Chapter 4

Biological evaluation of extracts of the selected *Diospyros* species.

Collection of plant material

The roots of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* were collected from local area of Ramghat located in the Western Ghat. The plants were identified by Prof S R Yadav, Taxonomist, Shivaji University, Kolhapur, Maharashtra and authenticated from Regional Research Institute, Bangalore, India. The voucher specimens, *D. oocarpa* (ADDO-1-12), *D. nigrescens* (ADDN-2-12) and *D. candolleana* (ADDC-3-12) were deposited in the herbarium of Visveswarapura Institute of Pharmaceutical Sciences, Bangalore.

Extraction of plant material

The roots of *Diospyros oocarpa, Diospyros nigrescens* and roots of *Diospyros candolleana* were collected & washed thoroughly under running tap water and they dried at 30-45ºC and powdered. The 500 gm of powdered material was extracted with 95% Ethanol using Soxhlet Apparatus. Extract was filtered and concentrated at low temperature to give the solid mass of EtOH extract (49.47 gm). Finally the extract was preserved in a closed container and kept in dessicator.

4.1. Evaluation of Acute Oral Toxicity– Acute Toxic Class Method[418,394,384]

The acute toxicity study of extract of roots of *Diospyros oocarpa, Diospyros nigrescens, Diospyros candolleana* was performed in accordance to the OECD guidelines-423. The female albino rats were used during investigation. The animals were

fasted for overnight prior to the experimental procedure. They were administered with test extract, orally and were observed for toxicity and death for 24 hours.

A. Principle of the test

It is a stepwise procedure using least number of animals per step to obtain sufficient information acute toxicity of the test substance to be able to classify it. definite doses is given orally to a group of experimental animals in steps. At each step three animals of a single sex (normally females) are used. Mortality of the animals dosed at one step will determine the next step with next higher or the next lower dose level.

B. Description of the method

a. Selection of animal species:

Preferably female rat are used in accordance to literatures of conventional LD_{50} tests, since females are generally slightly more sensitive. Healthy young adult Females employed, which are nulliparous and non-pregnant. For this study we employed female, nulliparous rats of 10 weeks old weighing 180-200 gm (190±10gm).

b. Housing and feeding conditions:

The temperature in the experimental animal room should be 22ºC (+ 3ºC) with relative humidity of at least 30% and more than 70% For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal. 12 hrs light and dark cycle was maintained. The animals were fed with standard laboratory chow diet and drinking water provided *ad libitum*.

c. Preparation of animals:

Animals were randomly selected and tagged for identification, and group in cages 5 days prior to commensing the study.

d. Preparation of doses:

An aqueous suspension of the drug using span 80% at varying doses (500, 1000, 2000 mg/) was prepared.

C. Procedure:

a. Administration of doses:

The test substance is administered in a single dose by gavage using a stomach tube or a suitable intubation canula. Animals were fasted overnight before dosing.

4.2. Cytotoxic Evaluation of the selected *Diospyros* species.

A. Chemicals and reagents:

Sl. No	Chemicals	Source
1.	7,12-diemthylbenz[a]anthracene (DMBA)	Sigma chemicals Co. USA
2.	12-O-tetradecanolyphorbol-13-acetate (TPA)	Sigma chemicals Co. USA
3.	Thiobarbituric acid	Himedia, Pvt. Ltd, Mumbai
4.	Diphenylamine	Himedia, Pvt. Ltd, Mumbai
5.	Di-sodium hydrogen phosphate	Himedia, Pvt. Ltd, Mumbai
6.	Potassium di-hydrogen phosphate	Merck Pvt. Ltd, Mumbai
7.	Sodium chloride	Himedia, Pvt. Ltd, Mumbai
8.	Tryphan blue solution (0.4%)	Himedia, Pvt. Ltd, Mumbai

All other chemicals used in the present study were of the analytical grade.

B. Instruments: Following instruments were used for the present study.

Sl. No	Equipment	Model & Make
1.	Micro pipettes	Accupipet Tarsons
2.	Micro centrifuge	Genei Pvt Ltd, Banglore
3.	Light Microscope	Pilot products
4.	CO_2 Incubator	Nuaire. NU-5500E
5.	Haemocytometer	Rohem, India
6.	Digital weighing balance	Ohaus Crop. Pine Brook, NJ USA
7.	Semi Auto analyzer	Star 21 Plus, Rapid Diagnositics
8.	Deep freezer	Blue Star
9.	Digital vernier caliper	Mitutoyo, Digimatic Caliper IP65
10.	Homogenizer	RQ 127A, REMI
11.	Sonicator	Fast Clean Ultrasonic Cleaner, Enertech Electronics
12.	UV Spectrophotometer	UV-1800, Shimazdu

4.2.1. Preliminary cytotoxicity screening of ethanolic extract of roots of *Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana* by Brine Shrimp Leathality assay

The preliminary pharmacological screening of ethanolic extract of roots of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* for cytoxicity was carried out by BSL assay as mentioned below.[164,193,105]

1. Saline was prepared by adding 38 g common salt per liter of water or sea water procured was used for hatching the eggs.
2. Saline was placed in small tank, shrimp eggs were added to one side of the divided tank i.e., in larger portion, and cover the tank. The lamp on the other side attracts young shrimp.
3. Allowed for two days for the shrimp to hatch and mature as nauplii (in warmer climates, hatching may take place sooner).
4. Different concentrations of test drugs 1000, 100, and 10 µg/ml were prepared for each extract; three vials at each concentration were prepared for a total of nine vials; weigh 20 mg of sample and add 2 ml of solvent (20 mg/2 ml); from this solution transfer 500, 50, or 5 µl to vials corresponding to 1000, 100, or 10 µg/ml, respectively. Solvent was evaporated. Alternatively, materials may be dissolved in DMSO (dimethylsulfoxide), and upto 50 µl may be added per 5 ml of brine before DMSO toxicity will affect the results.
5. Two days after (when the shrimp larvae are ready), about 4 ml of sea water is added to each vial, shrimps were counted to keep 10 shrimp per vial (30 shrimp per dilution), and the volume was adjusted with sea water to 5 ml/vial. Vials were placed

uncovered under the lamp. Care was taken to make sure that vials are not overheated by the lamp.

6. Twenty-four hour's later number of survivors were counted and recorded.[184]
7. The data was analyzed with the probit analysis to determine LC_{50}values and 95% confidence intervals.[62]
8. Percentage of mortality was calculated by following formula:

—— -----(a)

where, Mm_{ct} is mortality of individuals in time t [%]

N_{Mm} is average number of died individuals

N_0 is initial number of living individuals put into every concentration at the test start.

9. In case of 0 and 100 % of mortalities, the % of mortalities was then corrected by the following formulas (b & c) proposed by Ghosh.[99]

For 0 % mortality: 100 x (0.25×n) ------(b)

For 100 % mortality: 100 x (n− 0.25/n) ------(c)

where, n= no. of test animal in each group.

10. The Corrected % of mortality for each concentration was then transformed to probit by using Finney's[415] probit table (Table 4.1**).** The purpose of the probit transformation is to straighten the line so that the LC_{50} can be estimated more easily. The LC_{50} values (concentration of sample required to kill 50 % of brine shrimp) were calculated using graph by a plot of probit against the logarithm of the sample concentrations.

Table 4.1: Transformation of percentage of mortalities to probit.

S %	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

4.2.2. Pharmacological evaluation of for anti-tumor effect of ethanolic extract of roots of *Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana* against Dalton's Lymphoma Ascites by using *In-vitro* cytotocity test.

A. Preparation of Phosphate Buffered Saline (PBS) (pH 7.4)

238mg of disodium hydrogen phosphate, 19mg of potassium dihydrogen phosphate and 800mg of sodium chloride was dissolved in 100ml of distilled water.

B. Dalton's Lymphoma Cell line

Dalton's Lymphoma Ascites (DLA/DAL) cells were obtained from Amala Cancer Research Centre & employed for cytotoxicity study.

C. Preparation of DAL cells

0.2 ml of DAL cells retrieved from repository was washed by adding 0.8 ml of ice cold PBS pH 7.4 in 2 ml eppendorf tubes & centrifuged for 10 mins at 5000rpm. Procedure was repeated for 3 times. Viable cells were counted using tryphan blue dye exclusion. Cell population is adjusted to 1X 10^6 cells / 0.2 ml with PBS.

D. In-vitro cytotoxicity test

Principle:

The cytotoxic activity of ethanolic extract of roots of *Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana* against DAL cell line was examined *in- vitro* using tryphan blue dye exclusion assay. Trypan blue staining is a simple way to evaluate cell membrane integrity and thus assume cell proliferation or death. Trypan Blue is a blue acid dye, with two azo chromophore groups, which cannot cross into the cell

membranes of cells grown in culture. It is employed to estimate the number of viable cells present in a population.[66]

Procedure:

10 mg of each of the selected plant extracts and their isolates were taken in an Eppendorf tube of capacity 1 mL and diluted to six different concentrations using DMSO as a solvent and mixed with the help of a vortexing machine. The DAL cell suspension (l x 10^6 cells in 0.2 mL) was added to tubes containing various concentrations of the test extracts and isolates, and the volume was made up to 1 mL using phosphate buffered saline (PBS). Control tube contained only cell suspension in DMSO solvent. These assay mixtures were incubated for 3 hours at 37°C in CO_2 incubator with continuous supply of 5% CO_2. 5-FU was used as positive control. The cell viability was checked by trypan blue dye (1%). After incubation, 0.2 mL of assay mixture, 0.3 mL PBS and 0.5 mL tryphan blue solution (0.4%) were mixed well and kept aside for 5 min. The total number of viable and non-viable cells was counted by using a Neubauer chamber and the percentage viability was calculated.[342,431]

Percentage viability = Number of dead cells/sum of dead cells and live cells × 100.

The concentration of test sample required to produce 50% cytotoxicity (i.e. to kill 50% of cells) termed as LC_{50}, is generated by plotting percentage cytotoxicity against the corresponding log of test sample concentrations. The LC_{50} value was extrapolated from the trend line of the dose-response curve obtained.

4.2.3. Pharmacological evaluation of In-vivo chemopreventive effect of ethanolic extract of roots of *Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana* against DMBA induced skin carcinoma in mice.

The chemopreventive property of roots of *D. oocarpa*, *D. nigrescens* and *D. candolleana* was determined using two stage carcinogenesis induced by DMBA/TPA in mice.[393,139,57,255]

A. Dose selection

Dose of ethanolic extract of roots of *Diospyros oocarpa*, *Diospyros nigrescens* and *Diospyros candolleana* for the in-vivo chemopreventive study was selected on basis of previous reports and LD_{50} data from Acute Toxicity Study.

B. Animals:

Healthy adult female swiss albino mice weighing 25-30g were obtained from the CPCSEA approved breeder Venkateswara Enterprises Bangalore, they were housed in well ventilated cage at 12 hour day and night cycle with temperature between 23 ± 1^0C. The animals were allowed free access to standard laboratory pellet diet and drinking water *ad libitum.* The study protocol was approved by Institutional Animal Ethics Committee (IAEC), Visveswarapura Institute of Pharmaceutical Sciences, Bangalore, Karnataka (Annexure 1).

C. Carcinogens and their handling:

The carcinogenic materials were handled carefully according to SOP as follows 7,12-diemthylbenz[a]anthracene (DMBA) and 12-O-tetradecanolyphorbol-13-acetate (TPA) were procured from Sigma chemicals. Upon receipt, the bottles were placed in a

plastic freezer bag and stored in the freezer at -20^{0}C. Both the chemicals are weighed "by difference". A foil-wrapped scintillation vial and cap is weighed on an electronic balance and the weight is noted. Then the vial is taken to the fume hood and a diaper is placed over the work area. Gloves are worn. A small amount of chemical is placed into the vial with a wooden applicator stick and the cap replaced. The vial with the chemical is weighed and the quantity of chemical determined by subtraction. A calculation is performed to determine the amount of acetone to add to make a 10x stock solution and then to make a working solution. The stock and working solutions are stored in ziplock bags in the freezer. Contact materials are disposed in the infectious/biohazard waste. Unused solutions are arranged for proper disposal and incineration.[420,129]

D. Induction of experimental tumor:

The mice received a topical application of the carcinogen 7, 12- dimethylbenz [a] anthracene (DMBA) 200µl/mouse; twice a week to the dorsal skin. This is accomplished by holding the mouse by the tail and allowing it to hold onto the wire top of an empty mouse cage. Upon treatment, the mice were placed in cages where they remain for 1 week. After 1 week the bedding were discarded in the infectious/biohazard waste. Although DMBA is a carcinogen, this "initiating" dose does not cause skin tumors in the mice. Tumor development requires subsequent repetitive treatment with a tumor promoter. Hence, one week after carcinogen exposure, the mice received twice weekly treatments with the tumor promoter 12-O-tetradecanolyphorbol-13-acetate (TPA; 50µl/mouse). Although TPA is a strong mouse skin irritant and a tumor promoter of carcinogen treated skin, it is not a tumor promoter for human skin; human cells are not responsive to it. Skin tumors were counted weekly for 15 weeks.

E. Experimental Design:[393,316,423]

Table 4.2:

Group	Status	Treatment
I	Naive control (n=10)	Receives 100µl vehicle (acetone) applied topically twice weekly for 15 weeks.
II	Tumor control (n=10)	Receives DMBA 0.24 % 200 µl/ mouse applied topically for two applications in 1st week and 5 nM TPA 50 µl/ mouse twice weekly from 2nd week upto 15 weeks
III	Tumor + DOE **(***Diospyros oocarpa extract)* (n=10)	DMBA 0.24 % 200 µl/mouse was applied topically for two applications in 1st week and 5 nM TPA 50 µl/mouse twice weekly for 15 weeks and treated with *Diospyros oocarpa extract* (400mg/kg b.w.) p.o. per day, one hour before DMBA/TPA application.
IV	Tumor + DNE (*Diospyros nigrescens* extract) (n=10)	Applied topically DMBA 0.24 % 200 µl/ mouse for two applications in 1st week and 5 nM TPA 50 µl/mouse twice weekly for 15 weeks and treated with *Diospyros nigrescens extract* (400mg/kg b.w.)p.o per day, one hour before DMBA/TPA application.
V	Tumor + DCE (*Diospyros candolleana* extract) (n=10)	Recieved topical application of DMBA 0.24 % , 200 µl/ mouse for two applications in 1st week and 5 nM TPA 50 µl/mouse twice weekly for 15 weeks and treated with *Diospyros candolleana extract* (400mg/kg b.w.)p.o per day, one hour before DMBA/TPA application.

where, b.w. – Body weight, p.o.-Oral route.

Group 3, 4 & 5 were treated with test compound one week before the induction of tumor and continued upto 15 weeks one hr before the application of TPA.

