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Isolation of Fucoidan from seaweed *Turbinaria conoides* and evaluation of its anticancer potential on animal models

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Abstract

Background: In this study, Fucoidan a type of polysaccharide extracted from *Turbinaria conoides* was subjected to *invivo* acute toxicity, sub-acute toxicity study, and *invivo* anticancer study to find fucoidan effect on Sprague Dawley rat (SD rats). An acute toxicity test was executed on matured *Sprague Dawley* rats.

Result: They were administered with a single dose of 500mg/kg of fucoidan extract for 14 consecutive days and their general behavior, mental and physical changes along with mortality were observed and then the SD rats were subjected to the Sub-acute test and they were administrated with 50mg/kg, 100mg/kg, and 200mg/kg of fucoidan through oral gavage along with Tween 20 for 28 consecutive days and on the 29 day the animals were sacrificed and their vital organs were dissected and analyzed for noticeable signs of toxicity. Thus the study was extended to *invivo* anticancer analysis based on the results obtained from the acute and sub- acute toxicity studies to find the breast anticancer potential of the fucoidan. 250 mg/ml of test sample fucoidan was administered to 25mg/kg DMBA induced group animals (breast

carcinoma induced animals) and it was compared with 20mg/kg of standard drug Fluorouracil treated animals. From the results, it was found that test animals in Acute and Subacute analysis test groups injected with fucoidan extracted from *Turbinaria conoides* showed no significant changes in the test groups compared to the control groups on further analysis.

Conclusion SD rats injected with 25mg/kg of DMBA showed significant results on treating it with 250mg/kg of test compound fucoidan extracted from *Turbinaria conoides* compared with 20mg/kg of standard drug Fluorouracil thus it is concluded that our test compound contains the necessary pharmacological potential in it.

Keywords: Acute toxicity study, Breast cancer study, DMBA, Invivo study, *Turbinaria conoides*, Sub-acute toxicity study.

Background

Brown algae have a complex sulfated polysaccharide called fucoidan. Various types of brown seaweed have different fucoidan compositions and structural characteristics. Nevertheless, the main components of the substance are L-fucose and sulphate, with little amounts of D-galactose, D-mannose, D-xylose, and uronic acid [1]

Fucoidan has been thoroughly investigated ever since it was initially isolated from brown seaweed in 1913 [2]. Due to the popularity and increase in demand for pharmaceutical drugs derived from natural sources, it was not until the last decade or so have researchers focused more on the polysaccharides' broad range of physiological and biological activities [3,4,5] These include beneficial cytotoxicity [1], anti-inflammatory [6], antiviral [7] antioxidant [8] and anticoagulant activities [1]. Cancer is a serious disease with complex pathological pathways in the human body. The implementation of natural anticancer agents has been recognized as a possible alternative to conventional chemotherapeutic agents that are associated with minimal survival rates and unpleasant side effects [9]

Fucoidan was reported to suppress the growth of cancer cells *in vivo* and enhance the immune system to subdue the development of tumors [10]

Turbinaria conoides is a brown algae belonging to the Phaeophyceae family that grows in rocky substrates and is found predominantly in tropical marine areas. It's found in the Pacific Islands, Vietnam, China, the Indian and Pacific Oceans, Malaysia, Thailand, Japan, Indonesia, the Philippines, Singapore, New Zealand, and Australia, among other places. *Turbinaria* species are preferred by echinoids and herbivorous fishes in most tropical locations, resulting in a lower concentration of phenolics and tannins. In light of this, recent research developments have emphasized on the quest for antibacterial, anti-inflammatory, antioxidant, and anti-cancer properties in marine algae [11]

Thus our previous study confirmed the presence of fucoidan in our selected sample *Turbinaria conoides* and its *invitro* anticancer property. This study further aims to find the toxicity property through acute, sub-acute and anti-cancer study using the SD rats.

