57 black mice

5.3.5.3 Ki-67

Ki-67, nuclear non-histone protein expressed only in G2 and M phases of mitosis and undetectable in the resting stages of cell cycle [370]. There are large number of literatures suggesting the relationship between Ki 67 and tumor [371]. Our results correlate with the previous results, that Ki 67 protein was undetectable in groups I, III, IV, V and expressed in group II. The low levels of Ki 67 expression were observed in the normal oral epithelium and high expression represents the poor oral health [372].
5.4 CONCLUSION

The morphological, biochemical and antioxidant parameters in the *M. chamomilla* pre-treated groups showed an excellent response in maintaining the enzymes normal to the control mice. The histopathological studies of liver, kidney and tumor tissues and immune histochemical markers also indicate the anticancer activity of *M. chamomilla*. 