F. Collection of blood and organs:

Mice were anaesthetized using diethyl ether at the end of the study (after 15 weeks). Blood samples were collected by retro orbital puncture in sterile heparinized tubes. The plasma was separated and used for lipid peroxides and total protein estimation.

The mice were sacrificed by cervical dislocation and the dorsal skin was excised out for histological examination and for DNA estimation. The liver and spleen were excised immediately and weighed.

G. Preparation of tissue homogenate:

At the end of experiment animals were sacrificed by cervical dislocation, the tumor affected dorsal skin was quickly removed and thoroughly washed with chilled saline (0.85% pH 7.4). Then blot dried and weighed. A 10% w/v tissue homogenate was prepared in 0.15 M Tris HCl (pH 7.4).

H. Parameters for determination of chemopreventive effect:

The chemopreventive potential of Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana root extract was accessed by determination of survival time, change in body weight, cumulative number of tumors, tumor incidence, tumor burden, tumor yield, average latent period, tumor size, tumor volume, tumor mass.

i. Determination of survival time[242]:

At termination surviving animals of DMBA/TPA tumor bearing mice were counted and the mean survival time (MST) and the % increase in life span (% ILS) were calculated by the formula:

$$\% \text{ ILS} = \frac{(\text{MST of treated group} - \text{MST of control group})}{\text{MST of the Control Group}} \times 100$$

where, MST = Mean Survival time.

The MST and % ILS were recorded in Table No.11.

ii. Body Weight Analysis[266,58,83]:

All mice were weighed on the day of tumor induction, at weekly intervals, and at the end of study. Change in mean body weight was determined in grams and recorded in Table No.12.

iii. Cumulative number of tumors[139,83,388]:

The cumulative number of tumors in each week was measured till the end of experiment.

iv. Tumor incidence[83,388]:

Tumor incidence is defined as the number of mice carrying at least one tumor expressed as a percentage incidence, and calculated as follows.

$$\text{Tumor Incidence} = \frac{\text{No. of mice bearing tumors}}{\text{Total No. of mice}}$$

v. Tumor burden[266,83]:

Tumor burden is defined as the average number of tumors per tumor bearing mouse, and calculated as follows.

$$\text{Tumor Burden} = \frac{\text{Total No. of tumors}}{\text{Total No. of mice bearing tumors}}$$

vi. Tumor yield[139, 266,83]:

Tumor yield is defined as the average number of papillomas per mouse, and calculated as follows.

$$\text{Tumor Yield} = \frac{\text{Total No. of tumors}}{\text{Total No. of mice}}$$

vii. Average latent period[83,366]:

The lag between the application of the promoting agent and the appearance of 50% of tumors was determined. The average latent period was calculated by multiplying the number of tumors appearing each week by the time in weeks after the application of the promoting agent and dividing the sum by total number of tumors.

$$\text{Average Latent Period} = \frac{\Sigma fx}{n}$$

where, *f* is the number of tumors appearing each week

x is the numbers of weeks, and

n is the total number of tumors.

viii. Tumor size[83,366]:

The diameter of each tumor was measured at end of experiment by using digital Vernier caliper (Mitutoyo Digimatic caliper IP 65, Japan)

ix. Tumor volume per mouse[92]:

The Tumor Volume per mouse was calculated as follows

$$V = \frac{Dd^2\pi}{6}$$

where, V = tumor volume per mouse

D = bigger dimension of tumor

d = smaller dimension of tumor

x. Tumor mass[83,366,92]:

The weight of the tumors of each animal at the end of experiment was measured.

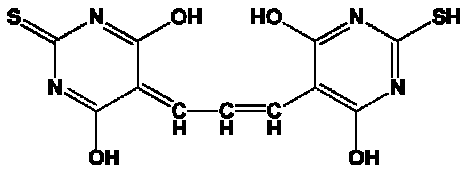
xi. Estimation of bio-chemical marker of oxidative stress:

a. Lipid peroxide level in plasma[83,366]:

The lipid peroxide levels in plasma were analyzed by the TBARS method76. Blood was drawn from each mouse in the conventional way and plasma was separated by centrifugation at 2000 rpm for 20 minutes. The determination of thiobarbituric acid reactive substances (TBARS) i.e. Malondialdehyde (MDA) as an index of lipid peroxidation was carried out.

Principle:

Free oxygen causes breakdown of poly unsaturated free fatty acid in biomembranes and the end product formed is MDA. This end product has high affinity to form complex with thiobarbituric acid.



The proposed method is based on the formation of pink/ purple coloured product by the reaction of MDA with TBA in an acidic medium on heating at 80^0C, One molecule of MDA forms complex with two molecules of TBA to give coloured product which is measured colorimetrically at 532 nm.

Reagents:

1. 1.15 M potassium chloride (KCl)
2. TBA reagent: 0.25 M HCl containing 15 % trichloroacetic acid, 0.375 % thiobarbituric acid and 0.055 % butylated hydroxy toluene and stored at 2-8^0C.

Method:

- To 0.2 ml of plasma, 1 ml of KCl and 2ml of chilled TBA reagent were added slowly with mild stirring and tubes were stoppered. Reaction mixture was then incubated at 80^0 C on a water bath for an hour.
- The tubes were then cooled on ice bath for 5 min and centrifuged at 5000 rpm at 4^0C for 10 min.
- The absorbance of pink chromogen formed was measured against blank (without lipid) at 532nm with UV-Visible spectrophotometer.

Calculation:

The amount of MDA was calculated using following formula and expressed as nmol MDA/ mg protein using molar extinction co-efficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$. (Table No. 16).

MDA (nmol/mg protein) = ( ) ________ ________ /

b. Total protein[83,366]:

Total protein in plasma was determined by modified Biuret, End point Assay using commercially available diagnostic kit.

Assay Principle:

The Peptide bond of protein reacts with cupric ion in alkaline solution to form a coloured chelate. The absorbance of each it measure at 578 nm. The biuret reagent contains sodium potassium tartarate which help in maintaining solubility of this complex at alkaline pH. The absorbance of the final color is proportional to the concentration of total protein in the sample.

$$\text{Protein} + Cu^{++} \xrightarrow{\text{Alkaline pH}} \text{Cu-Protein Complex.}$$

Reagent Composition:

Reagent 1: Total Protein reagent

Copper II sulphate	19 m mol/L
Potassium sodium tartarate	43 m mol/L
Potassium iodide	30 m mol/L
Sodium hydroxide	600 m mol/L

Reagent 2: Total protein Standard

Protein standard	6.0 g/dL

Procedure:

Table No. 4.3: Total protein Working Procedure

Pipette into tube marked	Blank	Standard	Test
Distilled water	10 µl	-	-
Plasma	-	-	10 µl
Reagent 2	-	10 µl	-
Reagent 1	1000 µl	1000 µl	1000 µl

The reagents and sample were mixed well and incubated at 37 °C for 5 min. Program the analyzer as per the above assay parameter. The estimation of total protein was carried out according to instructions given by manufacturer.

Calculation:

$$\text{Total Protein Concentration (g / dL)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times 6.5$$

Conversion Factor:

Total Protein Concentration in g/L = Total Protein Concentration in g/dL × 10

Total protein level in plasma is expressed as gm/dL. (Table No: 4.21).

xii. Estimation DNA content[83]:

DNA was estimated by the Burton method. Tissue homogenate was prepared as mentioned previously and centrifuged at 10 000 rpm for 10 minutes and washed with 5 mL cold 10% TCA. Thereafter, the homogenate was kept for 30 minutes at 4^0C and centrifuged at 10 000 rpm for 10 minutes. The pellet was collected and mixed with 5 mL cold 10% TCA. The mixture was centrifuged at 10 000 rpm for 10 minutes and further mixed with 5 mL alcohol ether (3:1) and centrifuged at 10 000 rpm for 10 minutes. The pellet was collected and mixed with 5 mL 1N KOH and incubated for 16 to 18 hours at 37^0C. The solution was mixed with 0.4 mL of 6N HCL and 5 mL of cold 5% TCA and allowed to precipitate at 4^0C for 30 minutes, then centrifuged at 10 000 rpm for 10 minutes. The pellet was collected and mixed with 5 mL 5% TCA, heated for 15 minutes at 90^0C, and cooled under tap water. Again, it was centrifuged at 10 000 rpm for 10 minutes, and the supernatant was collected. The 1 mL of supernatant was mixed with 2 mL diphenylamine reagent (diphenylamine : GAA : Conc. H_2SO_4, 1:100:2.5) and incubated for 16 to 18 hours at 37^0C, and the absorbance was recorded at 620 nm after setting zero with blank. The levels of DNA were expressed as mg/g of tissue sample.

xiii. Histological examination[438]:

Histopathological changes in the skin were analyzed by the following procedure. Dorsal skin of the animals excised out was immediately fixed in 10% formalin fixative for 24h. The tissues were then dehydrated in ascending series of alcohol, kept in 1:1 mixture of absolute alcohol and benzene and then in benzene for 1h each. Finally, tissue pieces were embedded in paraffin wax and 5 micron thick sections were cut and spread

on glass slides, stained with hematoxylin and eosin, slides mounted in DPX and viewed under light microscope and photographed.

4.3 Statistical Analysis

The results are expressed as Mean ± SE & are analyzed using one way ANOVA followed by student's 't' test. $P<0.05$ is considered as significant.

4.4 RESULTS AND DISCUSSION

A. Collection of Fruit of the three selected *Diospyros* species

In the present study, three *Diospyros* species were collected viz *D. oocarpa* from Amboli, *D. nigrescens* from Tillari ghat of Maharashtra and *D. candolleana* from Tillari ghat of Maharashtra and authenticated from Regional Research Institute, Bangalore, India.

B. Preparation of the extract and percentage yield

The plant material collected was air dried, powdered in Wiley mill and successively extracted with methanol by Soxhlet method and concentrated under vacuum to get residues.

Table No: 4.4. The yields of extracts after soxhlet extraction

Name of the plant	Quantity of Methanol extract in grams / kg of powder	Percentage yield of methanolic extract
D. oocarpa	26.87	2.68
D. nigrescens	28	2.8
D. candolleana	31.1	3.11

C. Acute Oral Toxicity– Acute Toxic Class Method

The methanolic extract of roots of all the three *Diospyros* species did not cause any mortality upto 2000 mg/kg and was considered as safe (OECD - 423 guideline unclassified). As per the OECD guideline no. 423 fixed dose method procedure; the LD_{50} dose for roots of *Diospyros oocarpa*, *Diospyros nigrescens* and *Diospyros candolleana* extract was found to be above 2000 mg/kg body weight; hence, $1/5^{th}$ of the LD_{50} dose of the extract (400 mg/kg body weight) was taken to evaluate pharmacological activity.

D. Preliminary cytotoxicity screening of ethanolic extract of roots of *Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana* by Brine Shrimp Lethality (BSL) assay.

The *Diospyros* genus is known to contain toxic principles and are often reportedly used as fish and arrow poison[48]. Most of the *Diospyros* species exhibit interesting biological and pharmacological activities. Several species are reported for their biocidal properties: termicidal (*D. sylvatica*[201], *D. virginiana*[447]) insecticidal (*D. ebenum, D. kaki*[215]), anti-feedant (*D. kaki*[215,247] *D.abyssinica*[135]), antiprotozoal (*D. assimilis*[124], *D. nirgriesence*[83], *D. oocarpa*[83], *D. candolleana*[83]), anthelmintic (*D. nirgriesence*[83], *D. oocarpa*[428], *D. candolleana*[428]), cytotoxic (*D. assimilis*[124], *D. kaki*[291]), antileishmanial (*D. montana*[441,396], *D. burmanica*[254]), piscicidal (*D. diepenhorstii*[284], *D. lanceofolia*[126], *D. cordifolia*[309]), molluscucidal (*D. usambarensis*[44], *D. zombensis*[295]), antimycobacterial (*D. mespiliformis*[296], *D. glandulosa*[28], *D. rhodocalyx*[28]) etc. These toxic properties are attributed to the presence of naphthoquinones and naphthalene derivative in large quantities in *Diospyros* genus.

The methanolic extracts of roots of *D. oocarpa, D. nigrescens, D. candolleana* and their isolates were assessed for *In-vitro* cytotoxicity studies by Brine Shrimp Lethality (BSL) assay described by Mclaughlin et al.[193] The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxicity, antibacterial, pesticidal anti-tumor properties and various other pharmacological actions.[106,369]

Table 4.5: Results of the BSL bioassay of Methanolic extracts of roots of *Diospyros oocarpa, Diospyros negrisens* and *Diospyros condolleana.*

Conc. (µg/mL)	Log Conc	Methanol ext. of root of *D. oocarpa*			Methanol ext. of root of *D. negrisens*			Methanol ext. of root of *D. condolleana*		
		No. of dead Shrimp	% Mortality	Probits	No. of dead Shrimp	% Mortality	Probits	No. of dead Shrimp	% Mortality	Probits
25	1.39	0	2.5	3.03	1	10	3.72	1	10	3.72
50	1.69	2	20	4.16	2	20	4.16	2	20	4.16
100	2.0	5	50	5.0	6	60	5.25	5	50	5.0
250	2.39	6	60	5.25	8	80	5.84	7	70	5.52
500	2.69	7	70	5.52	9	90	6.28	9	90	6.28
750	2.87	8	80	5.84	10	97.5	6.97	9	90	6.28
1000	3.0	10	97.5	6.97	10	97.5	6.97	10	97.5	6.97
LC_{50} (µg/mL)		**165.9**			**102.3**			**123.0**		

Table 4.6: Results of the BSL bioassay of isolates of the *Diospyros.*

Conc. (µg/mL)	Log Conc	ADDOR-02			ADDOR-03			ADDOR-05			ADDOR-06			ADDOR-08			ADDOR-09			ADDNR-02			ADDCR-04		
		NDS	% M	P	NDS	% M	P	NDS	% M	P	NDS	% M	P	NDS	% M	P	NDS	% M	P	NDS	% M	P	NDS	% M	P
5	0.69	0	2.5	3.03	0	2.5	3.03	0	2.5	3.03	0	2.5	3.03	0	2.5	3.03	0	2.5	3.03	0	2.5	3.03	0	2.5	3.03
10	1.0	0	2.5	3.03	0	2.5	3.03	0	2.5	3.03	1	10	3.72	2	20	4.16	2	20	4.16	3	30	4.48	2	20	4.16
50	1.69	2	20	4.16	2	20	4.16	2	20	4.16	3	30	4.48	5	50	5.0	5	50	5.0	6	60	5.25	5	50	5.0
100	2.0	5	50	5.0	4	40	4.75	6	60	5.25	5	50	5.0	7	70	5.52	8	80	5.84	8	80	5.84	7	70	5.52
200	2.3	8	80	5.84	7	70	5.52	8	80	5.84	8	80	5.84	9	90	6.28	9	90	6.28	9	90	6.28	8	80	5.84
500	2.69	10	97.5	6.97	10	97.5	6.97	10	97.5	6.97	10	97.5	6.97	10	97.5	6.97	10	97.5	6.97	10	97.5	6.97	10	97.5	6.97
LC_{50} (µg/mL)		**77.6**			**89.1**			**75.8**			**66.0**			**43.6**			**41.7**			**37.1**			**48.9**		

NDS = No. of dead Shrimp; %M = Percentage Mortality; P = Probits

ADDOR-02= 5-Hydroxy-4-methoxy-2-naphthaldehyde
ADDOR-03= 4-Hydroxy-5-methoxy-2-naphthaldehyde
ADDOR-05= Plumbagin
ADDOR-06= 4-Hydroxy-3,5-dimethoxy-2-naphthaldehyde
ADDOR-08= Diospyrin
ADDOR-09= 8'-Hydroxyisodiospyrin
ADDNR-02= Habibone
ADDCR-04= Diosindigo A

Table 4.7: LC_{50} values of methanol extracts of root of *Diospyros oocarpa, Diospyros negrisens* and *Diospyros condolleana* and their isolates from BSL Assay.