Methodology

Sample collection, Identification and extraction

Turbinaria conoides (Phaeophyta) seaweed samples was collected from Mandapam coastal area in Ramashwaram, TamilNadu, and India. Scientist Dr. M.U.sharief examined it and identified it as *Turbinaria conoides (*J. Agardh*)* kuietz-Sargassaceae. The collected sample was cleaned, air dried, powdered and the powdered sample was used for extraction of fucoidan using the standard procedure followed by [12]. The extracted fucoidan was used for all the procedures followed in the experiment.

Experimental Animals.

Animal study was done following the OECD Guidelines 423 using the Sprague Dawleys rats. SD rats were procured from Biogen, Bangalore and the work was carried out at C.L. Baid Metha College of Pharmacy, Department of Pharmacology, Thoraipakkam, Chennai- 600 097, Tamilnadu,

India. The experimental protocols using animals were authorized by Institutional Animal Ethics Committee (IAEC), CPCSEA, and New Delhi and received approval number IAEC no: 14/321/Po/Re/S/01/CPCSEA dated 11.10.2019. All of the animals were kept in a regular habitat (22°±3°C).The animals had unlimited access to food and water, as well as a conventional pellet diet (Sai Meera foods, Bangalore). Fucoidan was used in Ethnomedicine and recent researchers [13, 14] have found it non-toxic on administrating high dosages of 500mg/kg of fucoidan extract mixed with tween 20grouping of the animals were shown in the (Table. 1).

Table 1: Grouping of animals in an acute Toxicity study

S.NO	GROUP	SPECIFICTION	NO.OF RATS
1.	Group-I	Vehicle control –Sprague Dawley rats received normal water	6
2.	Group- II	High dose –received 500mg/kg of fucoidan extract from *Turbinaria conoides*	6

The table shows the grouping of animals for acute toxicity study with high dosage of fucoidan administration to SD rats

Acute Toxicity Study

An acute toxicity study was performed for 14 consecutive days on SD rats to find any toxic effect of fucoidan. Since, fucoidan was considered a traditional medicine, without any mortality even on administrating high dosage [13, 14]. So the dosage of 500mg/kg of fucoidan was administrated through oral gavage to 6 randomly selected matured rats, were grouped into 2 groups control and the high dose group and control group.

The animals administered 500mg/kg of fucoidan mixed with tween 20 were monitored individually for the first 30 minutes after medication. They were observed closely for the first four hours for any hazardous indications, then for the next 24 hours, and every day after that for a total of 14 days. Individually, the animals were observed and recorded for any toxic indications or pre-terminal deaths. Individual body weights for all of the animals were measured once a week to see any significant changes. Weekly, the colour and consistency of the animal's feces, mucous membranes (nasal), and changes in fur and skin, and eyes were studied.

***Invivo* Sub- acute Toxicity study**

For the *invivo* sub -acute toxicity study, 6 to 8 weeks old Males and female rats with a weight (of 150-200gm) were selected and they were segregated into (6 female animals/group) 4 groups as follows. Group-I Vehicle control group- *Sprague Dawley* rats received normal water. Group -II was Low- dose – *Sprague Dawley* rats received 50mg/kg of fucoidan extract from *Turbinaria conoides,* Group-III Mid -dose – *Sprague Dawley* rats received 100mg/kg of fucoidan extract from *Turbinaria conoides,* Group- IV High -dose – Sprague Dawley rats received 200mg/kg of fucoidan extract from *Turbinaria conoides* was administrated for 28 consecutive days. The animals were monitored throughout the study. On days 0, 7, 14, 21, and 28 of the trial, changes in body weight were noted along with health and clinical indicators of toxicity. All of the animals fasted the night before blood was drawn after the study period. Under ether anesthesia, blood samples from the abdominal aorta were drawn into two different types of tubes: one with EDTA and the other without additives. The anticoagulated blood was promptly examined for hematological conditions (tube with EDTA) and the other tube was centrifuged at 3000rpm for 10 min at 4°C to obtain the serum for biochemical analysis. Also, the liver and kidneys were dissected for histopathological studies.

