7. Evaluation of bioactives of *S. racemosa on* H4TG (rat hepatoma cell) induced hepatocellular carcinoma in rats

7.1. Material, chemicals and reagents

7.1.1. Chemicals and reagents

The drugs used in the study were ketamine injection (Troikaa Pharmaceuticals Ltd., India), ciprofloxacin injection (Torrent Research Center, India), diazepam injection (Intas Pharmaceuticals Ltd., India) and antibiotic ointment (Neon Laboratory, India). The reference standard 5-fluorouracil (5-FU) was obtained from HCG Hospital, Ahmedabad, India. All the kits for hepatic marker enzymes were obtained from Euro diagnostics, Chennai, India. For determination of alpha-feto protein (AFP) rat ELISA kit (Mybiosource Inc., USA) was used. Other chemicals used in the experiment were of analytical grade.

7.1.2. Cell line and culture medium

H4TG (rat hepatoma cells) cell line was obtained from National Centre for Cell Science (NCCS), Pune. H4TG epithelial and adherent cell line originated from the rat hepatoma cells comprising of an integrated hepatitis B virus genome. The cells were matained in DMEM, supplemented with 10% FBS, penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin-B (5 µg/mL) in a humidified atmosphere of 5 % CO_2 at 37°C.

7.2. Animals

Healthy male Wistar Albino rats (180-270g) were procured from Cadila Pharmaceuticals Limited, Ahmedabad, India. They were housed in microloan boxes with standard laboratory diet and water *ad libitum*. The animals were maintained as per the guidance of CPCSEA, Ministry of Social Justice and Empowerment, Government of India; and the study was conducted after obtaining Institutional Animal Ethical committee approval (Registration number: ARL/PT/019/2014).

7.2.1. Dose of animals

Oral test doses were selected 50 and 100 mg/kg for ESME, and 5 mg/kg for the isolated compounds for the animals. *S. racemosa* bark was found to safe and non-toxic as per the literature review (Vijayabaskarn et al., 2010; Venkidesh et al., 2011; Wakchure et al., 2011) and no toxic effects were observed at above selected doses in our experimental study.

7.3. Induction of HCC *in vivo*

Experimental design

The animals were acclimatized and divided into eight groups each having six animals and total 48 male Wistar Albino rats (180-270g) were used in the experiment.

Group I: Normal control

Group II: Sham control (without inoculation of rat hepatoma cells)

Group III: Disease control

Group IV: ESME (50mg/kg/day for 14 days)

Group V: ESME (100mg/kg for 14 days)

Group VI: Isolated compound-I (5mg/kg for 14 days)

Group VII: Isolated compound-II (5mg/kg for 14 days)

Group VIII: 5-FU (75 mg/kg once a week)

42 male wistar albino rats (180-270g) were anesthetized with a hind limb injection of ketamine (75-100mg/kg) and diazepam (2-6 mg/kg). After anesthesia laporatomy was performed on each rat and left hepatic lobe was exposed on a sterile compress (Group II). 1×10^6 rat hepatoma cells were inoculated in to the left hepatic lobe in the hepatic capsule. A pale whitish mark has been observed at the point of injection under the hepatic capsule. A gentle compression was applied with cotton applicator for 15 seconds to avoid bleeding and reflux of the cells. Then the abdominal incisions were closed with two layer technique followed by administration of ciprofloxacin injection (1-2mg/kg) and application of antibiotic ointment topically. Animals were returned for the duration of follow-up. After 24 h of hepatoma cells inoculation, the hepatoma bearing rats were randomized in to six groups each having 6 animals (Group III-VIII). After 28 days HCC was confirmed in the animals with the help of biochemical parameters and tumor marker test. During the entire experimental period, the body weight changes of the animals were recorded. At the end of experiment 56^{th} day, animals were fasted overnight, weighed, blood was collected from retro orbital plexus and the serum was separated out. The animals were sacrificed by exposing them to CO_2. Liver were immediately removed and suspended in ice-cold saline. Blood was analysed for hematological parameters with the help of hematology analyzer. Serum was analysed for biochemical parameters such as aspartate amino transferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein (TP), total bilirubin (BL) and tumor marker alpha-feto protein (Singh, B. N., 2009; Guo et al., 2011). 5-FU is extensively used

in the treatment of HCC and therefore used as a positive control in the present experimental study (Abdel-Hamid and Morsy, 2010; Chang et al., 2012).

7.3.1. Body weight and relative liver weight analysis

The body weight and the relative liver weight expressed in g/100g body weight were measured at the end of the experiment.

7.3.2. Assessment of hematological parameters and liver function tests

7.3.2.1. Collection of blood

Animals were fasted overnight, weighed and blood was collected in clean dry centrifuge tubes from the retro orbital plexus of the each animal and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation at 5000 rpm for 20 min at 4ºC and stored at -20ºC until the analyses were carried out.

7.3.2.2. Hematological studies

At the end of experiment blood was analysed for hematological studies. Red blood cells (RBC), White blood cells (WBC) and Hemoglobin (Hb) were estimated with help of hematology analyser. The RBC, WBC and Hb were expressed as **(*10^6/mm^3), (*10^3/mm^3)** and g/dl of blood respectively.

7.3.2.3. Liver function test

Serum was analysed for biochemical parameters such as aspartate amino transferase (AST), alanine transaminase (ALT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Kind and King, 1954), total protein (TP) (Lowry et al., 1951), total bilirubin (BL) (Mallay and Evelyn, 1937) through kit based assay (Euro diagnostic system Pvt. Ltd, Chennai).

7.3.2.3.1. Alanine amino transferase (ALT/SGPT)

Serum glutamic pyruvic transaminase (SGPT) called as Alanine amino transferase (ALT/ALAT) belongs to transferase class of enzymes. It is found to be distributed in the liver and to lesser extent in kidney and muscles. In hepatitis of different etiologies, ALT is an important indicator not only in the diagnosis of the ailment but also in assessing the prognosis and process of the disease. As a liver specific enzyme ALT is only significantly elevated in hepatobiliary diseases.

Principle

ALT catalyses the reaction between alpha-ketoglutaric acid and L-alanine to form glutamate and pyruvate, in the presence of lactate dehydrogenase (LDH), reacts with NADH giving lactate and NAD. The rate of NADH consumption is directly proportional to the AST activity in the sample which is determined.

L-alanine + 2-oxoglutarate ⟶ L-glutamate + pyruvate

Pyruvate + NADH + H^+ ⟶ L-lactate + NAD^+

Reagent I: Tris pH 7.5, L-alanine, lactate dehydrogenase (LDH)

Reagent II: 2-oxoglutarate, NADH

Working reagent: Four parts of reagent I mixed with one part of reagent II.