Sample	Linear regression	R^2	LC_{50} (µg/ml, 24 h)
Methanol extract of root of *Diospyros oocarpa*	y = 2.065x + 0.168	0.932	**165.95**
Methanol extract of root of *Diospyros negrisens*	y = 1.999x + 1.064	0.987	**102.32**
Methanol extract of root of *Diospyros condolleana*	y = 1.925x + 1.008	0.982	**123.02**
ADDOR-02 (5-Hydroxy-4-methoxy-2-naphthaldehyde)	y = 2.005x + 1.205	0.951	**77.62**
ADDOR-03 (4-Hydroxy-5-methoxy-2-naphthaldehyde)	y = 1.920x + 1.258	0.932	**89.12**
ADDOR-05 (Plumbagin)	y = 2.028x + 1.207	0.957	**75.85**
ADDOR-06 (4-Hydroxy-3,5-dimethoxy-2-naphthaldehyde)	y = 1.830x + 1.676	0.961	**66.06**
ADDOR-08 (Diospyrin)	y = 1.848x + 1.966	0.980	**43.65**
ADDOR-09 (8'-Hydroxyisodiospyrin)	y = 1.877x + 1.968	0.982	**41.68**
ADDNR-02 (Habibone)	y = 1.795x + 2.206	0.958	**37.15**
ADDCR-04 (Diosindigo A)	y = 1.762x + 2.040	0.970	**48.97**

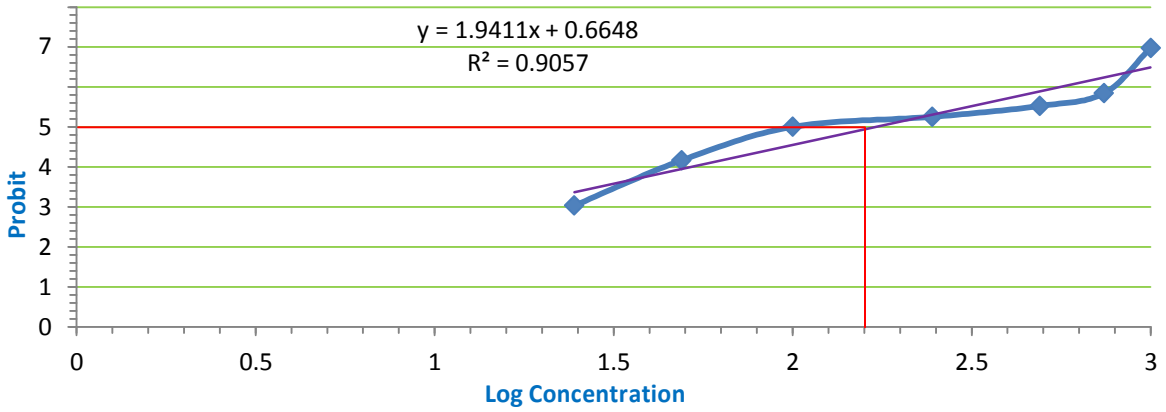
The assay carried out by treating the cells with various concentrations of the extract (25, 50, 100, 250, 500, 750 and 1000 µg/mL) and their isolates (5, 10, 50, 100, 200 and 500 µg/mL). The percentage cytotoxicity progressively increased with

concentration in a dose dependent manner for all the test samples and the corresponding LC_{50} values recorded (Table 4.5, 4.6 and 4.7).

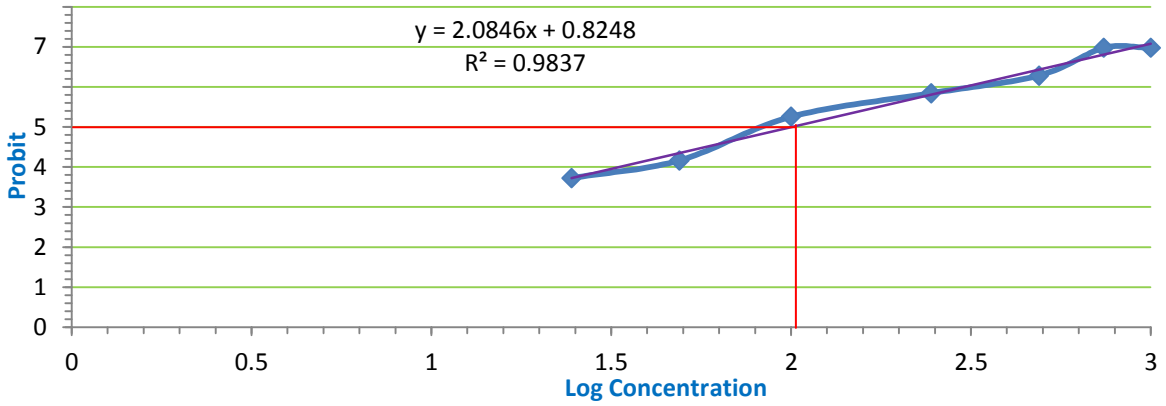
Lethality assessment of all the three Diospyros root extracts on brine shrimps (nauplii) showed good cytotoxic properties. The cytotoxicity of the extracts increased in accordance with concentration. From the Linear regression and Probit analysis after 24 h, it was observed that the methanolic extract of *D. negrisens* roots with LC_{50} value of 102.32µg/mL, exhibited significant cytotoxicity. While the methanolic extracts of roots of *D. oocarpa* and *D. condolleana* exhibited mild cytotoxic effects with LC_{50} values 165.95 and 123.02 µg/mL respectively. The *Diospyros* extracts contained naphthaquinone and naphthaldehyde derivatives and flavonoids, which are known to be effective in free radical mediated diseases such as cancer.[95,453]

All the isolates obtained *Diospyros* roots extracts under study, exhibited good cytotoxicity against brine shrimps. ADDOR-03 (4-hydroxy-5-methoxy-2-naphthaldehyde) LC_{50} 89.12µg/mL least effective, while ADDOR-02 (5-hydroxy-4-methoxy-2-naphthaldehyde) ADDOR-05 (Plumbagin) and ADDOR-06 (4-hydroxy-3,5-dimethoxy-2-naphthaldehyde) exhibited significant cytotoxicity with LC_{50} 77.62, 75.85 and 66.06 µg/mL respectively. ADDNR-02 (Habibone) exhibited highest activity among the isolates with LC_{50} value of 37.15µg/mL, while ADDOR-08 (Diospyrin), ADDOR-09 (8'-Hydroxyisodiospyrin) and ADDCR-04 (Diosindigo-A) also exhibited highly significant cytotoxicity with LC_{50} 43.65, 41.68 and 48.97 µg/mL respectively.

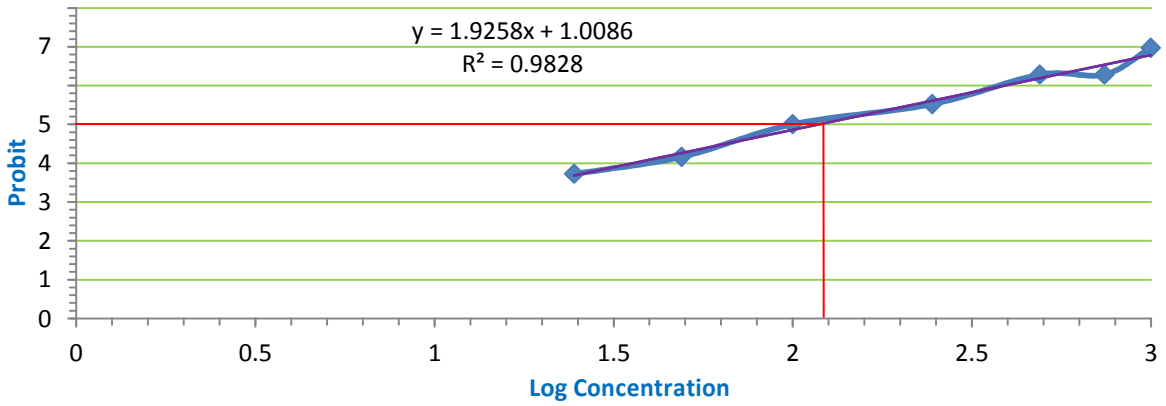
Graph 4.1: Plot of log doses versus probits for calculation of LC_{50} of methanol extract of root of *Diospyros oocarpa*.



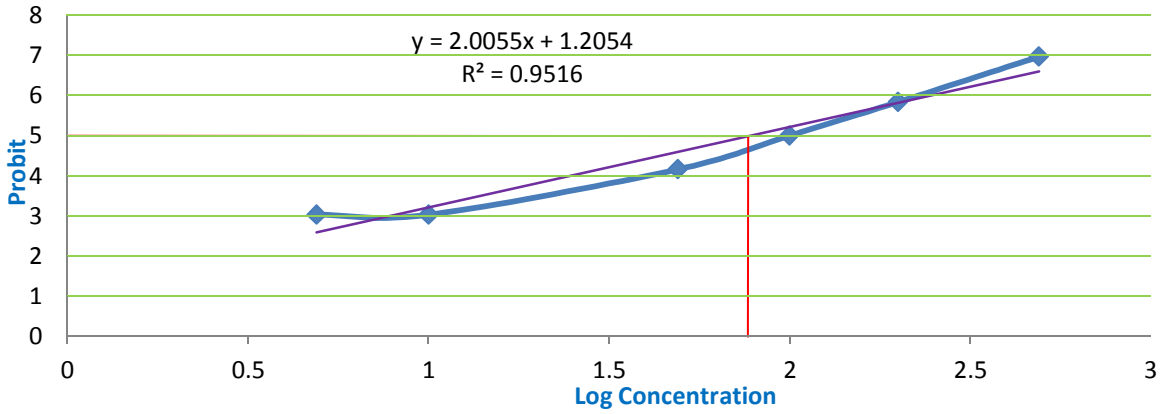
Graph 4.2: Plot of log doses versus probits for calculation of LC_{50} of methanol extract of root of *Diospyros negrisens*.



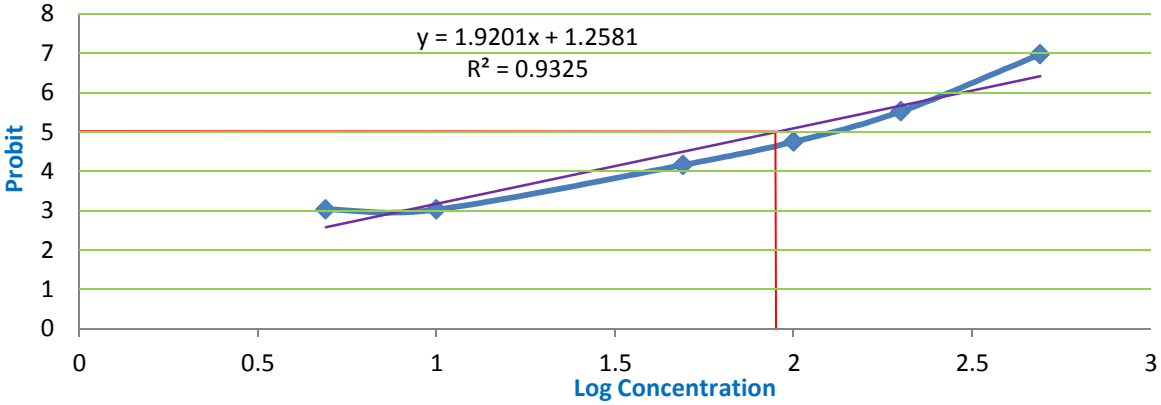
Graph 4.3: Plot of log doses versus probits for calculation of LC50 of methanol extract of root of *Diospyros condolleana*.



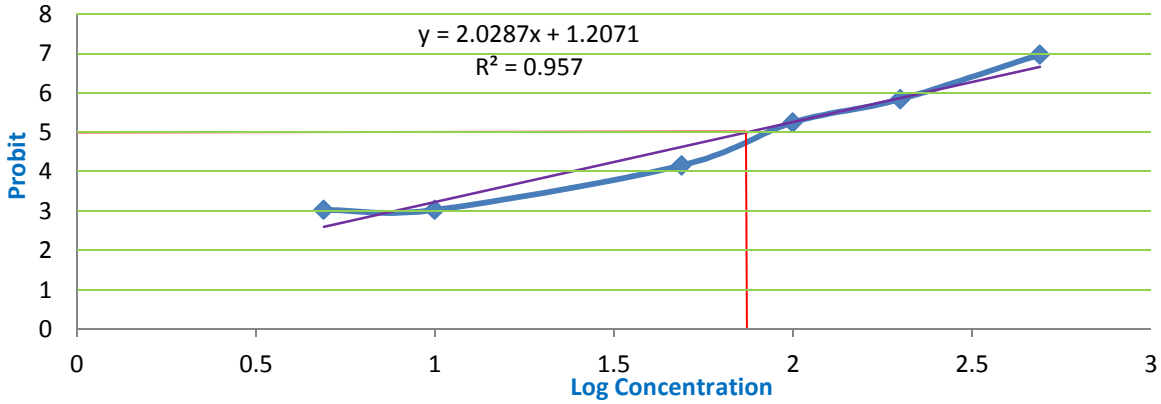
Graph 4.4: Plot of log doses versus probits for calculation of LC50 of ADDOR-02 (5-Hydroxy-4-methoxy-2-naphthaldehyde).