***Invivo* Anti-cancer study**

After the toxicity assessment, *invivo* anticancer potential of fucoidan was determined in selected female Sprague Dawley rats. Six rats were caged in each group. Group-I was taken as the control vehicle group, Group-II was administered with 25 mg/kg DMBA mammary carcinoma induced animals, Group -III was induced with 250mg/ml of the test compound fucoidan was treated , and group-IV consists of 20mg/kg of standard drug fluorouracil treated group. Mammary Carcinoma was induced in overnight fasted animals by a single dose (25 mg/kg body weight) of DMBA (7, 12-Dimethylbenz (a) anthracene) by mixing it with 0.5 ml of olive oil and 0.5 ml of saline by gastric intubation. Rats also received a single dose of alpha-tocopherol (200 mg/rat) 1 hour after DMBA administration via an intragastric tube [15] After 12 weeks DMBA administration, 250 mg/kg of the test compound fucoidan was administered. Based on the results obtained from the sub-acute toxicity study, it is found that our test compound showed significant anticancer activity without any adverse effect, so the dosage was reduced to find its anticancer potential in the selected dosage. After following the standard experimental regimen, the animals were sacrificed by exposure to mild diethyl ether anesthesia treatment. Blood was collected in a centrifuge tube with EDTA by heart puncture method and serum was separated by centrifugation at 1000 rpm for 10 minutes and utilized for various biochemical assays. After that, mammary tissue, liver, and kidney were excised right away and washed thoroughly using ice-cold physiological saline and blotted. The organs such as mammary tissue, liver, and kidney were excised immediately and thoroughly washed with ice-cold physiological saline and blotted dry. A part of the tissues from mammary tissue, liver, and kidney was spliced and fixed in 10 % formalin for histopathological analysis.

Laboratory Investigation

The blood drawn from the experimental animals were exposed to hematological and biochemical analysis. The EDTA treated whole blood was examined for complete blood count analysis like haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), packed cell volume (PCV), and platelets and it was performed by using SYSMEX Kx – 21(Eraba, Transasia) automatic haematology analyser. Serum was analyzed with serum liver markers of Aspartate transaminase (AST), Alanine transaminase (ALT) and Lactate dehydrogenase (LDH) levels were analyzed along with that serum renal functions were analyzed by the level of Urea (mg/dl), creatinine (mg/dl), Uric acid level (mg/dl), total protein (g/dl), and albumin (g/dl) and SOD enzymatic assay, Catalase (CAT) enzymatic assay, Glutathione Peroxidase activity GPX, Lipid peroxidation assay (LPO) were analyzed.

Furthermore, mammary gland, liver, and kidney tissues from all treatment groups were examined histopathologically using the hematoxylin and eosin dye method. They were dissected, stored in 10% normal saline for 48 hours, and then gradually dehydrated by passing through various ethyl alcohol-water mixes (50, 80, 95%, and lastly incubated in alcohol). Finally embedded in paraffin and cleaned with xylene. For microscopic examination of cells for necrosis, fatty alterations, hyaline degenerations, and ballooning deterioration, samples were cut into ultrathin slices by an ultra-microtome, stained with hematoxylin and eosin dye, and mounted in a neutral deparaffinization xylene (DPX) solution. An Axiostar Plus microscope (Zeiss-Germany) was used to take micrographs with a Canon 10.1-megapixel digital camera.

Statistical analysis

The statistical analysis software Graphpad Prism version 5 was used for all calculations. Mean SD was used to express experimental results. To calculate statistical significance, one-way analysis of variance (ANOVA) and the post hoc Dennett's test were used.

Values with p 0.05 were regarded as statistically significant.

Results

Acute Toxicity study

In the acute toxicity study, the test groups given a high dose of 500 mg/kg of fucoidan extract from *Turbinaria conoides* showed no symptoms of toxicity, mortality, or behavioral abnormalities when compared to the control groups. The weight of the test group administering a high dose of 500 mg/ml of fucoidan was monitored on the 1st, 7th, and 14th days of the study and showed 250.3± 4.14, 255.4±2.12, and 255.2± 1.05. The results showed no significant difference between the test and control groups. Likewise, the water intake of the fucoidan-treated group was observed on the 1st, 7th, and 14th days, and it was found to be 69.5±3.04, 64.5±6.0, and 69.6±2.04. The results showed no significant difference between the test and the control group. Similarly, the food intake of the SD rats exposed to fucoidan extract and normal diet groups was statistically calculated, and the test group showed 74.0±2.24 on the I day, 76.4±2.10 on the 7 day, and 76.6±2.40 on the 14 day. Therefore, these findings revealed that a greater oral dosage of the fucoidan extracted from *Turbinaria conoides* confirmed that the tested SD rats did not experience any substantial transience, suggesting that the medicine still had a high margin of safety. (Table. 2, 3, 4).