Procedure: Working reagent 1000 µL and sample of serum 100 µL were transferred in to cuvette and immediately mixed. After one minute initial absorbance was measured at 340 nm 37˚C. Then 1, 2 and 3 min from first reading absorbance was measured at 340 nm. Average change in absorbance per min has been recorded (ΔA/min). ALT was measured by following formula,

$$\text{ALT/SGPT (U/L)} = (\Delta A/\text{min}) \times 1746$$

7.3.2.3.2. Aspartate amino transferase (AST/SGOT)

Aspartate amino transferase (AST) also called as SGOT. The AST is a cellular enzyme found in highest concentration in liver cells, heart muscle and skeletal muscle. Although an elevated level of AST in the serum is not specific of the hepatic disease, is used mainly to diagnose and to verify the course of this disease with other enzymes like ALT, ALP.

Principle

AST catalyses the reaction between alpha-ketoglutaric acid and L-aspartate to form glutamate and oxaloacetate, in the presence of malate dehydrogenase (MDH), reacts with NADH giving malate and NAD. The rate of NADH consumption is directly proportional to the AST activity in the sample which is determined.

L-Aspartate + 2-oxoglutarate ⟶ oxalacetate +L-glutamate

oxalacetate + NADH + H^+ ⟶ L-malate + NAD^+

Reagent I: Enzyme

Reagent II: Substrate

Working reagent: Four parts of reagent I mixed with one part of reagent II.

Procedure: Working reagent 1000 µL and sample of serum 100 µL were transferred in to cuvette and immediately mixed. After one minute initial absorbance was measured at 340 nm at 37 ˚C. Then 1, 2 and 3 min from first reading absorbance was measured at 340 nm. Average change in absorbance per min has been recorded (ΔA/min). AST was measured by following formula,

$$\text{AST/SGOT (U/L)} = (\Delta A/\text{min}) \times 1746$$

7.3.2.3.3. Alkaline phosphatase (ALP)

ALP is an enzyme of the Hydrolase class which acts in alkaline medium. It is found in high concentration in the liver, biliary tract and bones. Elevated level of ALP associated with liver and bone diseases.

Principle

P-nitrophenyl phosphate is converted to p-nitrophenol and phosphate by ALP. The increase in absorption at 510 nm is proportional to the ALP concentration in the sample (Figure 7.1).

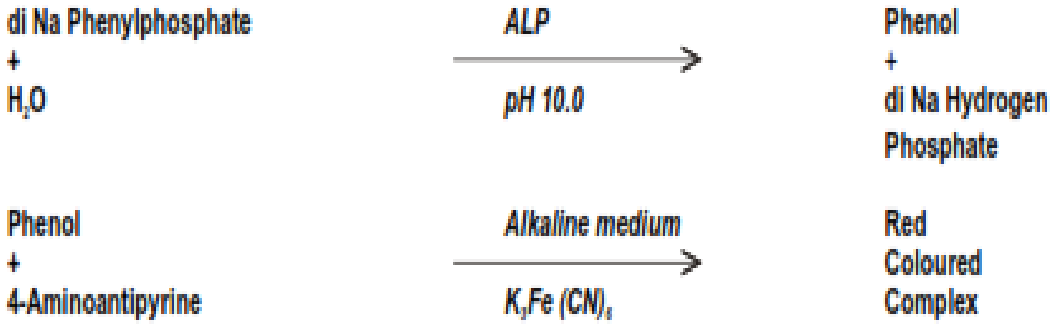


Figure 7.1: Principle reaction behind estimation of ALP

Table 7.1: Assay procedure for estimation of ALP

Addition sequence	Blank (B)	Standard (S)	Control (C)	Test (T)
Distilled water ate	1.5	1.0	1.0	1.0
Buffered reagent	1.0	1.0	1.0	1.0
Substrate reagent	0.1	0.1	0.1	0.1
Mixed well and incubated for 3 min. at 37^0C				
Serum	-	-	-	0.05
Phenol standard	-	0.05	-	-
Mixed well and incubated for 15 min. for 37^0C				
Colour reagent	1.0	1.0	1.0	1.0
Serum	-	-	0.05	-

Mixed well after the addition of each reagent and the absorbance of all test tubes was measured against purified water using spectrophotometer at 510nm and calculated for alkaline phosphatase as follows (Table 7.1):

$$\text{Serum alkaline phosphatase activity in KA units} = \frac{\text{Abs. test} - \text{Abs. control}}{\text{Abs. standard} - \text{Abs. blank}} \times \mathbf{10}$$

7.3.2.3.4. Total protein

Proteins are pretreated with copper ion in alkali solution. Aromatic amino acid present in the sample reduce the phosphomolybdatephosphotungstic acid present in the folin-ciocalteau reagent converted in to blue colour. Absorbance was taken at 750 nm (Lowry et al., 1951).

Different dilutions of bovine serum albumin (BSA) stock solution were prepared in the concentration range 20 to 100 µg/mL. In these different dilutions, 3 mL of alkaline copper sulphate solution was added. This solution was mixed well and incubated at room temperature for 10 minutes. Then 3 mL of folin-ciocalteau reagent was added to each test tube and incubated for 30 min. The absorbance was noted at 750 nm on UV Spectrophotometer. The standard curve of absorbance against protein concentration was plotted. From this curve absorbance of unknown sample and the concentration of the unknown sample were determined.

7.3.2.3.5. Total bilirubin

Bilirubin is the breakdown product of hemoglobin. It is transported from the spleen to the liver and excreted into bile. Hyperbilirubinemia results from increase in bilirubin concentration in plasma. Causes of hyperbilirubinemia: Total bilirubin: genetic error, increase hemolysis, neonatal jaundice, drugs. Direct bilirubin: hepatic cholestasis, hepatocellular damage, genetic error.

Principle

Bilirubin is converted to colored azobilirubin by diazotized sulfanilic acid and measured photometrically. Bilirubinglucuromide and free bilirubin loosely bound to albumin found in the serum. Bilirubinglucuromide reacts directly in water whereas free bilirubin requires solubilization in the dimethylsulfoxide to react. The intensity of colour formed is proportional to the bilirubin concentration in the sample.

Reagents:

Reagent 1: Sulfanilic acid and hydrochloric acid

R 2: Sulfanilic acid and hydrochloric acid & DMSO

R3: Sodium nitrite

Table 7.2: Assay procedure for estimation of bilirubin

	Blank	Total BL	Blank	Direct BL
R1(D)mL	----	----	1.5	1.5
R2(T)mL	1.5	1.5	----	----
R3(µl)	----	50	----	50
Sample(µl)	100	100	100	100

All the reagents were pipette out; incubated from 5 min at 15-25ºC and absorbance were observed at 555 nm. Total and direct bilirubin content was measured by following formula (Table 7.2).

mg/dl of bilirubin = (Absorbance of sample – Absorbance of blank) * factor

For total bilirubin factor=19.1, direct bilirubin factor=14

7.3.3. Tumor marker test

Tumor marker AFP is a single chain glycol protein with a molecular weight of approximately 70,000 daltons and alpha electrophoretic mobility. AFP is a major serum protein found in yolk sac and embryo liver (Sell, 1983; Premalatha and Sachdanandam, 1999).