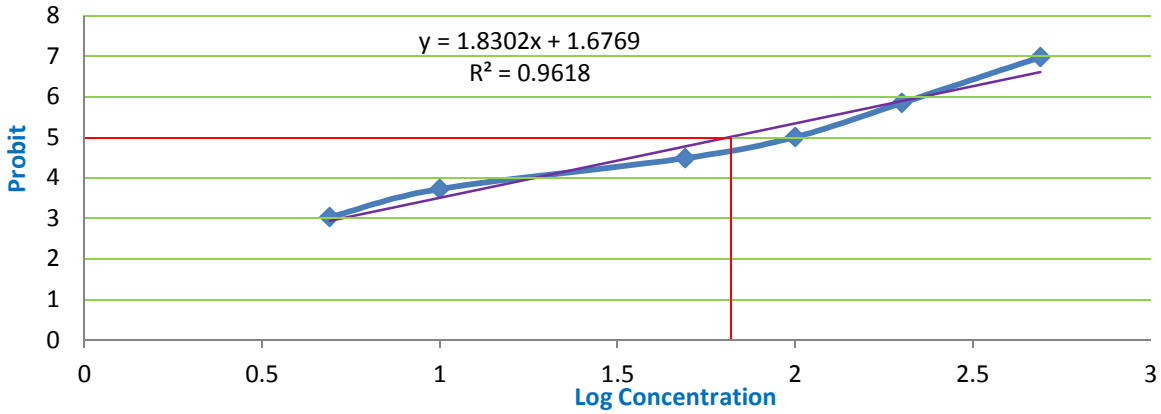
Graph 4.5: Plot of log doses versus probits for calculation of LC50 of ADDOR-03 (4-Hydroxy-5-methoxy-2-naphthaldehyde).



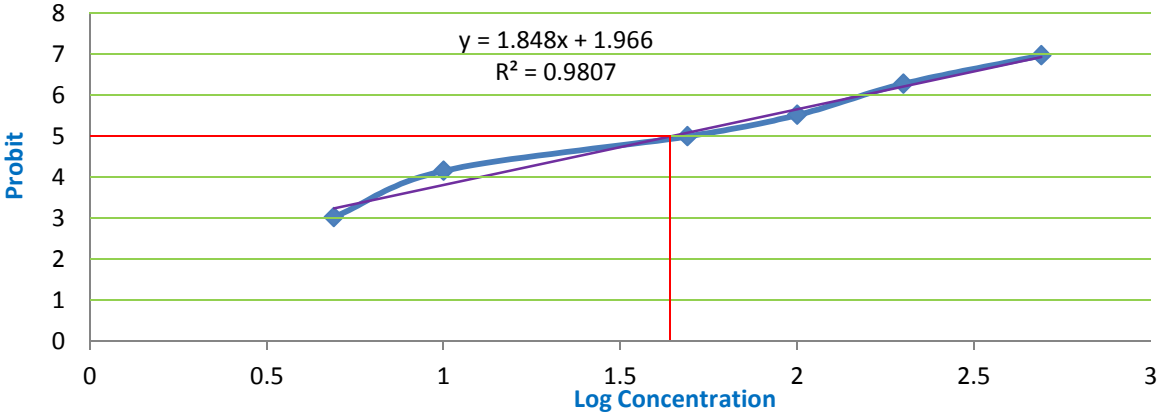
Graph 4.6: Plot of log doses versus probits for calculation of LC50 of ADDOR-05 (Plumbagin).



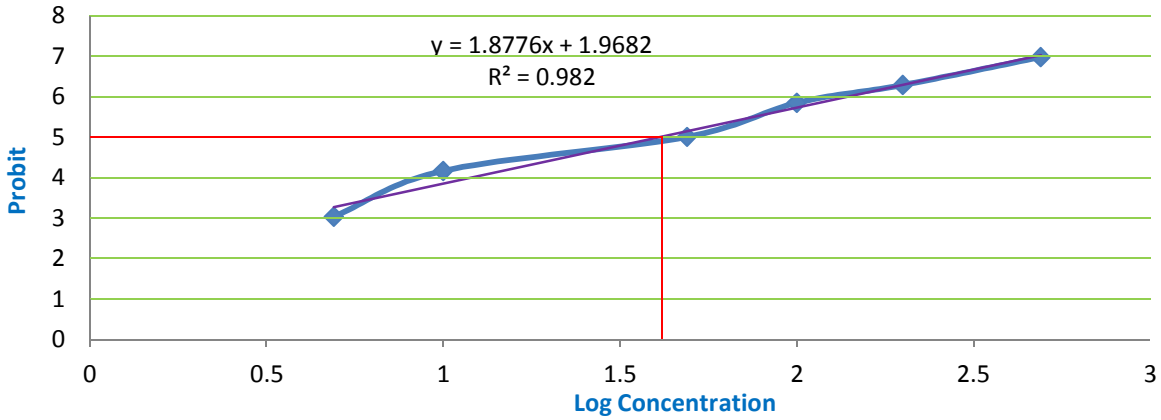
Graph 4.7: Plot of log doses versus probits for calculation of LC50 of ADDOR-06 (4-Hydroxy-3,5-dimethoxy-2-naphthaldehyde).



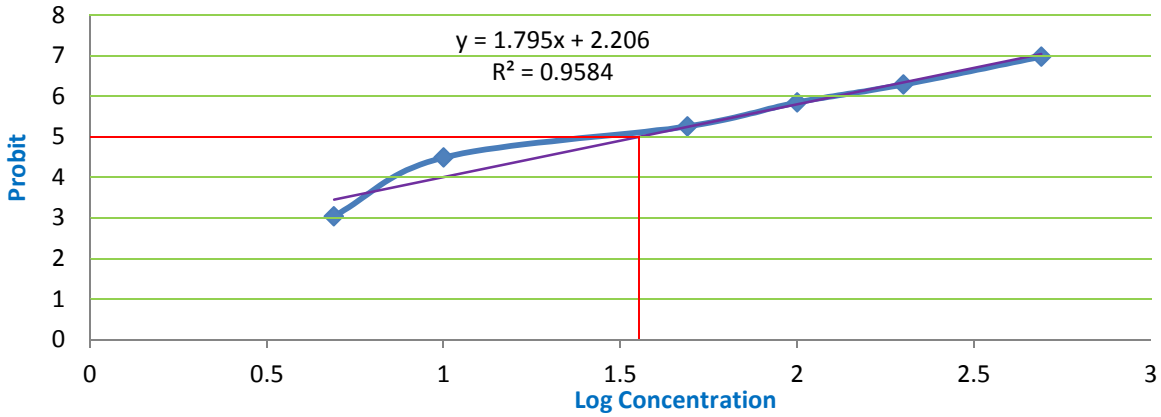
Graph 4.8: Plot of log doses versus probits for calculation of LC50 of ADDOR-08 (Diospyrin).



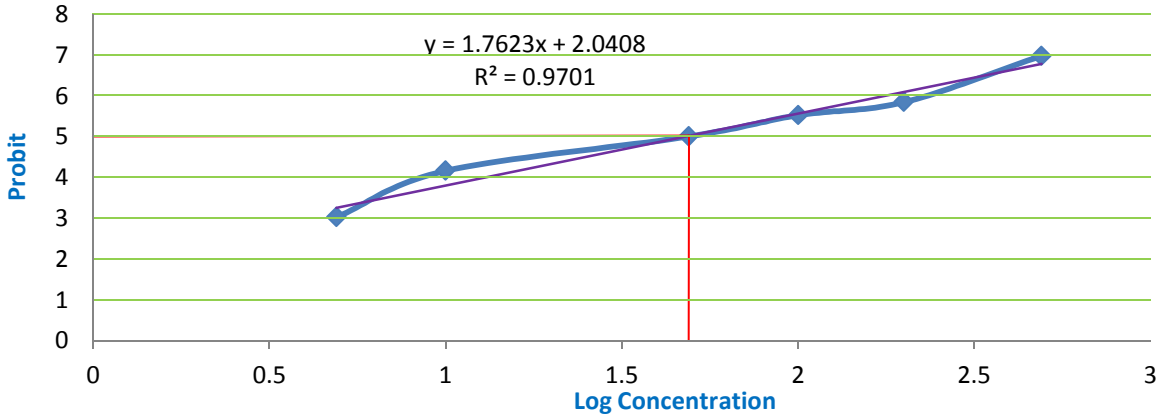
Graph 4.9: Plot of log doses versus probits for calculation of LC50 of ADDOR-09 (8'-Hydroxyisodiospyrin).



Graph 4.10: Plot of log doses versus probits for calculation of LC50 of ADDNR-02 (Habibone).



Graph 4.11: Plot of log doses versus probits for calculation of LC50 of ADDCR-04 (Diosindigo A).



E. Pharmacological evaluation of for anti-tumor effect of ethanolic extract of roots of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* against Dalton's Lymphoma by using In-vitro cytotocity test

The methanolic extracts of roots of *D. oocarpa*, *D. nigrescens*, *D. candolleana* and their isolates were assessed for *In-vitro* cytotoxicity studies by DLA assay. The assay carried out by treating the cells with various concentrations of the extract (10, 25, 50, 100, 200 and 400 µg/mL) and their isolates (5, 10, 25, 50, 100 and 200 µg/mL). The percentage cytotoxicity progressively increased with concentration in a dose dependent manner for all the test samples and the corresponding LC_{50} values recorded (Table 4.8, 4.9 and 4.10).

Table 4.8: Results of *In vitro* cytotoxic activity of methanol extracts of roots of *Diospyros oocarpa, Diospyros negrisens* and *Diospyros condolleana* on DLA cell lines.

Conc. (µgm/mL)	Log Conc.	Percentage of Dead Cells		
		Methanol ext. of root of *D. oocarpa*	Methanol ext. of root of *D. negrisens*	Methanol ext. of root of *D. condolleana*
10	1	14.3	11.2	17.1
25	1.39	22.8	21.8	24.9
50	1.69	35.6	33.8	38.7
100	2	44.3	45.7	49.3
200	2.3	59.1	51.8	60.2
300	2.47	69.9	60.8	66.8
400	2.6	80.6	82.6	75.6
LC_{50} (µg/mL)		**104.7**	**123.0**	**93.3**

Figures in the table indicate the percentage of dead cells after culture with the test samples.

Table 4.9: Results of *In vitro* cytotoxic activity of isolates of the *Diospyros* on DLA cell lines.

Conc. (µgm/mL)	Log Conc.	Percentage of Dead Cells							
		ADDOR-02	**ADDOR-03**	**ADDOR-05**	**ADDOR-06**	**ADDOR-08**	**ADDOR-09**	**ADDNR-02**	**ADDCR-04**
5	0.69	12.9	11.7	11.3	11.3	20.4	23.5	18.4	14.6
10	1.0	20.8	19.4	23.2	18.8	33.4	41.2	29.5	30.3
25	1.39	31.2	28.1	46.5	31.2	42.6	59.7	37.9	41.5
50	1.69	45.2	37.6	69.1	43.8	64.5	70.3	55.4	51.7
100	2.0	57.6	55.3	76.5	63.1	74.6	82.6	67.3	62.1
200	2.30	75.9	76.8	85.1	79.2	95.4	98.7	88.7	88.1
LC_{50} (µg/mL)		**57.5**	**67.6**	**30.2**	**52.5**	**24.5**	**16.9**	**33.1**	**37.1**

ADDOR-02= 5-Hydroxy-4-methoxy-2-naphthaldehyde
ADDOR-03= 4-Hydroxy-5-methoxy-2-naphthaldehyde
ADDOR-05= Plumbagin
ADDOR-06= 4-Hydroxy-3,5-dimethoxy-2-naphthaldehyde
ADDOR-08= Diospyrin
ADDOR-09= 8'-Hydroxyisodiospyrin
ADDNR-02= Habibone
ADDCR-04= Diosindigo A

Table 4.10: LC_{50} values of methanol extracts of root of *Diospyros oocarpa, Diospyros negrisens* and *Diospyros condolleana* and their isolates from DLA Assay.

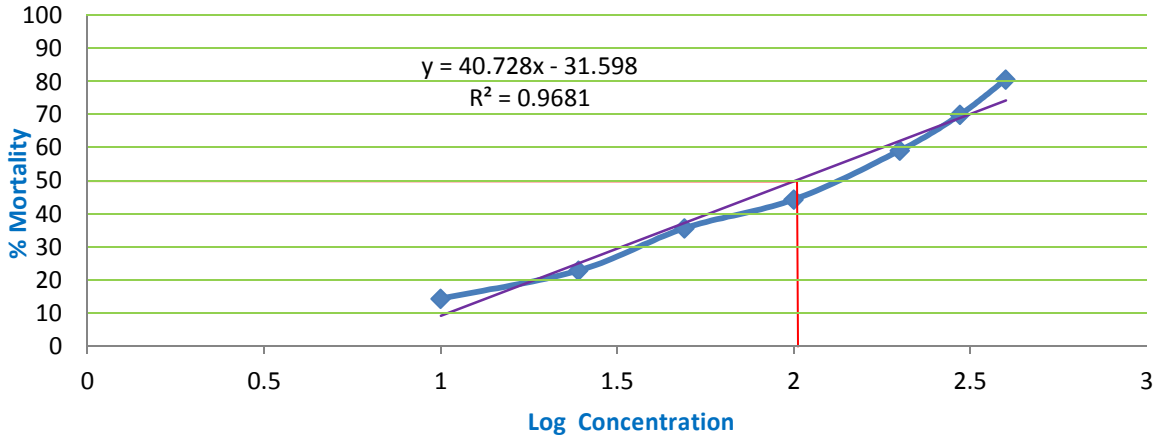
Sample	Linear regression	R^2	LC_{50} (µg/ml, 24 h)
Methanol extract of root of *Diospyros oocarpa*	y = 40.72x - 31.59	0.968	**104.71**
Methanol extract of root of *Diospyros negrisens*	y = 39.54x - 32.02	0.934	**123.02**
Methanol extract of root of *Diospyros condolleana*	y = 36.57x - 22.76	0.987	**93.32**
ADDOR-02 (5-Hydroxy-4-methoxy-2-naphthaldehyde)	y = 38.50x - 17.60	0.974	**57.5**
ADDOR-03 (4-Hydroxy-5-methoxy-2-naphthaldehyde)	y = 38.67x - 20.31	0.937	**67.6**
ADDOR-05 (Plumbagin)	y = 48.79x - 21.81	0.974	**30.2**
ADDOR-06 (4-Hydroxy-3,5-dimethoxy-2-naphthaldehyde)	y = 42.50x - 23.02	0.972	**52.5**
ADDOR-08 (Diospyrin)	y = 45.62x - 13.81	0.977	**24.5**
ADDOR-09 (8'-Hydroxyisodiospyrin)	y = 45.03x - 5.417	0.995	**16.9**
ADDNR-02 (Habibone)	y = 42.22x - 14.30	0.969	**33.1**
ADDCR-04 (Diosindigo A)	y = 41.47x - 14.63	0.963	**37.1**

All three *Diospyros* extracts as well as their isolates exhibited dose dependant cytotoxicity on DLA cells *in vitro.* The methanolic extract root of *D. negrisens* roots exhibited least cytotoxicity on DLA cells, LC_{50} value of 123.0 µg/mL. While the

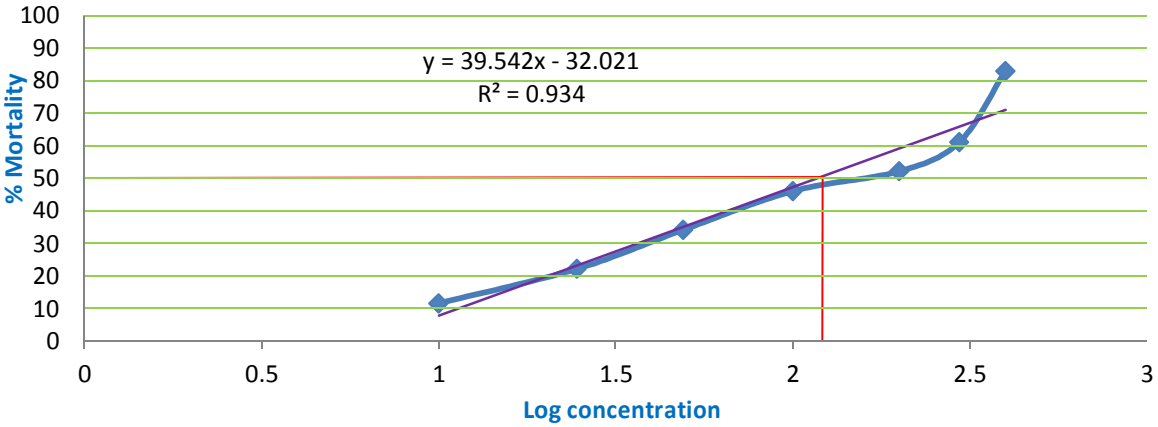
methanolic extracts of roots of *D. oocarpa* and *D. condolleana* exhibited good cytotoxic effects with LC_{50} values 104.7 and 93.3 µg/mL respectively.