This study estimated the median lethal dose (LD_{50}), or the dose that would kill roughly 50% of the skilled population. As a result, it was determined that fucoidan extracted from *Turbinaria conoides* was not toxic and that the LD_{50} value for oral toxicity was 500 mg/kg body weight.

Table.2 Change in the body weight (mg/kg) of control and test groups after the treatment of the fucoidan extracted from *Turbinaria conoides*.

Dose	**Days**		
	1	**7**	**14**
CONTROL	330.1±65.70	330.7± 09.71	340.6 ±2.10
HIGH DOSE 500mg/kg	250.3± 4.14	255.4 ±2.12	255.2 ± 1.05
P value (p)*	NS	NS	NS

N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 6 values are mean ± S.D

Table 3: Water intake (ml/day) of control and test groups after the treatment of the fucoidan extracted from *Turbinaria conoides*

Dose	**Days**		
	1	**7**	**14**
Control	60 ± 1.62	60±1.10	60.1±1.04
High Dose 500 mg/ml	69.5±3.04	64.5±6.07	69.6±2.04
P value (p)*	NS	NS	NS

N.S- Not Significant, (p > 0.01), *(p >0.05), n = 6 values are mean ± S.D)

Table 4: Food intake (mg/day) of control and test groups after the treatment of the fucoidan extracted from *Turbinaria conoides*

ɒse	Days		
	1	**7**	**14**
CONTROL	74.4±1.54	72.2±1.62	73.7±1.06
HIGH DOSE	74.0±2.24	76.4±2.10	76.6±2.40
P value (p)*	NS	NS	NS

N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 6 values are mean ± S.D

Sub-Acute Oral Toxicity Study

In the sub-acute oral toxicity studies, The SD rats received 3 different concentration (50mg/kg, 100mg/kg and 200mgkg) of fucoidan extracted from *Turbinaria conoides* for 28 consecutive days Animals administered with a low dose of 50mg/kg in the group II animals were found to be 331.05± 6.22 on day 28. Group-III animals administered with a mid-dose of 100mg/kg were observed to be 265.8±1.22 on the 28th day. Group-IV animals administered with a high dose of 200mg/kg was observed to be 287.1 ± 3.60 and it was compared with the control group. The bodyweight of the test groups with mid-dose 50mg/kg showed increased weight compared to the control group while a decrease in the body weight of the test group treated with 100mg/kg and high dose treated with 200mg/kg was observed compared to control group (Table 5).

Similarly the water and food intake of the control and test group was observed in the control group and in the test groups for 28 days. The intake of water in the control group –I was 84.5±2.46 on 28th day. Group-II animals with low dosage of 50mg/kg of fucoidan extract the water consumption was found to be 90.2±2.10 on 28th day. Group –III test animals administered with mid dose 100mg/kg of test fucoidan extract from compound found to consume 95.4±1.42 on 28th day and the group-IV test animals administered with high dose of 200mg/kg of test compound found to consume 86.4±1.38 on 28th day. (Table: 6) The results indicates that all the animals have drucked sufficient amount of water as per their need. (Table: 7) depicts the amount of feed in taken by the animals as per its need. Throughout the course of the sub-acute toxicity study, there were no anomalous deviations, deaths, or bodily alterations. Similarly, there was no noteworthy comparative alteration observed in laboratory research to comparison groups that amply demonstrate allowing Fucoidan extracted from *Turbinaria conoides* wasn't fatal to the bodily parts like the kidney, liver, and spleen.