AFP was determined in the serum using the assay based on quantitative sandwich enzyme immunoassay technique. Antibody specific for AFP has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any AFP present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for AFP is added to the wells. After washing, avidin conjugated Horseradish Peroxidase is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of AFP bound in the initial step. The color development is stopped and the intensity of the color is measured at 450 nm.

7.3.4. Tumor assessment

Tumor assessment was based on morphology and histology of the liver tissues. Liver tissues were excised, weighed and examined macroscopically on the surface for gross visible persistent nodules. The persistent nodules were identified by their grayish white color and demarcation. The persistent nodules and their incidence were described on basis of number of rats with nodules per total number of rats and the number of nodules per total volume of liver. The nodule dimensions were measured in two perpendicular directions using calipers to obtain the average diameter. The

persistent nodules were categorized into three groups according to their diameter (Less than 1, Between 2-3 and more than 3) (Moreno et al., 1991).

7.3.5. Histopathological examination

A small portion of liver was fixed in phosphate buffered 10% formalin, embedded in paraffin and used for histopathological studies. Five-µm-thick sections were cut, deparaffinized, hydrated, stained with hematoxylin and eosin, examined and photographs were taken with a digital stereomicroscope. The sections were observed blindly for tubular cell swelling, interstitial edema, tubular dilation and necrosis in all treatments.

7.3.6. Statistical analysis

The results were expressed as mean±SEM. Statistical significance between more than two groups was determined by one way ANOVA followed by Tukey's multiple comparison tests as appropriate using a computer based fitness programme (Prism, graphpad). Difference were considered to be statistically significant at $P<0.05$.

7.4. Results and discussion

7.4.1. Body weight and relative weight

The changes in body weight of experimental groups of animals are shown in Table 7.3. Disease control group showed significant reduction in body weight as compared to normal control group. Treatment group showed significant increase in body weight as compared to disease control group. The remarkable reduction in body weight observed in the H4TG induced animals group could be an indirect index of the declining hepatic function due to development of HCC or following exposure to hepatocarcinogen. The weight loss could be predominantly due to cancer cachexia, resulting in wasting of skeletal muscle and adipose tissue, with relative sparing of visceral proteins thereby implying poor prognosis and short survival period (De wys, 1986).

Table 7.3: Effect on change in animal body weight in HCC

Group	Dose	Body weight (g)		
		0 Day	28th Day	56th Day
Normal control	----	233.3±8.02	244±8.41	262.5±9.92
Sham control	----	230±2.29	239±2.45	257.1±2.08
Disease control	----	229.3±12.41	233±11.80	240.3±12.85*
ESME	50mg/kg	232±6.92	238±7.28	256.1±6.07#
	100mg/kg	232.5±6.55	234.8±6.92	250.3±7.22#
Isolated comp. 1	5mg/kg	234±6.37	238.3±6.34	256±6.31#
Isolated comp. 2	5mg/kg	229.5±2.75	233.8±2.71	248.3±2.15#
5-FU	75mg/kg	226.8±8.38	231.8±8.07	251.1±8.08 #

Table 7.3: Effect of ESME, isolated compound 1 and 2 and 5-FU on Animal body weight; N= 6 animals in each group; Values are expressed as mean±SEM; *P value <0.0001 as compared to control group. #P value <0.005 as compared to disease control group.

7.4.2. Liver weight

The significant increase in liver weight was observed in the disease control group which could be due to formation of nodules and tumors in the liver because of uncontrolled cell proliferation due to development of HCC. In the treatment group decreased liver weight could indicate the amelioration capacity and protective efficiency of ESME (100mg/kg) and isolated compounds 1 and 2 (5mg/kg) towards anti-proliferative action against H4TG induced HCC.

Also for 5-FU, the results were consistent with previously reported research indicating that 5-FU is cytotoxic to the proliferating cells by arresting them in the G0-G1 phase of the cell cycle (Chang, et al., 2012). The results are shown in Table 7.4.

Table 7.4: Effect on change in liver weight in HCC

Group	Dose	Liver weight (g)	Relative liver weight (g)
Normal control	----	4.59±0.28	1.75±0.07
Sham control	----	4.76±0.22	1.85±0.09
Disease control	----	8.19±0.18*	3.39±0.19*
ESME	50mg/kg	7.07±0.16$^{\#}$	2.76±0.03$^{\#}$
	100mg/kg	6.80±0.15$^{\#}$	2.73±0.10$^{\#}$
Isolated comp. 1	5mg/kg	5.75±0.08$^{\#}$	2.24±0.05$^{\#}$
Isolated comp. 2	5mg/kg	5.61±0.10$^{\#}$	2.26±0.04$^{\#}$
5-FU	75mg/kg	3.46±0.11$^{\#}$	1.38±0.07$^{\#}$

Table 7.4: Effect of ESME, isolated compound 1 and 2 and 5-FU on liver weight and relative liver weight; N= 6 animals in each group; Values are expressed as mean±SEM; *P value <0.0001 as compared to control group. $^{\#}$P value <0.005 as compared to disease control group.

7.4.3. Hematological study

In disease control group the RBC and Hb levels were decreased with concomitant increase in WBC as compared to normal control group whereas treatment group maintained the normal level of hematological parameters as compared to disease control group which is shown in Table 7.5. The decreased RBC count may be due to destruction of erythrocytes or the result of adverse effect on erythropoitic tissue namely bone marrow (Sreelatha et al., 2011; Wang et al., 2012).

Table 7.5: Hematological parameters

Design of treatment	Dose (mg/kg)	RBC (*10^6/mm^3)	Hb(g/dl)	WBC (*10^3/mm^3)	Platelet (*10^3/ml)
Normal control	----	7.65±0.04	12.56±0.44	10.73±0.15	0.98±0.07
Sham control	----	7.19±0.19	13.99±0.48	8.3±0.84	0.94±0.08
Disease control	----	5.63±0.13*	9.45±0.15*	13.71±0.30*	0.90±0.05
ESME	50	7.38±0.47	13.95±0.54#	9.96±0.42#	0.98±0.08
	100	7.2±0.36	13.70±0.88#	8.36±0.83#	0.85±0.07
Isolated comp. 1	5mg/kg	7.55±0.14	13.9±0.74#	8.11±0.99#	0.81±0.05
Isolated comp. 2	5mg/kg	7.46±0.12	12.71±0.57#	9.56±0.35#	0.85±0.05
5- FU	75mg/kg	7.22±0.34	13.36±0.82#	8.85±0.68#	0.96±0.12

Table 7.5: Effect of ESME and isolated compound 1 and 2 on RBC, HB, WBC Platelets; N= 6 animals in each group; Values are expressed as mean±SEM; *P value <0.0001 as compared to control group. #P value <0.005 as compared to disease control group.