Among the isolates, ADDOR-08 (Diospyrin) and ADDOR-09 (8'-Hydroxyiosdiospyrin) possessed highly significant activity with LC_{50} 24.5 and 16.9 µg/mL respectively. While the isolates ADDOR-05 (Plumbagin), ADDNR-02 (Habibone) and ADDCR-04 (Diosindigo-A) showed good cytotoxic with LC_{50} 30.2, 33.1 and 37.1 µg/mL respectively. All the three naphthaldehydes ADDOR-02 (5-hydroxy-4-methoxy-2-naphthaldehyde) (LC_{50} 57.5 µg/mL), ADDOR-03 (4-hydroxy-5-methoxy-2-naphthaldehyde) (LC_{50} 67.6 µg/mL) and ADDOR-06 (4-hydroxy-3,5-dimethoxy-2-naphthaldehyde) (LC_{50} 52.5 µg/mL) exhibited mild cytotoxicity.

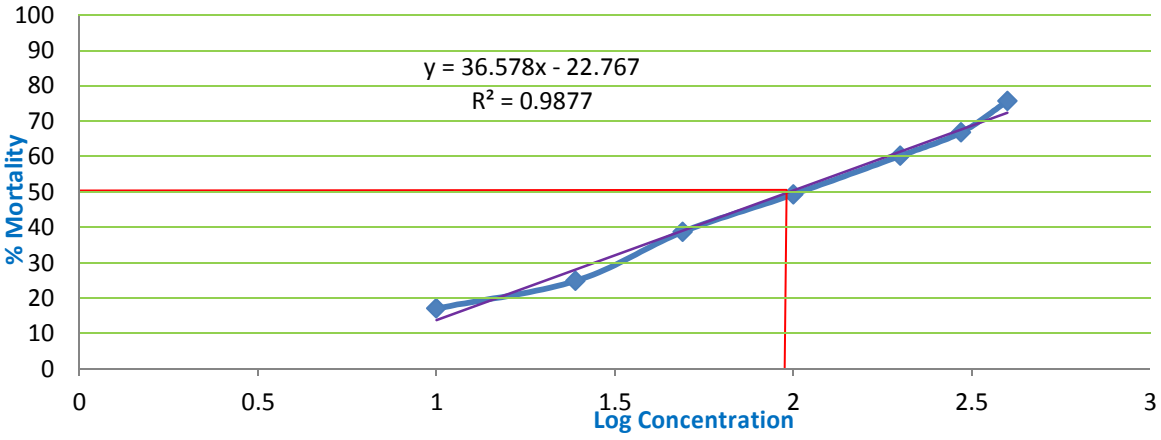
Graph 4.12. Determination of LC_{50} of methanol extract of *D. oocarpa* (Roots) on DLA Cells.



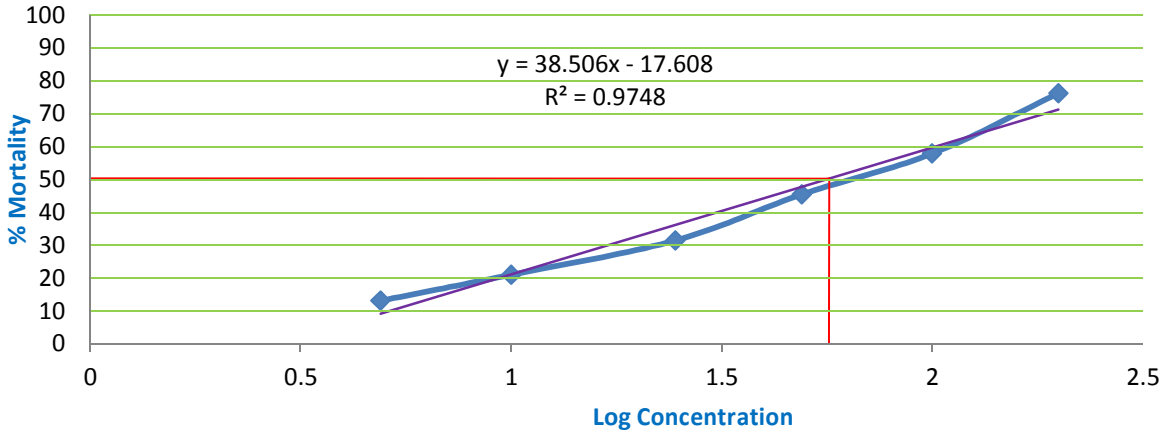
Graph 4.13. Determination of LC_{50} of of methanol extract of *D. negrisens* (Roots) on DLA Cells.



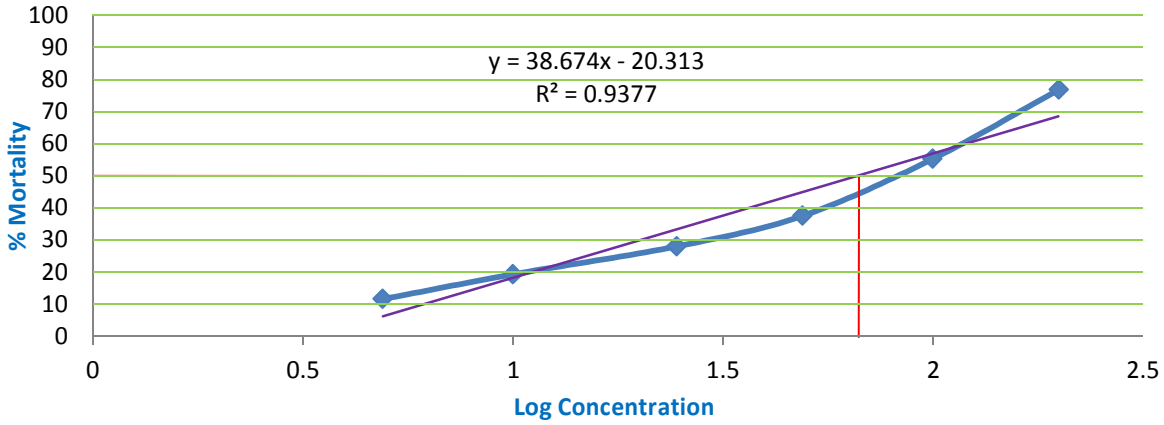
Graph 4.14: Determination of LC_{50} of methanol extract of *D. condolleana* (Roots) on DLA Cells.



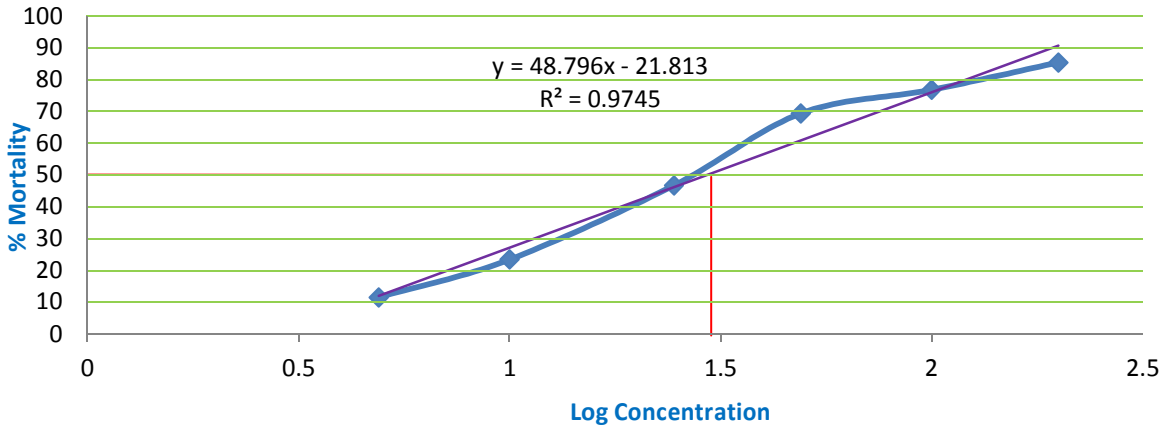
Graph 4.15: Determination of LC_{50} of methanol extract of ADDOR-02 (5-Hydroxy-4-methoxy-2-naphthaldehyde) on DLA Cells.



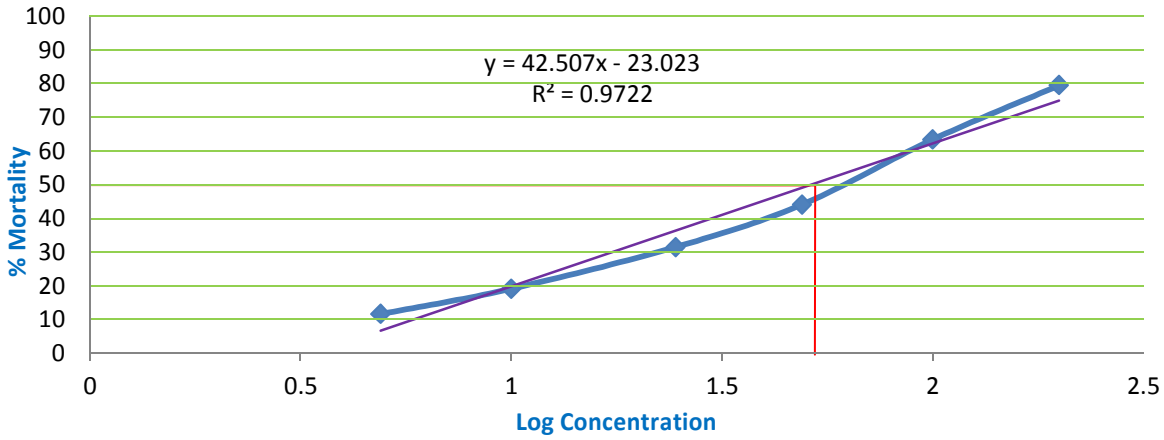
Graph 4.16: Determination of LC_{50} of methanol extract of ADDOR-03 (4-Hydroxy-5-methoxy-2-naphthaldehyde) on DLA Cells.



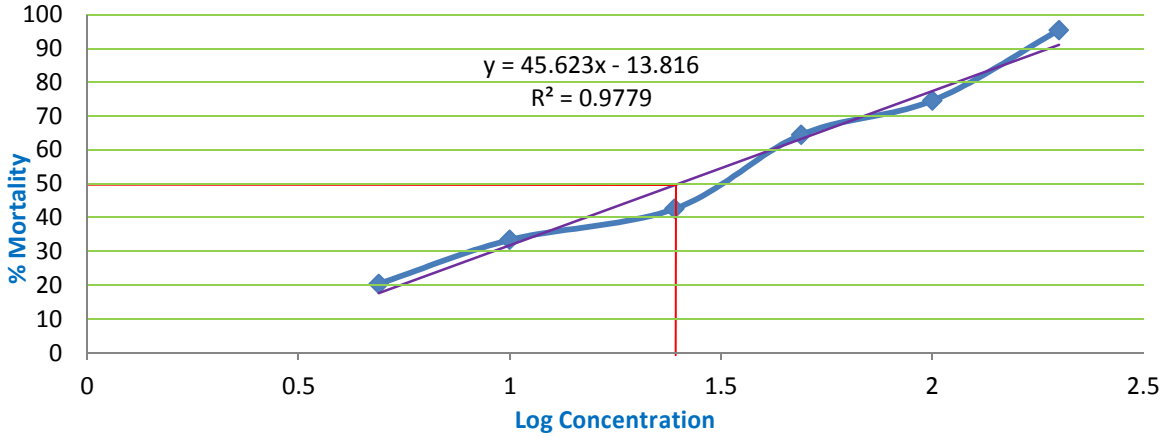
Graph 4.17: Determination of LC_{50} of methanol extract of ADDOR-05 (Plumbagin) on DLA Cells.



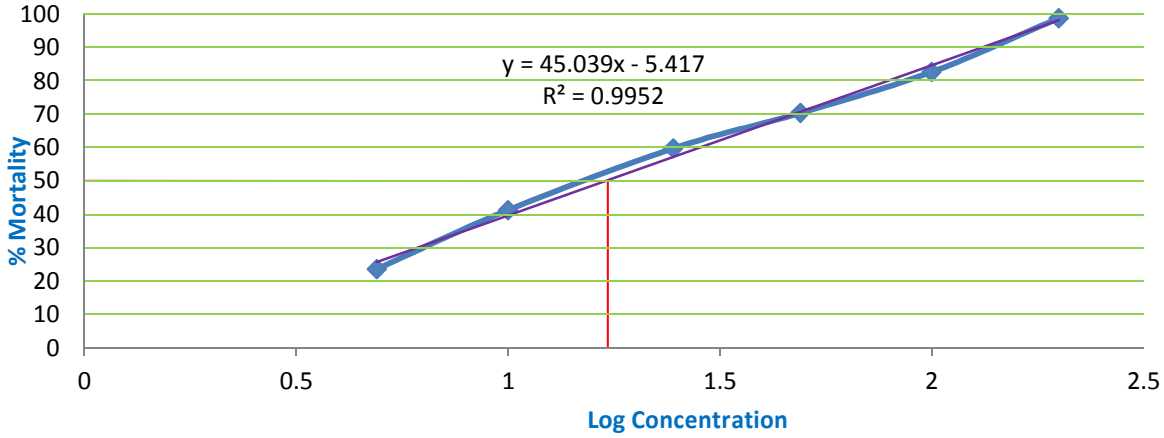
Graph 4.18: Determination of LC_{50} of methanol extract of ADDOR-06 (4-Hydroxy-3,5-dimethoxy-2-naphthaldehyde) on DLA Cells.