Analysis of Hematological parameters

The test substance Fucoidan was administered to the control and test groups after sub-acute toxicity experiments were finished. Animals were tested for hematological effects at low dose concentrations of 50 mg/kg, mid-dose concentrations of 100 mg/kg, and high dose concentrations of 200 mg/kg. In both the control and test groups of animals, blood was drawn from the carotid artery and subjected to a hemoglobin test, which included measuring corpuscular volume, packed cell volume, mean cell hemoglobin concentration, and red blood cell count (RBC) in all four groups. Animals treated at low doses of 50 mg/kg, intermediate doses of 100 mg/kg, and high doses of 200 mg/kg showed no significant changes. The observation's specifics

are provided in (Table.8).

Analysis of Liver Function Parameter

The levels of glucose, triglycerides, cholesterol, alkaline phosphatase, alanine aminotransferase, total proteins, albumin, and creatinine in the liver following fucoidan induction were measured in all test group SD rats treated with low dose concentrations of 50 mg/kg, mid-dose concentrations of 100 mg/kg, and high dose concentrations of 100 mg/kg of the test compound fucoidan. The statistical information is shown in (Table: 9). According to the findings, there was a drop in glucose, triglycerides, cholesterol, ALT, and ALP levels in the test groups compared to the control groups as the test sample concentration was increased. The outcome suggests that our test chemical may have therapeutic potential.

Analysis of Biochemical Parameters

The control and the test group animals treated with low dose 50mg/kg, mid-dose100mg/kg, and high dose 200mg/kg of the test compound fucoidan extracted from *Turbinaria conoides* was subjected to biochemical analysis to find the blood urea nitrogen (BUN), Lactate dehydrogenase, Total proteins, albumin, and creatinine levels using the auto-analyzer. The statistical information from the results is shown in (Table .10) and demonstrates a drop in a number of biochemical indicators, demonstrating that our extract had no negative effect on the SD rats.

Table: 5 Body weight of Sprague Dawley rats group exposed to the fucoidan extracted from *Turbinaria conoides*.

Dose	Day 1	Day 7	Day 14	Day 21	Day 28
Control	302.1±1.14	302.2 ± 1.20	302.3± 0.22	302.4 ± 2.40	302.5 ± 2.22
Low Dose (50mg/kg)	330.1 ± 10.3	330.4 ± 1.14	330.4± 1.4	331 ± 03.11	331.05± 6.22
Mid Dose (100mg/kg)	264.3± 4.20	264.5 ± 2.27	265.7 ± 2.29	265.8 ± 8.30	265.8 ± 1.22
High Dose (200mg/kg)	287± 12.21	287 ± 2.14	287±2.16	287 ± 1.28	287.1 ± 3.60
P value (p)*	NS	NS	NS	NS	NS

Table: 6 Intake of water in Sprague Dawleys rats group exposed to the fucoidan extracted from *Turbinaria conoides*.

Dose	Day 1	Day 7	Day 14	Day 21	Day 28
Control	84.2 ± 1.14	84±1.26	84.6±1.30	84.6±4.06	84.5±2.46
Low Dose 50mg/kg	90.2±1.08	90.2±4.40	90.4±3.24	90.6±1.20	90.2±2.10

Mid Dose 100mg/kg	95.3±1.10	95.3±1.14	95.2±1.12	95.4±1.33	95.4±1.42
High Dose 200mg/kg	86.3±1.10	86.4±1.12	86.3±1.21	86.1±1.33	86.4±1.38
P value (P0)*	NS	NS	NS	NS	NS

Table: 7 Food consumed by Sprague Dawleys rats group exposed to the fucoidan extracted from *Turbinaria conoides*.