7.4.4. Liver function tests

All the H4TG induced animals (disease control group) had a significant incidence of HCC at the day of 28th and end of experiment at the day of 56th as evidenced by increase in hepatic enzymes with decrease in total protein. Treatment group showed significant decrease in hepatic enzymes with increase in total protein as compared to disease control group (Table 7.6). Liver damage caused in diseased control group generally reflects instability of liver cell metabolism which leads to distinctive changes in the serum enzymes activities. AST, ALT, ALP and BL are the representative of liver function. Their increased levels are the indicators of liver damage and implicative of the cellular leakage and improper membrane integrity. The elevation of ALT activity is repeatedly credited to HCC and is usually accompanied by a rise in AST. Analysis of hepatic marker enzymes AST and ALT demonstrates mechanisms of cellular injury, subsequent release of proteins, their extracellular turnover and mechanisms of neoplastic processes (Iqbal et al., 2004). The elevation of AST and ALT at 28th and 56th day in the disease control group were significantly higher than those in normal control group ($P<0.0001$). In contrast, treatment group showed significantly reduction in AST and ALT levels compared to disease control group ($P<0.005$). No significant differences in the hepatic enzymes were observed in normal control and sham control group. Increase in ALP reflects the pathological alteration in biliary flow. Elevation in BL may be attributed to leakage of plasma membrane and loss of functional integrity of cell membranes in liver. Increased ALP level may reflect cholestatis, which is partial or

complete blockade of bile ducts. Since bile duct transport bile from the liver to gall bladder and intestine, inflamed or damaged liver may result in the leakage of ALP into blood stream. ALP level is frequently elevated in bile obstruction, intra hepatic cholestasis and fatty liver or liver tumors. Elevation of ALP is one of the sign of suggesting space occupying lesions in the liver. Tumor development leads to tissue damage that invokes the release of ALP in the circulation and cause the elevation of ALP in the serum of tumor bearing animals (Varley, 1988; Delvin, 1997; Martin and Friedman, 1998; Abdel-Hamid, 2001; Rajesh and Perumal, 2012). In the present study, treatment with ESME and isolated compound 1 and 2 decreased the activities of these enzymes which were elevated in the animals due to development of HCC and the levels were normalized (Table 7.6). This suggested that the ESME and isolated compound 1 and 2 played a role in parenchymal cell regeneration in the liver, thus protecting membrane integrity, thereby decreasing enzyme leakage. As evident in present study, administration of ESME, isolated compound 1 and 2 and 5-FU significantly restored the levels of hepatic enzymes due to hepatoprotective effects thus preventing the HCC damage caused by H4TG induced HCC, thereby suppressing the leakage of enzymes through plasma membranes. Graphical representation of the estimation of hepatic enzymes was shown in Figure 7.2-7.6.

Table 7.6: Liver function tests

Group	Dose (mg/kg)	AST(U/L) Day			ALT(U/L) Day			ALP(mg/dL) Day			TP(mg/dL) Day			BL(mg/dL) Day		
		0 Day	28th	56th	0 Day	28th	56th	0 Day	28th	56th	0	28th	56th	0	28th	56th
NC	---	65.46±2.84	68.95±3.64	67.98±3.28	23.88±0.99	27.28±0.78	28.96±1.12	5.50±0.1	5.30±0.09	5.18±0.25	6.13±0.15	6.17±0.17	6.15±0.15	0.56±0.01	0.56±0.002	0.56±0.002
SC	---	67.34±2.94	71.77±3.86	72.51±3.73	22.805±1.74	28.115±1.89	30.205±2.28	5.48±0.11	5.43±0.07	5.38±0.08	5.87±0.24	5.85±0.23	5.88±0.24	0.55±0.004	0.56±0.006	0.56±0.004
DC	---	**66.37±5.39**	**183.17±1.38**	**243.61±1.39***	**21.11±1.54**	**94.74±1.53**	**168.14±1.45***	**5.54±0.1**	**9.06±0.38**	**10.73±0.51***	**5.78±0.05**	**4.07±0.1**	**2.40±0.05***	**0.55±0.006**	**0.74±0.006**	**1.01±0.02***
ESME	50	65.72±3.78	184.46±2.34	106.38±4.48#	24.83±1.79	93.45±1.41	68.23±3.08#	5.49±0.009	8.88±0.41	7.67±0.006#	5.82±0.08	4.08±0.07	4.63±0.04#	0.60±0.1	0.77±0.007	0.56±0.007#
	100	66.3±4.14	186.65±1.57	95.56±0.89#	23.73±1.78	91.59±0.87	58.58±1.87#	5.46±0.06	9.18±0.34	6.73±0.09#	5.97±0.09	4.12±0.13	4.48±0.08#	0.57±0.006	0.77±0.008	0.55±0.009#
IS-1	5	68.39±3.24	183.67±3.74	92.10±1.34#	22.63±1.78	96.93±0.87	50.76±1.86#	5.45±0.1	8.62±0.28	6.53±0.21#	5.90±0.04	3.95±0.07	5.45±0.11#	0.56±0.005	0.77±0.008	0.55±0.005#
IS-2	5	62.56±5.61	185.95±2.76	90.12±1.23#	25.85±0.73	95.60±1.38	53.96±2.68#	5.58±0.13	8.38±0.33	6.47±0.1#	6.02±0.1	3.97±0.09	5.35±0.07#	0.56±0.004	0.77±0.005	0.56±0.007#
5-FU	75	68.70±4.46	184.70±2.04	75.70±1.15#	21.60±1.72	96.90±1.52	46.10±2.22#	5.33±0.07	8.65±0.34	5.52±0.11#	5.90±0.085	4.13±0.18	5.70±0.11#	0.56±0.02	0.76±	0.55±0.003#

Table 7.6: Effect of ESME and isolated compound 1 and 2 and 5-FU on AST, ALT, ALP, TP, TB; N= 6 animals in each group; Values are expressed as mean±SEM; *P value <0.0001 as compared to control group. #P value <0.005 as compared to disease control group.