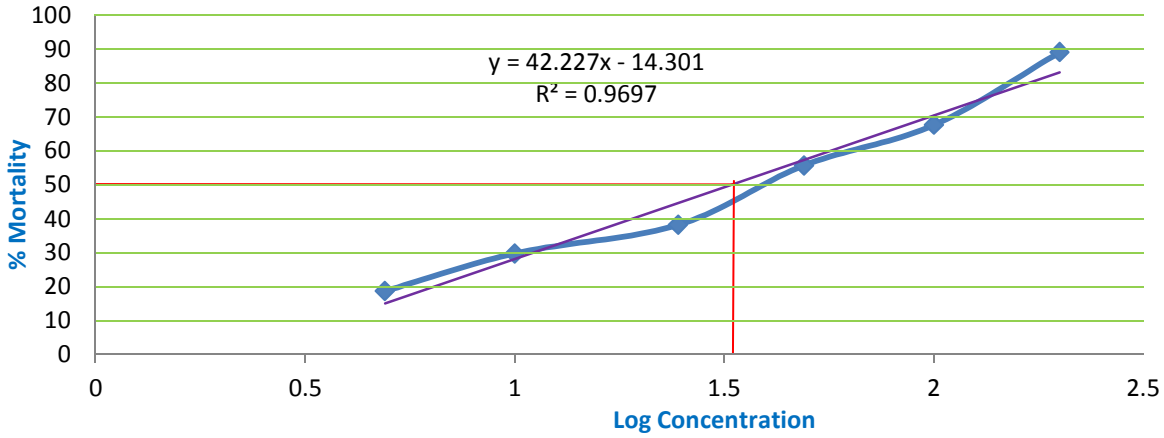
Graph 4.19: Determination of LC_{50} of methanol extract of ADDOR-08 (Diospyrin) on DLA Cells.



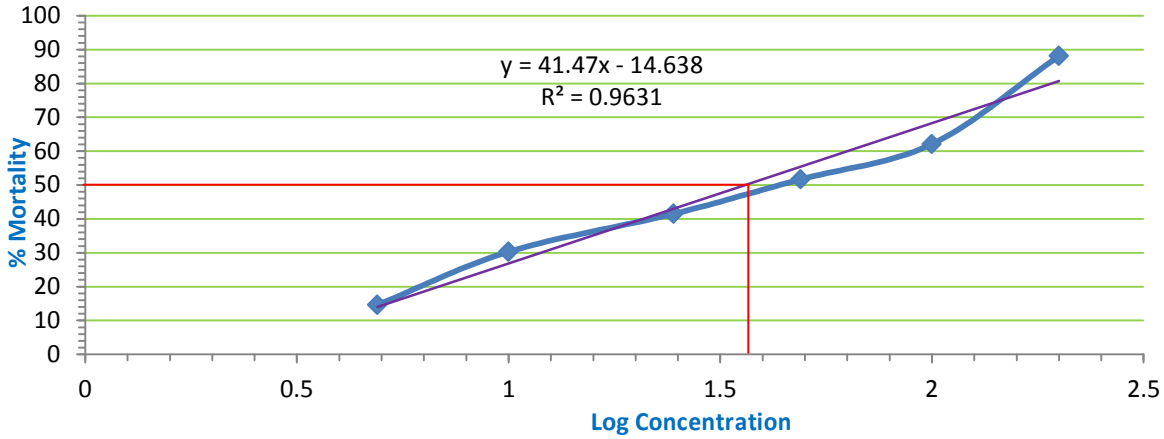
Graph 4.20: Determination of LC_{50} of methanol extract of ADDOR-09 (8'-Hydroxyisodiospyrin) on DLA Cells.



Graph 4.21: Determination of LC_{50} of methanol extract of ADDNR-02 (Habibone) on DLA Cells.



Graph 4.22: Determination of LC_{50} of methanol extract of ADDCR-04 (Diosindigo A) on DLA Cells.



F. Pharmacological evaluation of In-vivo chemopreventive effect of ethanolic extract of roots of *Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana* against DMBA induced skin carcinoma in mice.

a. Mortality rate of animals

There was a 10% mortality rate in animals which are exposed to DMBA/TPA. DMBA/TPA mortality was prevented in animals which are treated with *D. oocarpa, D. nigrescens* and *D. candolleana* root extract.

b. Induction of tumor

Mice which received DMBA 0.24 % 200 µl/ mouse topically for two applications in 1st week and 5 nM TPA 50 µl/ mouse twice weekly from 2nd week up to 15 weeks produced 100% tumor induction in surviving animals.

c. In-vivo chemopreventive effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract

The various parameters such as mean survival time, change in body, liver, and spleen weight, tumor incidence, tumor burden, tumor yield, cumulative number of tumors, average latent period, tumor size, tumor mass, tumor volume per mouse, lipid peroxides and total protein levels in plasma, histopathological changes and DNA content in the skin samples were assessed.

Fig No: 4.1. Tumor induction in mice after topical application of DMBA followed by TPA and effect of DO extract on tumor incidence.

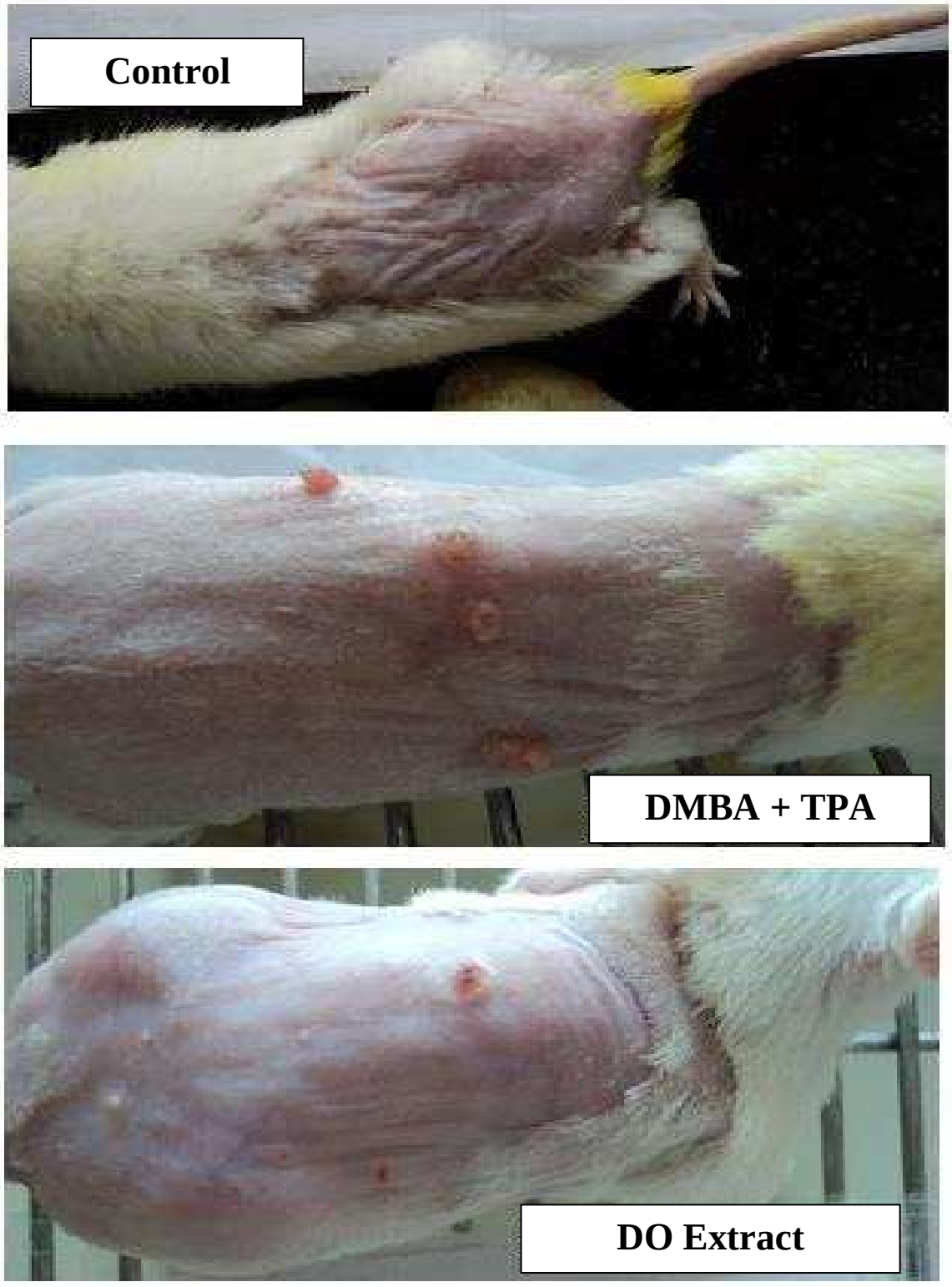
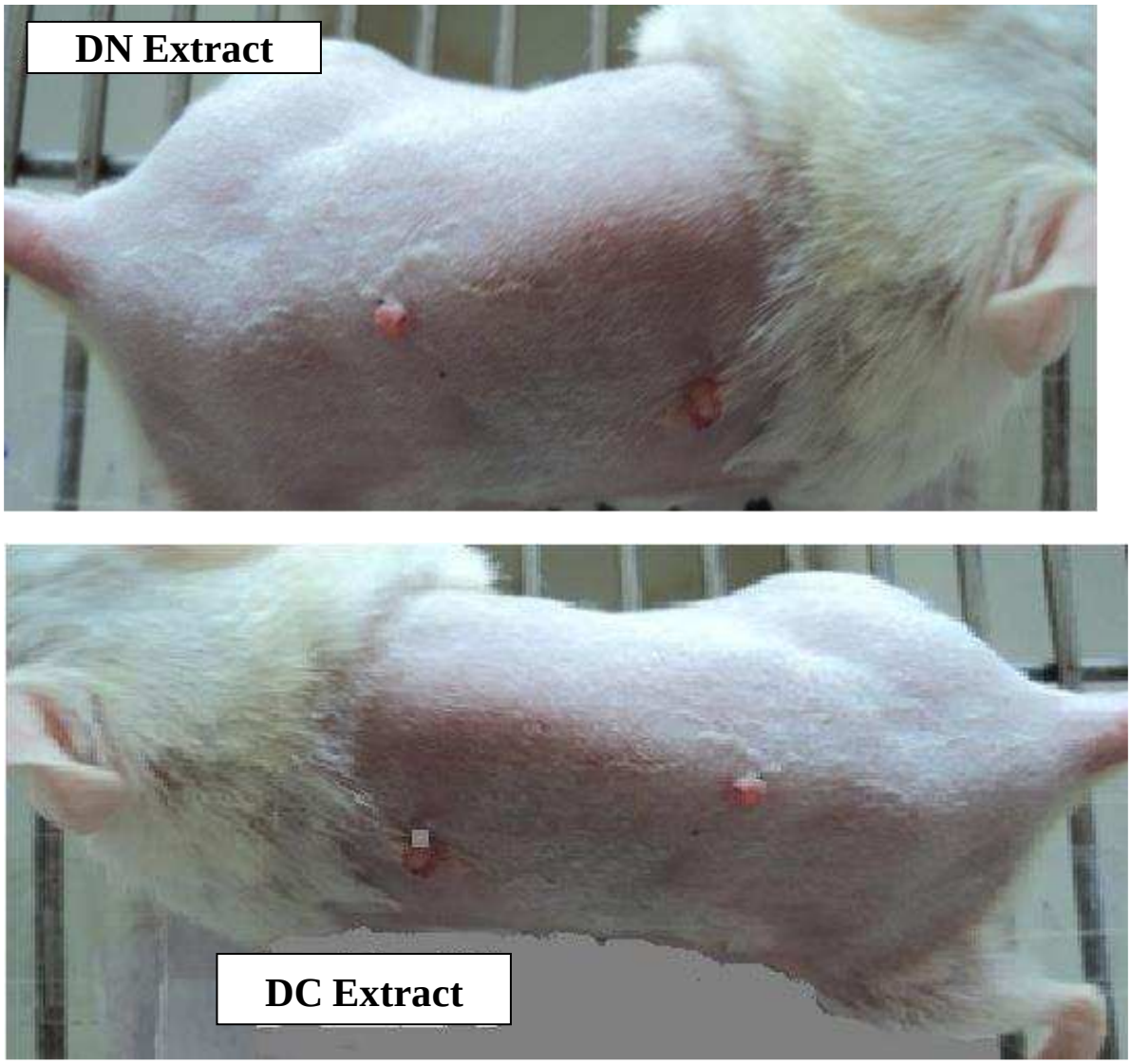


Fig No: 4.2. Tumor induction in mice after topical application of DMBA followed by TPA and effect of DN & DC extract on tumor incidence.



d. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on mean survival time

As shown in Table No: 4.11, topical application of DMBA followed by TPA resulted in decrease (8.67%) in the mean survival time of animals when compared to naïve control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally in these animals has prevented the DMBA/TPA induced

decrease in mean survival time. The administration of *D. oocarpa, root* extract orally (400mg/kg body weight) leads to 3.24% increase in life span and *D. nigrescens* (400mg/kg body weight) also increased the percentage of life span to 2.76% *and D. candolleana root* extract orally (400mg/kg body weight) leads to increase in 2.85 percentage of life span.

e. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on body weight

As shown in Table No: 4.12, topical application of DMBA followed by TPA resulted in significant decrease in the body weight of animals when compared to naïve control animals. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract (400mg/kg body weight) has prevented the DMBA TPA induced decrease in body weight when compared to DMBA/TPA treated group.

f. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on organ weight

As shown in Table No: 4.13, the weight of liver and spleen of mice in DMBA/TPA treated group was increased when compared to naïve control. However, it was nonsignificant. Concomitant administration *D. oocarpa, D. nigrescens* and *D. candolleana* root extract (400 mg/kg body weight) orally has prevented the DMBA/TPA induced increase in liver & spleen weight.

Table No: 4.11. Effect of *Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana root* extract on mean survival time in mice exposed to DMBA/TPA topical application.