Dose	**Day 1**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Control	378.6± 1.20	378±1.32	378.8±2.30	378.8±2.33	378.2±2.11
Low Dos (50mg/kg)	356.1±1.42	356.2±1.30	356.4±1.40	357.2±3.41	357.3±1.22
Mid Dose (100mg/kg)	401.4±1.24	401.3±1.13	401.4±2.42	402.3±1.21	402.7±2.42
High Dose (200mg/kg)	406.1±1.20	406.2±2.30	406.4±3.32	406.2±3.52	407.4±6.24
P value (p)*	NS	NS	NS	NS	NS

Table: 8 Hematological parameters of Sprague dawley rats group exposed to *Fucoidan extracted from Turbinaria conoides*

Category	**Control**	**Low dose**	**Mid dose**	**High dose**	**P-value (p)***
Haemoglobin(g/dl)	13.4±0.06	13.4±0.23	13.6±0.16	13.6±0.60	N.S
Total WBC ($\times 10^3$ µL)	11.5±0.04	11.5±0.03	11.6±0.04	11.60±1.46	N.S
Neutrophils (%)	25.2±0.02	25.3±0.08	25.5±1.04	25.6±2.12	N.S
Lymphocyte (%)	70.1±1.21	70.2±1.12	70.2±1.66	70.7±1.76	N.S
Monocyte (%)	0.01±0.02	0.01±0.04	0.01±0.06	0.01±0.07	N.S
Eosinophils (%)	.04±0.23	.04±0.25	.04±0.41	.04±0.42	N.S
Platelets cells10^3/µl	233.13±2.16	233.14±4.30	233.12±1.30	234.4±3.14	N.S
Total RBC 10^6/ µl	6.64±0.01	6.64±0.70	6.64±0.07	6.64±0.04	N.S
PCV%	44.1±0.2	44.10±1.0	44.6±1.12	44.1±2.04	N.S
MCHC g/dL	35.8±1.10	35.8±0.32	35.8±1.50	36.3±1.30	N.S
MCV fL(µm^3)	56.1±1.01	56.1±3.11	56.9±1.12	56.9±1.14	N.S

Group	Treatment	Glucose (mg/dL)	iglycerides (mg/dL)	holesterol (mg/dL)	PT/ALT (U/L)	ALP (U/L)
I	Control	189.02±01.15	48.21±2.32	74.98±1.04	34.12±1.24	162.02±02.18
II	Low dose (50mg/kg)	162.20±01.22	44.22±1.10	66.08±0.28	28.12±1.42	140.44±01.18
III	Mid dose (100mg/kg)	151.02±02.12	42.10± 1.08	64.04±0.13	26.11±1.62	136.12±0.28
IV	High dose (200mg/kg)	143.10±1.22	40.12±06.22	62.11±0.84	24.12±1.72	130.54±06.24

Table: 9 Liver Function analysis of Sprague Dawley rats exposed to fucoidan extracted from *Turbinaria conoides*

Subacute Histopathology Assessment

The animals were sacrificed once hematological and biochemical investigations were finished. Their internal organs, such as the kidneys, liver, and spleen, were taken out, and the tissues were kept for histological study by being fixed in the 10% formaldehyde solution. Selected organs from the control and test groups underwent examination. The papillary ducts and glomerular capsule were both clearly evident in the kidney tissue, which was found to be normal. Liver cells were discovered to be normal, showing erythrocytes, hepatocytes, and the major vein. The spleen was found to have a crimson pulp sinusoid and more lymphocytes and neutrophils than usual. According to the histopathology results, all test groups given the modest dose of 50mg/kg, 100mg/kg and High dose 200mg/kg of the test compound fucoidan extracted from *Turbinaria conoides* discovered their kidney, liver, and spleen tissues to be in normal condition.(Fig. 1-3)

***Invivo* Anticancer activity**

Based on the results obtained in the Acute and sub-acute toxicity analysis our study was extended to *invivo* anticancer analysis to find the breast anticancer potential of the fucoidan extracted from *Turbinaria conoides* using the Sprague Dawley rats. In this study 250 mg/ml concentration of test sample fucoidan was fixed based on the results obtained from the acute and sub- acute toxicity studies. A detailed description on the concentration of all the other groups are given in the (Table 10).