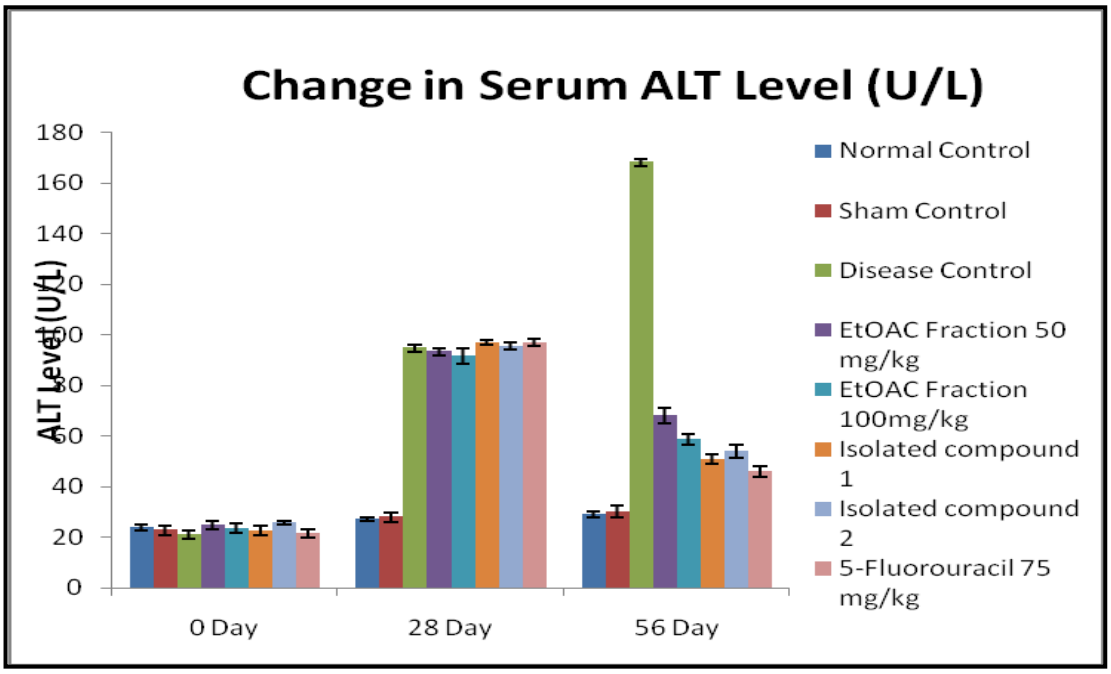


Figure 7.2: Effect of ESME (50 and 100 mg/kg) and isolated compound 1 and 2 (5mg/kg) and 5-FU on ALT level

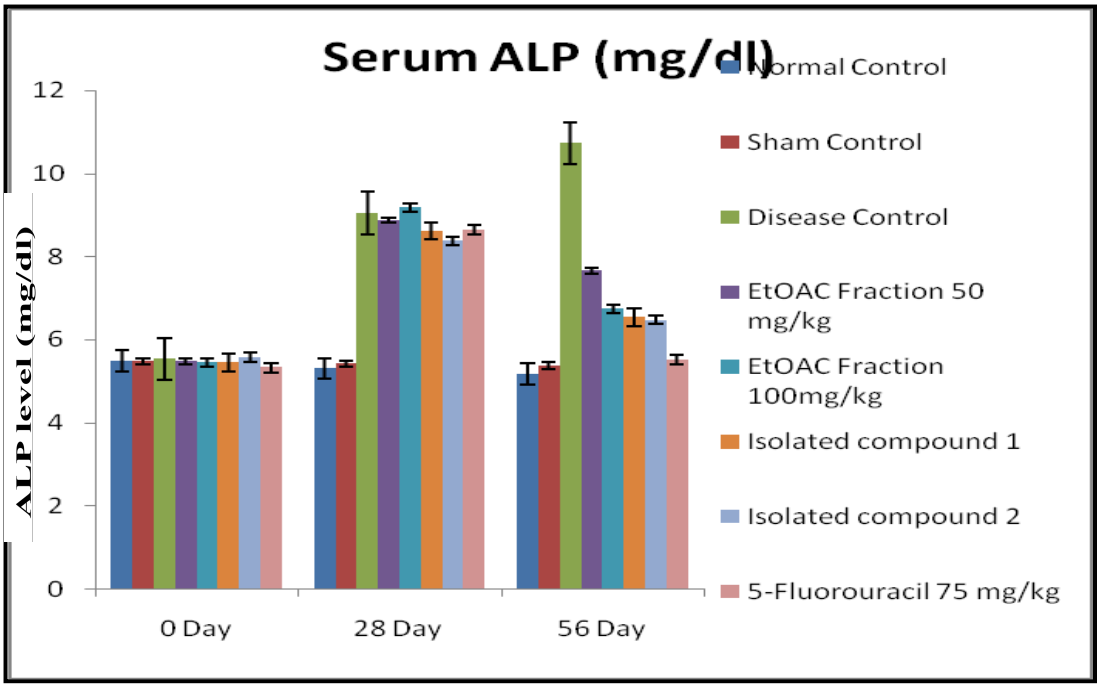


Figure 7.3: Effect of ESME (50 and 100 mg/kg) and isolated compound 1 and 2 (5mg/kg) and 5-FU on ALP level