Mean Survival Time (days)					
Sl. No	**Control**	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	105	105	90	64	85
2	105	105	92	84	74
3	105	105	86	105	95
4	105	105	95	105	105
5	105	105	105	105	105
6	105	105	105	105	105
7	105	105	105	105	105
8	105	105	105	105	105
9	105	105	105	105	105
10	105	14	105	105	105
Mean ± SEM	**105 ± 0.0**	**95.9 ± 9.1**	**99.3 ± 2.427**	**98.8 ± 4.394**	**98.9 ± 3.478**

g. Data are expressed as Mean ± S.E.M. (n=10).

Table No: 4.12. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on body weights of animals in mice exposed to DMBA/TPA topical application.

Body weight (g)										
Sl. No.	**Control**		**DMBA/TPA**		**DMBA/TPA + DOE**		**DMBA/TPA + DNE**		**DMBA/TPA+ DCE**	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
1	26.7	29.8	27.6	26.3	32.9	34.7	31.5	37.2	30.0	36.0
2	30.0	28.8	23.5	28.2	28.1	27.3	30.3	27.1	29.0	30.0
3	28.5	27.3	26.4	24.4	23.8	26.0	26.6	34.6	26.0	32.0
4	33.3	34.7	31.2	23.6	27.4	26.4	25.6	29.4	27.0	29.0
5	30.5	30.3	29.6	25.9	33.8	33.5	31.6	30.4	31.0	32.0
6	26.8	25.8	33.8	21.8	36.5	32.0	28.1	28.5	29.0	30.0
7	22.5	26.5	27.4	30.9	19.8	32.8	29.6	31.5	30.0	32.0
8	36.3	33.7	30.2	29.0	27.0	33.0	30.7	32.0	29.0	32.5
9	21.3	28.7	30.3	24.5	29.8	32.5	25.3	25.0	26.0	28.0
10	27.3	31.0	26.9	26.1	26.8	30.8	27.8	31.5	28.0	30.5
Mean ± SEM	**28.32± 1.435**	**29.66± 0.919**	**28.69± 0.921**	**26.07*± 0.958**	**28.59± 1.554**	**30.90#± 1.001**	**28.71± 0.748**	**30.72#± 1.118**	**28.50± 0.542**	**31.20#± 0.708**

Data are expressed as Mean ± S.E.M. (n=10). *$p<0.01$ when compared to control and $^{\#}p<0.01$ when compared to DMBA/TPA .

Table No: 4.13 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on liver and spleen weights in mice exposed to DMBA/TPA topical application.

Liver weight (g) and spleen weight (mg)										
Sl. No.	**Control**		**DMBA/TPA**		**DMBA/TPA+DOE**		**DMBA/TPA+DNE**		**DMBA/TPA+DCE**	
	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen
1	1.58	113.7	1.85	70.00	0.99	79.00	1.54	130.00	1.28	125.00
2	1.32	71.0	1.76	200.00	1.25	135.00	1.34	100.00	1.36	132.00
3	1.17	170.7	1.78	90.00	1.28	80.00	1.55	120.00	1.15	135.00
4	1.35	110	1.48	110.00	1.31	110.00	1.38	110.00	1.65	145.00
5	1.22	150	1.55	150.00	1.34	70.00	1.55	110.00	1.40	105.00
6	1.93	90	1.50	110.00	1.25	90.00	1.23	120.00	1.23	110.00
7	1.65	120.5	1.62	115.00	1.50	132.00	1.10	130.00	1.15	125.00
8	1.40	122	1.70	122.00	1.46	128.00	0.95	110.00	1.25	135.00
9	1.56	115	1.85	125.00	1.60	117.00	1.57	115.00	1.46	100.00
10	1.32	110	---	---	1.30	120.00	1.48	110.00	1.50	125.00
Mean ± SEM	**1.450± 0.073**	**117.3± 8.798**	**1.677± 0.048**	**121.3± 12.33**	**1.328± 0.053**	**106.1± 7.667**	**1.369± 0.068**	**115.5± 3.023**	**1.343± 0.051**	**122.2± 4.767**

Data are expressed as Mean ± S.E.M.

h. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on DMBA -TPA induced skin carcinogenesis

1. Effect of *Diospyros oocarpa, Diospyros nigrescens and Diospyros candolleana root* extract on tumor incidence

Topical application of DMBA followed by TPA, produced skin papillomas, which started appearing from the eighth week onwards and continued till fifteenth week. The tumor incidence was 100% in surviving animals exposed to DMBA/TPA. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally delayed the appearance of papillomas i.e., tumor appeared from tenth, eleventh & tenth week onwards respectively and the percentage of tumor incidence was reduced to 40%, 50% & 50% respectively.

2. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor burden

As shown in Table No: 4.14, topical application of DMBA followed by TPA resulted in significant increase in the tumor burden (papillomas per papilloma-bearing mice) when compared to naïve control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally resulted in significantly decreased the tumor burden when compared to DMBA/TPA group.

3. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor yield

As shown in Table No: 4.15, topical application of DMBA followed by TPA resulted in significantly increase in tumor yield (the average number of papillomas per mouse) when compared to naïve control. Concomitant administration of *D. oocarpa, D.*

nigrescens and *D. candolleana* root extract orally resulted in significant decrease in the tumor yield when compared to DMBA/TPA group.

4. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on cumulative number of tumors

Topical application of DMBA followed by TPA resulted in significant increase in the cumulative number of tumors when compared to naïve control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally significantly decreased the cumulative number of tumors when compared to DMBA/TPA group.

5. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on average latency period

As shown in Table No: 4.16, topical application of DMBA followed by TPA resulted in significant decrease in the average latency period (i.e, time lag between the application of the promoter and the appearance of 50% of tumors) when compared to control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally resulted in significant increase in the average latency period when compared to DMBA/TPA group.

6. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor size

Topical application of DMBA followed by TPA resulted in significant increase in the tumor size when compared to control. Simultaneous administration of *D. oocarpa, D.*

nigrescens and *D. candolleana* root extract orally resulted in significant decrease in the tumor size when compared to DMBA/TPA group. (Table 4.17)

7. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor mass

As shown in Table No: 4.18, topical application of DMBA followed by TPA resulted in significant increase in the tumor mass when compared to naïve control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally resulted in significant decrease in the tumor mass when compared to DMBA/TPA group.

8. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor volume per mouse

As shown in Table No: 4.19, topical application of DMBA followed by TPA resulted in increase in the tumor volume per mouse when compared to naïve control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana root* extract orally resulted in decreased tumor volume per mouse when compared to DMBA/TPA group.

9. Effect of AME on lipid peroxides in plasma

As shown in Table No: 4.20, topical application of DMBA followed by TPA resulted in significant increase in the plasma lipid peroxides level when compared to naïve control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally lead to significantly decrease in the lipid peroxides level when compared to DMBA/TPA group.

Table No: 4.14 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor burden in mice exposed to DMBA/TPA topical application.

Tumor burden					
Sl. No.	**Control**	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	0	2	2	1	1
2	0	3	2	1	0
3	0	2	0	0	1
4	0	1	0	1	1
5	0	4	0	3	2
6	0	3	0	0	0
7	0	6	1	0	0
8	0	5	0	0	0
9	0	3	0	1	1
10	0	--	1	0	0
Mean ± SEM	**0.0 ± 0.0**	**3.2 ± 1.521***	**0.6 ± 0.267$^{\#}$**	**0.7 ± 0.300$^{\#}$**	**0.6 ± 0.221$^{\#}$**
Tumor burden	**0**	**3.2**	**1.5**	**1.4**	**1.2**

Date are expressed as Mean ± S.E.M. (n=10). *$p<0.001$ when compared to control and $^{\#}p<0.001$ when compared to DMBA/TPA d group.

Table No:4.15 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor yield in mice exposed to DMBA/TPA topical application

Tumor yield					
Sl. No.	**Control**	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	0	2	2	1	1
2	0	3	2	1	0
3	0	2	0	0	1
4	0	1	0	1	1
5	0	4	0	3	2
6	0	3	0	0	0
7	0	6	1	0	0
8	0	5	0	0	0
9	0	3	0	1	1
10	0	--	1	0	0
Mean ± SEM	**0.0 ± 0.0**	**3.2 ± 1.521***	**0.6 ± 0.267#**	**0.7 ± 0.300#**	**0.6 ± 0.221#**
Tumor Yield	**0**	**3.2**	**0.6**	**0.7**	**0.6**

Date are expressed as Mean ± S.E.M. (n=10). *$p<0.01$ when compared to control and $^{\#}p<0.05$ when compared to DMBA/TPA group.

Table No: 4.16 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on average latent period in mice exposed to DMBA/TPA topical application.

No. of Weeks after TPA Application	Average latent period (weeks)				
	Control	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
6	0	1.2	0	0	0
7	0	0	7	6	5
8	0	1.142	0	0	0
9	0	1	6.5	4.5	6
10	0	0.909	6	3.33	4.5
11	0	0	6	1.833	2.33
12	0	0.923	6	1.714	1.2
13	0	0	6.25	0	0
14	0	2.66	0	0	0
Mean ± SEM	**0.0 ± 0.0**	**0.56 ± 0.2115***	**2.196 ± 1.818$^{\#}$**	**1.241 ± 0.535$^{\#}$**	**1.359 ± 0.5848$^{\#}$**

Data are expressed as Mean ± S.E.M. (n=14). * $p<0.05$ when compared to control group and $^{\#}p< 0.05$ when compared to DMBA/TPA group.

Table No: 4.17 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor size in mice exposed to DMBA/TPA topical application

Tumor size (mm)					
Sl. No.	**Control**	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	0	2.285	1.795	1.99	1.856
2	0	3.725	4.91	2.423	0
3	0	8.67	0	0	2.682
4	0	5.14	0	3.98	1.923
5	0	2.973	0	4.35	3.823
6	0	15.36	0	0	0
7	0	4.71	2.35	0	0
8	0	7.95	0	0	0
9	0	9.56	0	2.63	3.342
10	0	----	3.32	0	0
Mean ± SEM	**0.0 ± 0.0**	**6.708± 1.381***	**1.238± 0.5633$^{\#}$**	**1.537 ± 0.5566$^{\#}$**	**1.363± 0.4894$^{\#}$**

Data are expressed as Mean ± S.E.M. (n=10). *$p<0.001$ when compared to control and $^{\#}p< 0.001$ as compared to DMBA/TPA group.

Table No: 4.18 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor mass in mice exposed to DMBA/TPA topical application

Tumor mass (mg)					
Sl. No.	**Control**	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	0	35.25	27.69	30.7	28.62
2	0	57.47	75.76	37.38	0
3	0	133.77	0	0	41.32
4	0	79.31	0	61.41	29.56
5	0	45.87	0	67.12	60.34
6	0	237	0	0	0
7	0	72.67	32.52	0	0
8	0	120.65	0	0	0
9	0	132.32	0	40.58	55.68
10	0	----	56.83	0	0
Mean ± SEM	**0.0 ± 0.0**	**101.6 ± 20.90***	**19.28 ± 8.867$^{\#}$**	**23.72 ± 8.587$^{\#}$**	**21.55 ± 7.815$^{\#}$**

Data are expressed as Mean ± S.E.M. (n=10). **p<0.001 when compared to control. $^{\#}$p< 0.001 when compared to DMBA/TPA.

Table No: 4.19 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on tumor volume per mouse in mice exposed to DMBA/TPA topical application.

Tumor volume per mouse (mm^3)					
Sl. No.	**Control**	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	0	4.771	58.277	2.61	32.00
2	0	674.71	2.442	4.12	0
3	0	32.51	0	0	62.62
4	0	341.37	0	33.02	45.85
5	0	20.63	0	43.11	23.56
6	0	13.059	0	0	0
7	0	21.207	35.50	0	0
8	0	36.58	0	0	0
9	0	42.35	0	9.52	10.56
10	0	----	10.10	0	0
Mean ± SEM	**0.0 ± 0.0**	**131.9 ± 76.41**	**10.63 ± 6.35**	**9.24 ± 4.95**	**17.46 ± 7.20**

Data are expressed as Mean ± S.E.M. (n=10).

Table No: 4.20 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on lipid peroxides in plasma in mice exposed to DMBA/TPA topical application.

Lipid peroxides in plasma (nM)					
Sl. No.	**Control**	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	0.807	9.961	1.346	2.480	2.652
2	1.346	4.711	1.211	1.673	3.120
3	1.884	1.884	1.634	1.269	1.102
4	2.423	4.038	1.826	1.673	2.523
5	3.903	16.96	1.961	2.115	1.852
6	1.230	10.12	1.850	3.654	2.689
7	2.354	16.85	1.658	2.482	3.212
8	1.856	8.956	1.925	1.562	1.150
9	2.160	9.235	2.126	1.952	1.268
10	1.214	---	3.256	1.659	1.364
Mean ± SEM	**1.918 ± 0.2785**	**9.191 ± 1.750***	**1.879 ± 0.1767$^{\#}$**	**2.052 ± 0.2171$^{\#}$**	**2.093 ± 0.2646$^{\#}$**

Data are expressed as Mean ± S.E.M. *$p<0.001$ when compared to control. $^{\#}p<0.001$ when compared to DMBA/TPA.

Table No: 4.21 Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on total protein levels in plasma in mice exposed to DMBA/TPA topical application.

Total protein in plasma (g/dL)					
Sl. No.	**Control**	**DMBA/TPA**	**DMBA/TPA + DOE**	**DMBA/TPA + DNE**	**DMBA/TPA + DCE**
1	9.10	5.32	7.15	6.93	9.32
2	7.09	6.06	7.73	7.63	8.32
3	7.49	6.05	8.02	10.44	7.89
4	9.91	6.92	9.08	8.03	8.65
5	8.95	7.25	9.13	8.37	7.82
6	7.52	6.59	10.05	8.42	9.22
7	8.28	4.65	8.65	8.65	10.10
8	7.65	5.56	9.12	9.23	8.62
9	9.32	6.32	8.85	7.96	7.32
10	8.50	----	7.98	8.2	7.56
Mean ± SEM	**8.381 ± 0.2948**	**6.080 ± 0.2710***	**8.576 ± 0.2692$^{\#}$**	**8.386 ± 0.2988$^{\#}$**	**8.482 ± 0.2774$^{\#}$**

Data are expressed as Mean ± S.E.M. *$p<0.001$ when compared to control, $^{\#}p<0.001$ when compared to DMBA/TPA.

10. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on total protein levels in plasma

As shown in Table No: 4.21, topical application of DMBA followed by TPA resulted in significant decrease in total protein levels in plasma when compared to naïve control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally resulted in significant prevention of the decrease in plasma total protein when compared to DMBA/TPA group.

11. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on DNA content in skin

As shown in Table No: 12, topical application of DMBA followed by TPA resulted in significant lower/ levels of DNA in skin tissue homogenate when compared to control. Simultaneous administration of *D. oocarpa, D. nigrescens* and *D. candolleana* root extract orally significantly elevated the levels of DNA in Skin tissue homogenate when compared to DMBA/TPA treated group.