Determination of Body Weight

Each group of animals were administration of the selected dose with selected dose and they were observed for the body weight. The weight of the test animals was recorded after the intubation of test compound fucoidan extracted from *Turbinaria conoides*. The final body weight of the Group-I control animal was found to weigh 331.6±2.88gms, Group-II animals induced with 25 mg/kg DMBA was found to be decreased to 321.6±2.88 whereas Group-III animals treated with 250 mg/kg of the test compound was found to be 338.3±2.88, and Group-IV

animals were treated with the 20mg/kg standard positive control drug fluorouracil showed 341.6±2.88.This shows that our test compound has the ability to revert the damaged cells and bring it back to the normal state.(Table.11)

Table 10 Table Represents the *Invivo* anticancer drug treatment dosage to the Sprague Dawley rats

***Invivo* anticancer Experiment Design**	
Group I	Control – Sprague Dawley rats received normal water and pelleted diet.
Group II	Mammary Carcinoma was treated in overnight fasted animals by a single dose of DMBA (7, 12-Dimethylbenz (a) anthracene) in olive oil 25 mg/kg body weight) by gastric intubation (Veena et al., 2006).
Group III	Mammary Carcinoma rats were treated with 250 mg/kg of fucoidan extracted from *Turbinaria conoides* for 25 continuous days by gastric intubation after the development of mammary carcinoma.
Group IV	Mammary Carcinoma induced rats treated with 20mg/kg fluorouracil standard drug for 25 continuous days by gastric intubation.

Table 11: Body weight of the Experimental animals treated with test compound Fucoidan extracted from *Turbinaria conoides*

Groups	**Treatment**	**Final Body weight (g)**
Group- I	Control	331.6±2.88
Group-II	DMBA 25mg/kg	321.6±2.88
Group-III	Test compound Fucoidan from *Turbinaria conoides* (250mg/kg)	338.3±2.88*
Group IV	Fluorouracil (20mg/kg)	341.6±2.88

Values are expressed as mean ± SD of six animals in each group* $p < 0.05$ significantly different when compared group II vs group III, IV. NS = Not significant.

Haematological assays

Haematological assays like Haemoglobulin (HB), Packed cell volume (PCV), Red Blood cell count (RBC), White Blood cell count (WBC) and Platelet count was performed in all four groups and the results were obtained for total Haemoglobulin (g/dl) level in the control group-I, DMBA 25mg/kg treated in group-II, group-III treated with the test compound Fucoidan from *Turbinaria conoides* (250mg/kg) and group-IV treated with 20mg/kg of fluorouracil are depicted clearly in the (Fig. 4)Thus, the results show that group-III treated with 250mg/kg test compound fucoidan was significantly different from group-II treated with the 25mg/kg of DMBA and standard drug fluorouracil treated group-IV.

Biochemical Assays

Serum Liver Marker Assay

The liver function was analyzed using the Biochemical parameter of serum analysis of Aspartate transaminase (AST), Alanine transaminase (ALT) and Lactate dehydrogenase (LDH) activity were observed in all 4 groups. Aspartate transaminase results were found to be 34.2±0.60 in control, 44.2±1.47 in group II, 37.1±1.36 in group III, and 32.03±0.55 in group IV. Alanine transaminase (ALT) showed 32.26±3.01 in control, 42.5±1.47 in group II, 35.5±1.35 in group III, and 31.6±0.85 in group IV and the results of Lactate dehydrogenase (LDH) was found to be 266.2±12.86 in control group I, 409.1±5.30 in group II, 326.3±17.9 in group III, and 302.5±11.84 in group IV. Thus from the results, it was found that our test compound fucoidan 250mg/kg showed a significant difference when compared with group III treated with the standard Fluorouracil 20 mg/kg drug. (Fig.5)

Serum Renal Function Markers

The renal functions were studied in the control and test group's animals by analyzing the level of Urea (mg/dl), creatinine (mg/dl), Uric acid level (mg/dl), total protein (g/dl), and albumin (g/dl). The level of Urea (mg/dl) in the control group was found to be 13.36±0.15, DMBA(25mg/kg) induced group II animals produced 26.96±1.70, group III treated with test compound fucoidan extract (250mg/kg) from *Turbinaria conoides* produced 15.86±2.63, and group IV animals treated with standard drug Fluorouracil (20mg/kg) produced 13.93±0.63. The amount of Creatinine (mg/dl) level in group I was found to be 0.53±0.05, 0.53±0.05 in group II, 0.56±0.05 in group III, and 0.53±0.05 in group IV. The level of Uric acid (mg/dl) was found to be 1.46±0.15 in the control group, 2.66±0.15 in group II, 1.76±0.15 in group III, and 1.43±0.05 in group IV was observed. Total protein (g/dl) level in control group I was found to be 6.06±0.23, 3.76±0.15 in group II, 4.50±0.30 in group III, 5.30±0.17 in group IV. While the level of albumin (g/dl) in group I was found to be 3.60±0.34, 1.60±0.20 in group II, 2.06±0.23 in group III, and 2.53±0.25 in group IV(Fig.6)