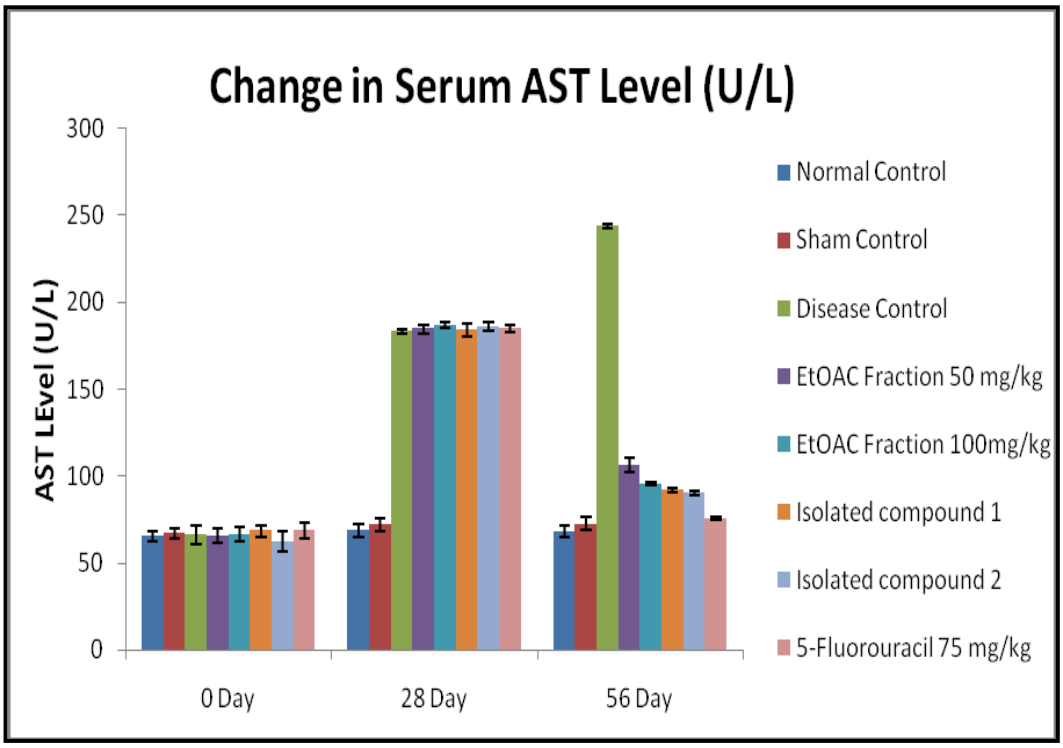


Figure 7.4: Effect of ESME (50 and 100 mg/kg) and isolated compound 1 and 2 (5mg/kg) and 5-FU on AST level

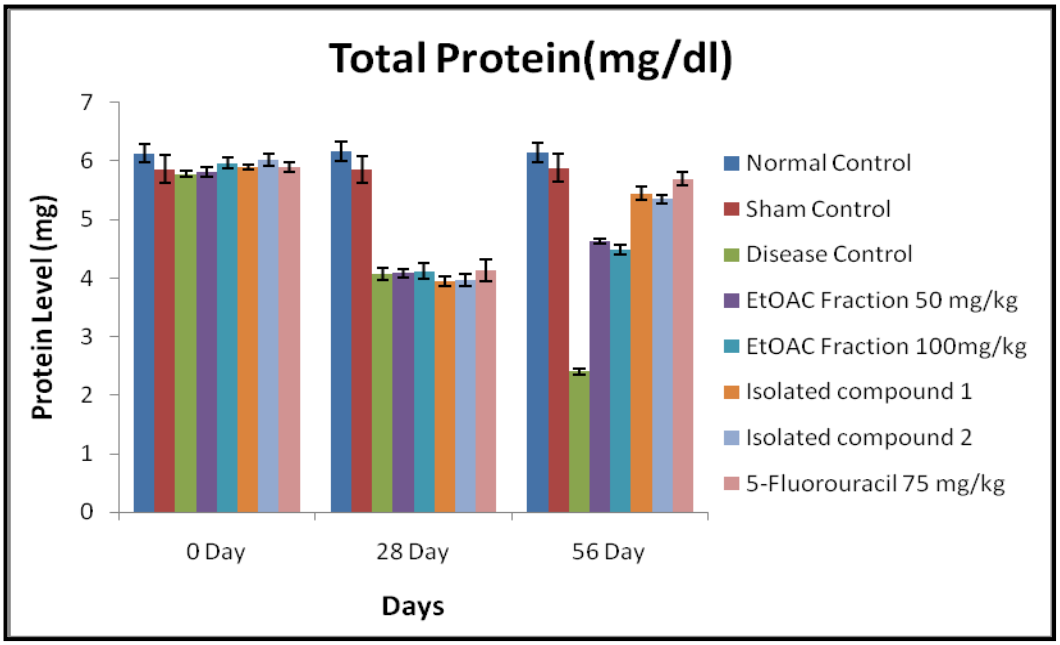


Figure 7.5: Effect of ESME (50 and 100 mg/kg) and isolated compound 1 and 2 (5mg/kg) and 5-FU on TP level

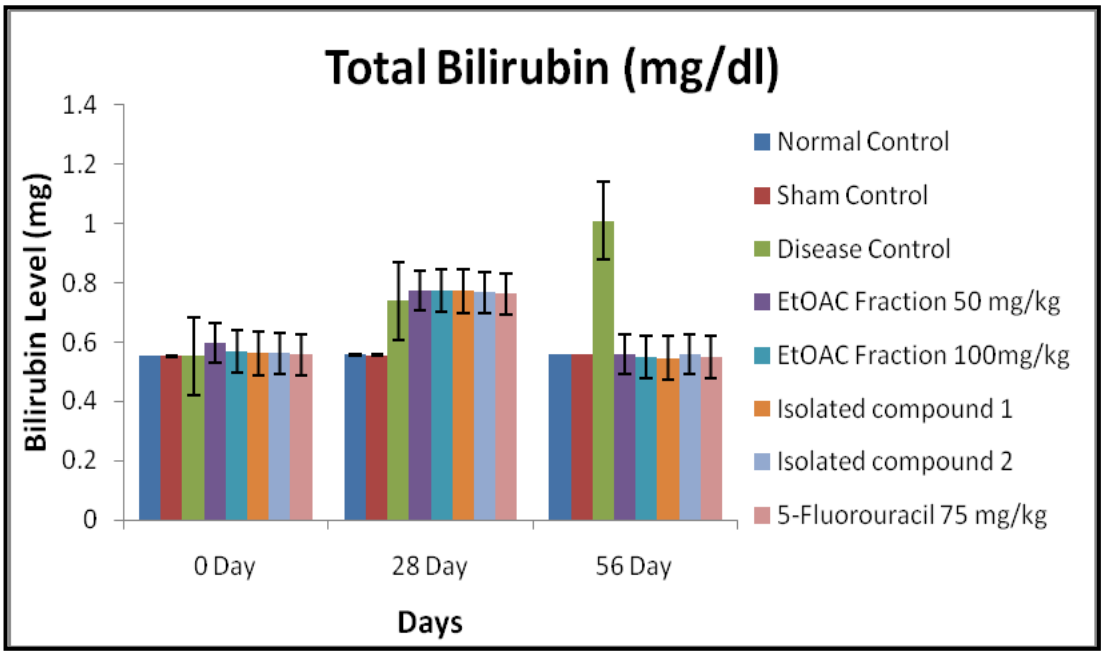


Figure 7.6: Effect of ESME (50 and 100 mg/kg) and isolated compound 1 and 2 (5mg/kg) and 5-FU on TB level

7.4.5. Tumor marker test for AFP

Tumor marker AFP is an immunomodulatory glycoprotein with an uncertain biological function, normally synthesized by the immature liver cells in the fetus and is progressively lost during development. AFP is a major serum protein found in yolk sac and embryo liver. In terms of size, structure and amino acid composition, it is similar to serum albumin, but it is detectable only in minute amounts in the serum of healthy adults (Sell and Leffert, 2008). AFP manifests high specificity for HCC. Elevation of AFP levels is observed due to development of HCC and on exposure to hepatotoxic agents. It is a predictive marker for tumor invasiveness and recurrence of HCC. Elevation of AFP levels has been observed in the 90% of the patients with hepatic cancer. It's detection in the serum during monitoring of HCC treatment is well acknowledge in patients with elevated AFP levels prior to therapy and is advocated by the European Association for the study of Liver (EASL) as an index to determine the extent of the cancer and the diagnosis of tumor response to the therapy (Abeler, 1971; Sell, 1983). In the present study, AFP levels were elevated in disease control group at 28th and 56th day of the study as compare to normal control group and sham control group. The levels is significantly declined on administration of ESME and isolated compound 1 and 2 in the diseased animals indicating their anticancer potential which is compared with standard drug 5-FU (Figure:7.7) ($P<0.005$). The results are shown in Table 7.7.