12. Effect of *Diospyros oocarpa, Diospyros nigrescens* and *Diospyros candolleana* root extract on histological changes in skin

As shown in the Fig. No: 4.3 (B), the skin histological sections of DMBA/TPA treated mice revealed pathological changes when compared to control, as indicated by dysplastic epithelium, infiltration of neutrophils and lymphocytes into stroma, papillomatous projections, disruption of basal lamina, dermal fibrosis, hyperkeratosis of the epidermis and deposition of keratinocyte pearls over the epidermis.

A

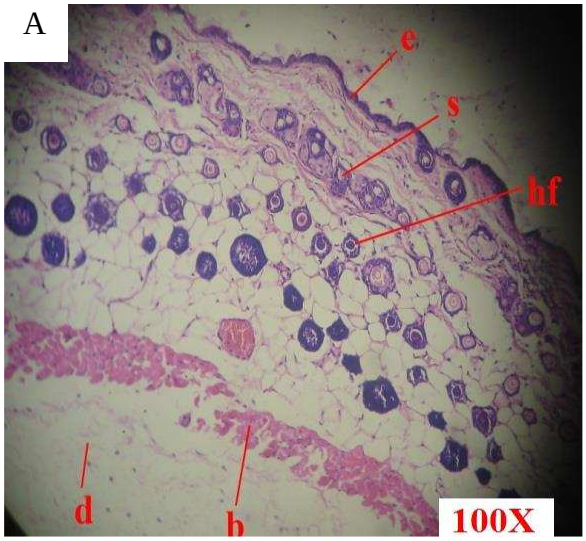


Figure: 4.3 (A) Photomicrograph showing histological section of normal skin in mice (group I) [showing e: epidermis, d: dermis, b: basal lamina, s: sebaceous glands, hf: hair folicles] at magnification x100.

B

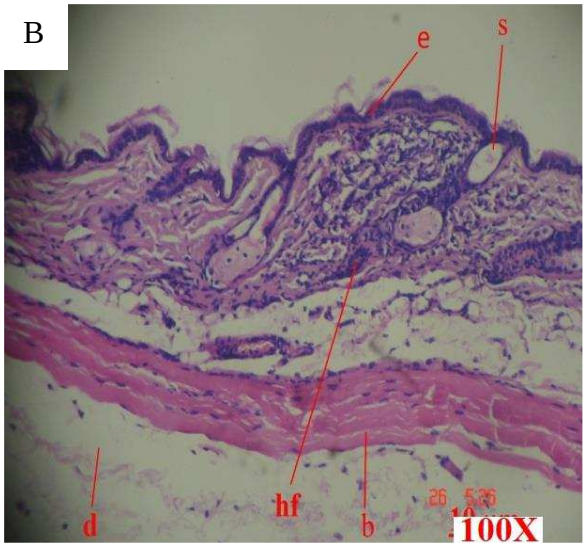


Figure: 4.3 (B) Photomicrograph showing histological section of DMBA/TPA induced skin tumor in mice (group II) [i: infiltration of neutrophils and lymphocytes into stroma, k: keratinocyte pearls, pp: papillomatous projections, hy: hyperkeratosis, df: dermal fibrosis, and dy: dysplastic epithelium] at magnification x100.

C

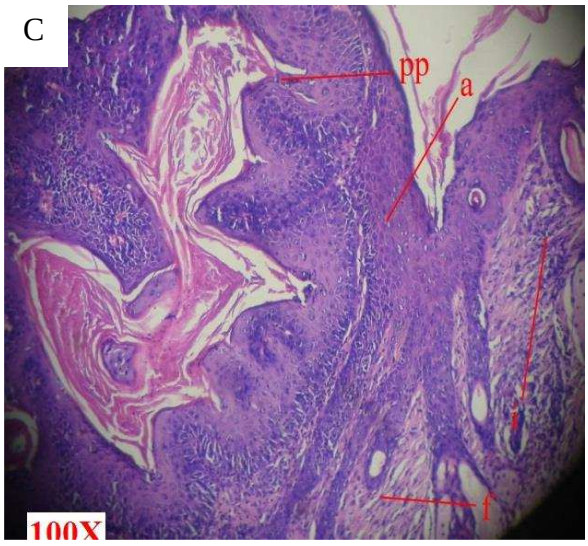


Figure: 4.3 (C) Photomicrograph showing histological section of DMBA/TPA induced skin tumor in mice administered with DOE orally (group-III).

D

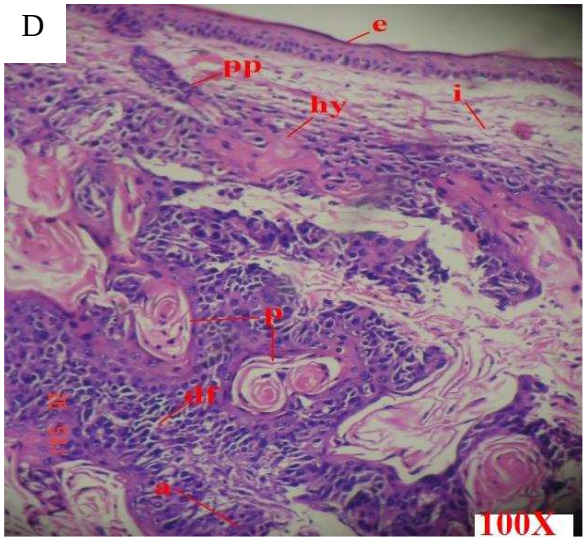


Figure: 1(D) Photomicrograph showing histological section of DMBA/TPA induced skin tumor in mice administered with DNE topically (group-IV) [i: infiltration of neutrophils and lymphocytes into stroma, k: keratinocyte pearls, dy: dysplastic epithelium] at magnification x100.

E

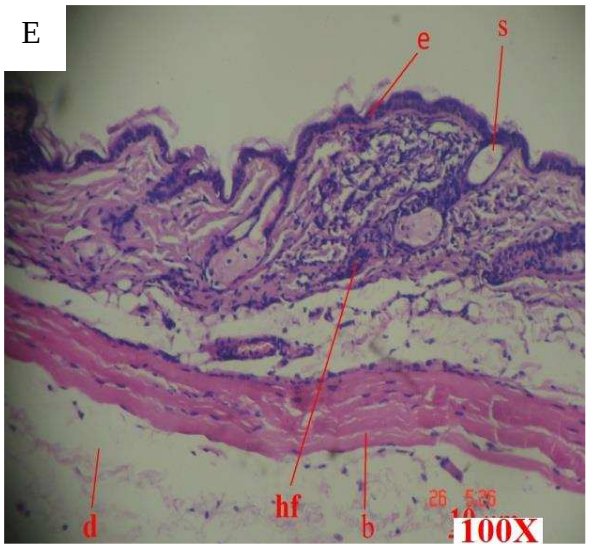


Figure: 4.3 (E) Photomicrograph showing histological section of DMBA/TPA induced skin tumor in mice administered with DCE topically (group-V) [i: infiltration of neutrophils and lymphocytes into stroma, k: keratinocyte pearls, dy: dysplastic epithelium] at magnification x100.

Oral administration of *D. oocarpa*, *D. nigrescens* and *D. candolleana* root extract prevented the histological changes induced by DMBA/TPA such as dysplastic adaptation of epithelium, dermal fibrosis, papillomatous projections, hyperkeratosis of the epidermis and deposition of keratinocyte pearls over the epidermis when compared to DMBA/TPA treated group (Fig: 4.3 C, D, E).

The extent of lesion induced by DMBA/TPA were much less in skin histological sections of mice treated with *D. oocarpa*, *D. nigrescens* and *D. candolleana* root extract as indicated by absence of keratinocyte pearls, absence of dermal fibrosis, intact basal lamina, absence of hyperkeratosis, mild dysplasia when compared to DMBA/TPA treated group (Fig. No: 4.3 C, D, E).

SUMMARY AND CONCLUSIONS

The thesis entitled "Studies on chemopreventive effect of three species of diospyros on DMBA induced skin carcinoma in mice." embodies the work on the extraction, isolation and characterization of secondary metabolites of *Diospyros oocarpa* (Roots), *Diospyros negrisens* (Roots) and *Diopyros condolleana* (Roots). The isolated molecules were characterized using UV, IR, ^{1}H NMR, ^{13}C NMR and HR-EIMS spectroscopic analysis. The plant extracts and the isolates were evaluated for *in-vitro* cytotoxic potentials by Brine Shrimp Lethality Assay (against Brine Shrimps) and tryphan blue dye exclusion assay (against DLA Cell lines). The *Diospyros* extracts were also evaluated for *in-vivo* chemopreventive effects DMBA induced skin carcinoma and the results are presented.

Several *Diospyros* species are found to be used in tribal medicine for its different pharmacological activities, especially in treatment of tumours, warts, wounds which may be considered to be indicative of cancer. The survey of literature and documentation of tribal knowledge revels that various parts like roots, leaves, bark and flowers of *Diospyros* species were employed for such problems.

Globally, Cancer continues to be one of the major causes of death and only a modest progress has been achieved in reducing the morbidity and mortality of this disease. An integrated approach, incorporated by experience and knowledge gained through scientific developments, is needed to manage cancer. Several plants and traditional compounds are being screened worldwide to validate their use as anti-cancerous drugs[12]. But only a small portion has been explored phytochemically. So, it is anticipated that plants can provide potential bioactive compounds for the development of new 'leads' to combat cancer diseases.

The present study is designed to evaluate the of three unexplored *Diospyros* species from Western Ghats of Karnataka, were selected and tested to justify their existing bioactivities, for their phytochemical and dynamic biological profiles i.e. isolation and characterization of their important constituents and their anticancer activity.

Conclusion: All the three *Diospyros* species, *D. oocarpa, D, negrisens* and *D. condolleana*; and some of their isolates showed good cytotoxic properties on Brine shrimps (BSL assay) and DLA cell lines. The three *Diospyros* extracts, also showed significant chemopreventive effects, against DMBA induced skin carcinomas in mice.

Chapter 1 -Introduction:

Nature has always been a hallmark to emplify the outstanding phenomenon of symbiosis. It has provided a plethora of remedies to cure all ailments of mankind. Unaware of the specific importance and distinct chemical nature of the compounds present in natural products, mankind has been using them in their medical system. It was only by the start of twentieth century that natural products have experienced a great surge as a strong branch of organic chemistry. Many factors have led to this change, which include the development of new and more powerful separation techniques, such as advanced chromatographic methods, electrophoresis etc.; better characterization techniques via modern spectroscopic methods and the change in the perception of society about chemicals. The elucidation of the biosynthetic pathways for natural products in several cases led to isolation and characterization of the enzymes involved and even to the recognition and cloning of the genes which code for these enzymes. This led to the opening up of new avenues for discovery of bioactive molecules.

Cancer is a grave clinical problem that poses significant social and economic challenges to the healthcare system. Albeit to the developments of imaging and molecular diagnostic techniques, cancer continues to affect millions of people globally. Globally, cancer is the second leading cause of death after heart diseases. Lung, colorectal and stomach cancer are among the five most prevalent cancers in the world for both men and women. According to the American Cancer Society and from the International Union Against Cancer, 12 million cases of cancer were diagnosed in 2011, with 7 million deaths worldwide; these numbers are expected to double by 2030 (27 million cases with 17 million deaths).

Plants have been a prime source of highly competent conventional drugs for the treatment of many forms of cancer. A number of promising agents such as flavopiridol, roscovitine, combretastatin A-4, betulinic acid and silvestrol are in clinical or preclinical development.

Review of literature:

Diospyros is an important member genus of the Ebanaceae family. *Diospyros* species are known to elaborate a series of naphthoquinones and pentacyclic triterpenoid saponins. Medicinally, *Diospyros* species are used as anthelmentic, anti-inflammatory, antibacterial, antifungal, antioxidant, anticancer, antiviral, molluscicidal, piscicidal and termite resistant activities. Many *Diospyros* species have been reported to exhibit interesting biological and pharmacological activities viz. termicidal, Antifeedant, insecticidal, piscicidal fungicidal molluscicidal and activities**.** A comprehensive biological and phytochemical review on *Diospyros* species that are so far examined, the metabolites isolated and their chemical structures were presented.

No scientific information was available regarding the chemical constituents of *D. oocarpa*. Previous studies on the stem bark of *Diospyros nigresens* report the isolation of some unusual anthraquinone glycosides, ellagic acid glycosides, diospyranonaphthoside, diospyranooleanolide, diospyrososide. While earlier studies on *Diospyros condolleana* reealed the presence of triterpenes namely α-amyrin, lupeol and betulin.

Materials and Methods:

All the three *Diospyros* extracts were assessed for several pharmacognostic standardization parameters to obtain the qualitative information about the purity and quality. The preliminary phytochemical investigation was carried out to check the chemical profile of the three *Diospyros* extracts. Further, the extracts were subjected to a sequence chromatographic techniques for isolation of metabolites, and their structures were characterized using UV, IR, ^{1}H NMR, ^{13}C NMR and HR-EIMS spectroscopical analysis.

The preliminary Cytotoxic screening of the three *Diospyros* roots extract was carried out by BSL assay. Also, an *in-vitro* cytotoxicity test was carried out against Dalton's lymphoma ascites to evaluate the antitumor effects of the three *Diospyros root extracts.*

The *in-vivo* chemopreventive effects of the *Diospyros* extracts was carried out against DMBA induced skin carcinoma in mice.

Results and Discussion:

Preliminary phytochemical investigation of the three species revealed the presence of flavonoids, triterpenoid naphthoquinones and naphthaldehydes as the major phytoconstituents.

The chemical examination of the chloroform extracts of roots of *D. oocarpa* ten compounds (ADDOR 1-ADDOR), *D. nigrescens* seven compounds (ADDNR1- ADDNR) and *D. candolleana* seven compounds (ADDCR1-ADDCR) respectively.

The Biological evaluation for cytotoxic potential revealed that all the three *Diospyros* extracts and most of their isolates possessed good cytotoxic properties. Among the extracts, *D. negrisens* extract highest activity against brine shrimps, while *D. condolleana* extract showed highest cytotoxicity to DLA cell line. Among the isolates, were Diospyrin, 8'-hydroxyisodiospyrin and Habibone significantly inhibited the Brine shrimps, while Diospyrin, 8'-hydroxyisodiospyrin, plumbagin, Habibone and Diosindigo A were showed highly potent cytotoxicity against DLA cell lines.

The treatment of DMBA induced skin carcinoma mice with the three *Diospyros* extracts considerably decresased tuumour incidence, tumour volume, tumour weight, tumour incidence and mortality rate; and increased lipid peroxide and DNA levels in plasma.