Table 7.7: Tumor marker test for AFP

Group	Dose	AFP(ng/mL)	
		28th Day	56th Day
Normal control	----	0.25±0.008	0.26±0.007
Sham control	----	0.275±0.004	0.283±0.003
Disease control	----	1.42±0.025	3.45±0.1*
ESME	50mg/kg	1.41±0.022	1.66±0.009#
	100mg/kg	1.40±0.025	1.39±0.06#
Isolated comp.1	5mg/kg	1.42±0.024	1.26±0.06#
Isolated comp.2	5mg/kg	1.39±0.024	1.35±0.05#
5-FU	75mg/kg	1.37±0.022	1.31±0.08#

Table 7.7: Effect of ESME and isolated compound 1 and 2 on AFP; N= 6 animals in each group; Values are expressed as mean±SEM; *P value <0.0001 as compared to control group. # P value <0.005 as compared to disease control group.

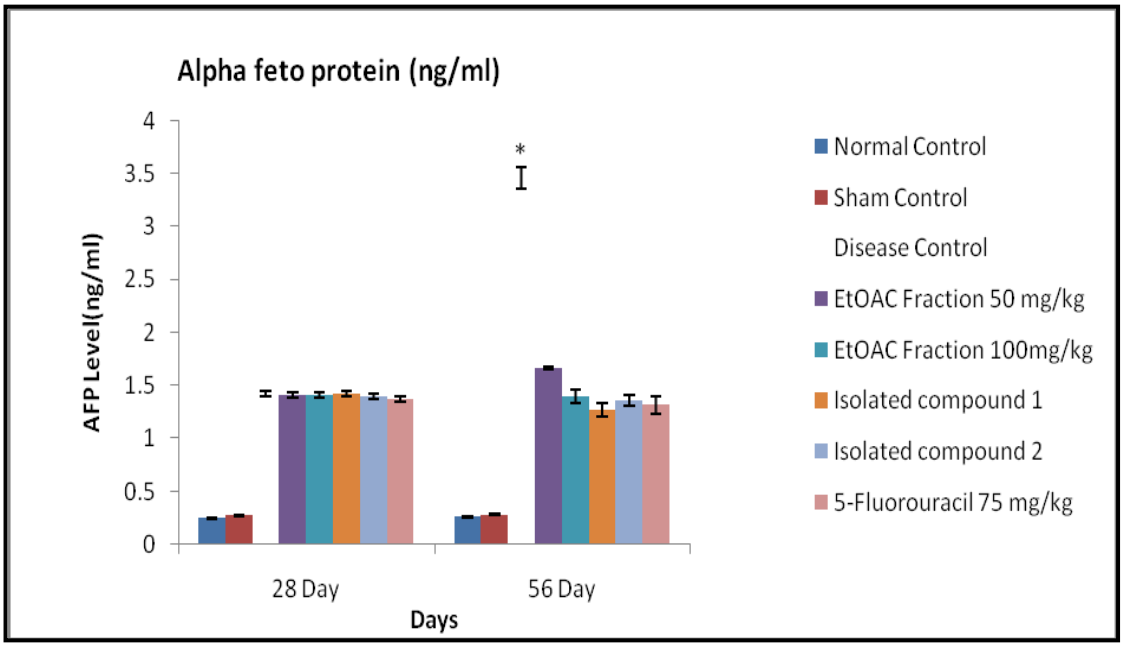


Figure 7.7: Effect of ESME (50 and 100mg/kg), isolated compounds (5mg/kg) and 5-FU on AFP level

7.4.6. Tumor assessment

Not all hepatocyte nodules become malignant during the life span of animals however several compelling evidence indicate that nodules are the precursors of hepatic cancer. Various experimental and observational studies correlate both frequency and size of nodular heprplasia to

hepatic carcinoma (Sell and Leffert 2008; farber and Sarma, 1987; Farber, 1990.). In the present study, there were no hepatocyte nodules in the livers of normal control group and sham control group. Macroscopic nodules have been observed in the H4TG induced HCC animals.

In the disease control group all the animals were showed the induction of HCC. The average size of tumor was found to be more than 3 mm. In the treatment group no of nodules per total volume of liver were found to be decreased and size of nodules were also reduced (Table 7.8). Thus the ESME and isolated compound 1 and 2 mediated inhibition of hepatic nodule formation and suppression of nodule growth as observed in the current study established the potential of the bioactives in the liver cancer carcinoma. The morphologic appearance of liver of different groups is shown in the Figure 7.8.

Table 7.8: Percent incidence of nodules, number and size of nodules

Design of treatment	**Dose (mg/ kg)**	**Number of rats bearing nodules/Total no of rats**	**% incidence of nodules**	**Number of nodules/total volume of liver**	**Size of nodules (mm)**
Normal control	----	------	-----	------	------
Sham control	----	------	-----	------	------
Disease control	----	6/6	100	22	>3
ESME	50	5/6	83.33	13	2-3
	100	5/6	83.33	10	1-2
Isolated comp. 1	5	4/6	66.67	7	0-1
Isolated comp. 2	5	4/6	66.67	6	0-1
5-FU	75	4/6	66.67	5	0-1

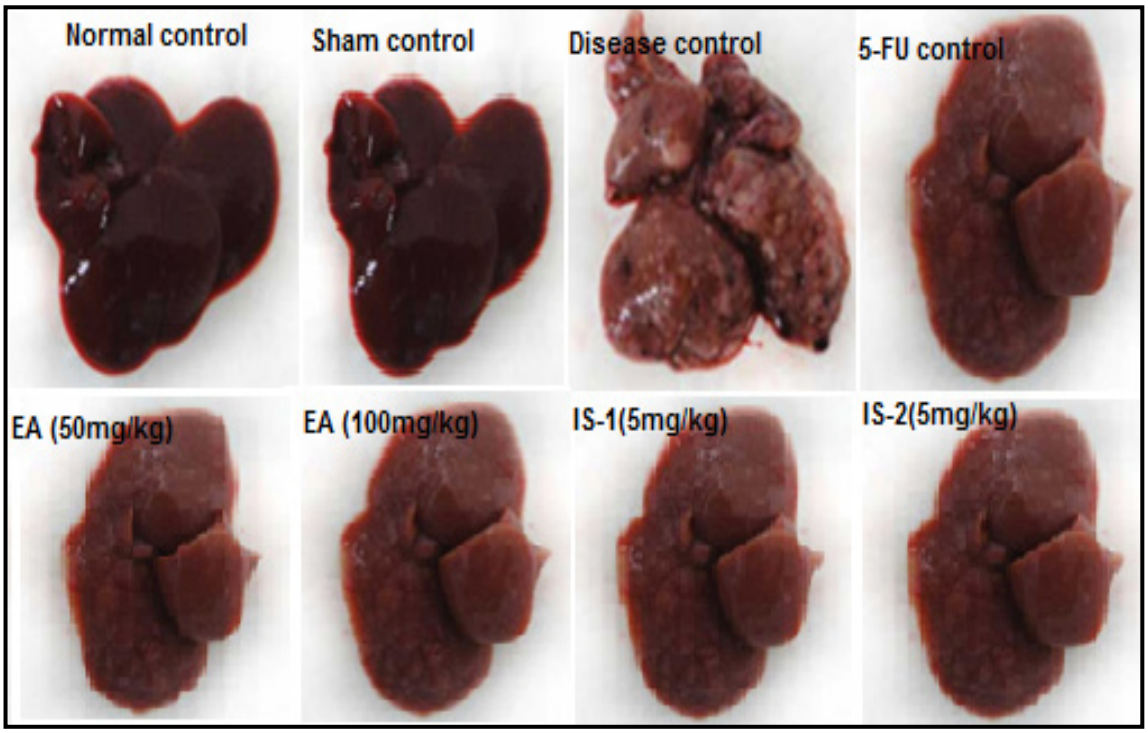
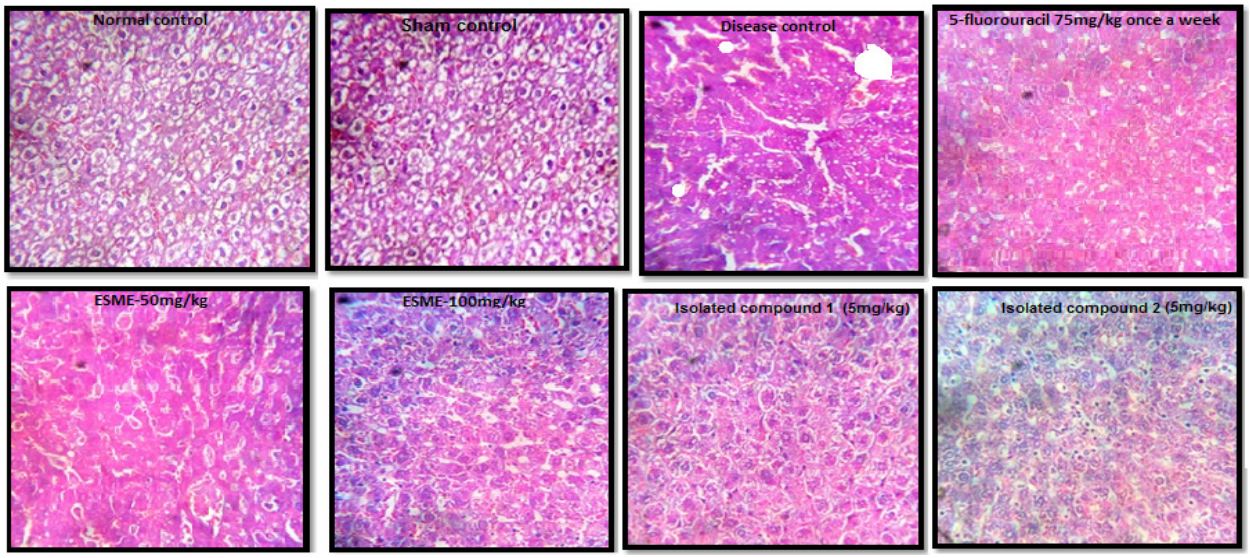


Figure 7.8: Morphological appearance of liver of normal control, sham control, disease control, treatment control (ESME, isolated compounds 1&2 and 5-FU)

7.4.7. Histopathological study

Liver sections from the normal and sham control group showed normal hepatic architecture, no evidence of hepatocyte injury or fibrosis or dysplasia or malignancy noticed. In contrast, liver sections from disease control animals showed extensive clusters of hepatocyte necrosis, loss of architecture, inflammatory infiltrates. The tumor cells resembling hepatocytes show pleomorphism and were seen as 2-8 celled wide trabeculae that were separated by endothelium lined sinusoidal spaces. The prophylactic group showed periportal inflammation with conspicuously dilated blood vessels and ballooning degeneration of mononuclear infiltrates associated with regenerative cellular changes of adjacent hepatocytes. The standard control group (5-FU) showed some loss of architecture and hepatocellular necrosis due to the cytotoxicity of the drug (Rajkapoor et al., 2006; Roomi et al., 2010). The histopathological evaluation of liver sections form different group supported the biochemical findings and is illustrated in the Figure 7.9.



NC & SC: H and E stained section of liver from a normal control rat showing normal architecture of hepatic cells (100x).DC: H and E stained section of liver cell lines injected rat showing loss of architecture, severe hepatocellular necrosis with the adjacent liver cells (100x). TC: H and E stained section of liver rat showing neoplastically transformed cells and hepatocytes maintaining near normal liver architecture and necrosis was healed which indicted the effectiveness of the treatment (100x). 5-FU control: H and E stained section of liver rat showing some hepatocellular necrosis due to the cytotoxicity of the drug.

Figure 7.9: Histological study of liver of normal control, sham control, disease control, treatment control (ESME, isolated compounds 1&2 and 5-FU)

7.5. Conclusion

The present study was undertaken to investigate the effect of ESME and isolated compounds 1 and 2 on H4TG induced hepatocellular carcinoma in the rats. H4TG induced HCC in disease control group was evidenced by increase in liver weight, increase level of hepatic enzymes like AST, ALT, ALP, total bilirubin and decrease in total proteins with elevation of tumor marker AFP. All these changes have been reverted bake to normal on administration of ESME and isolated compounds as compared with 5-FU, indicating a strong inhibition of hepatocellular carcinogenesis induced by H4TG cell. The potential anticancer activities of ESME and isolated compounds 1 and 2 against hepatic cancer *in vivo* were investigated first time in this experimental study. The results demonstrated that ESME and isolated compound 1 and 2 (stigmasterol and ursolic acid) have powerful anticancer activity against liver cancer without significant effect on normal cells and might serve as a novel therapeutic agent in the treatment or prevention of hepatocellular carcinoma.

7.6. References

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