The results showed that the level of Urea, uric acid, and creatinine in group III treated with test compound fucoidan (250mg/ml) was reduced compared with group II animals induced with mammary carcinoma DMBA (25mg/kg). While raise in Total protein and albumin level was observed in the group III treated with test compound fucoidan extract from *Turbinaria conoides*.

Serum enzymatic Antioxidant assay

SOD enzymatic assay

The control and experimental animals in groups I, II, III, and IV were analyzed for biochemical analysis using serum enzymatic antioxidant assays. Superoxide Dismutase (SOD) is a key enzyme that plays an important role in oxidative stress and scavenging free radicals. The sodium dismutase (SOD) scavenging activity in group I treated with control group was found to be 7.43±0.20, 3.60±0.10 in group II induced with 25mg/kg of DMBA, 5.5 ±0.30 in group III treated with 250 mg/kg fucoidan extract from *Turbinaria conoides* and 6.36±0.15 in group IV treated with 20 mg/kg of standard drug Fluorouracil. The results showed that our test compound fucoidan 250mg/ml treated group III showed a significant rise in sodium dismutase activity compared to the group-II induced with DMBA. *Catalase* is an antioxidant enzyme that plays an important role in protecting the cell by splitting the paired hydrogen peroxide molecules into an unpaired oxygen particle. The catalase enzyme activity in the control group I was found to be 37.03±1.33, 26.93±1.30 in group II mammary carcinoma induced by treating 25mg/kg of DMBA, 33.30±1.15 in group III treated with the 250mg/kg of test compound fucoidan, and 36.60±1.11 in group IV treated with the 20mg/kg of standard drug Fluorouracil. Our results showed a significant rise in catalase activity in group III treated with test compound 250mg/kg of fucoidan compared to group II treated with

DMBA. Glutathione Peroxidase Activity Oxidation of glutathione was found to be 8.50±0.30 in the control group I, 3.43±0.15 in group II mammary carcinoma induced by treating 25mg/kg of DMBA, 5.43±0.15 in group III treated with the 250mg/kg of test compound fucoidan, and 6.90±0.26 in group IV treated with the 20mg/kg of standard drug Fluorouracil. The results showed a significant increase in the group III animals treated with 250mg/kg of test compound fucoidan compared to group II induced by the mammary carcinoma DMBA. In lipid peroxidation assay was used to measure oxidative stress. The measured oxidative stress in the control group was found to be 28.96±3.30, 59.43±2.72 was found in group II mammary carcinoma induced by treating 25mg/kg of DMBA, 43.33±6.78 in group III treated with the 250mg/kg of test compound fucoidan, 33.46±1.36 in group IV treated with the 20mg/kg of standard drug Fluorouracil. The oxidizing activity of LPO on the test sample 250mg/kg fucoidan treated sample was found to drop down compared to the group II DMBA induced groups. The results obtained for the serum enzymatic antioxidant assay is represented in the (Fig. 7).

***Invivo* Anticancer Histopathology Study**

Liver Histopathology report

Animals were slaughtered once the *invivo* anticancer investigation was complete, and their organs, including the liver, kidney, and mammary gland from all groups, were examined. Sectioning of the control group's liver tissue revealed normal vasculature and hepatocytes in group I. Group II, which had been given DMBA (25 mg/kg), a breast cancer inducer, displayed aberrant lobules with a localized area of fatty alteration, coupled with inflammation, necrosis, and congestion, as well as partial loss of liver parenchymal cells. Fucoidan (250 mg/kg) treatment in Group III resulted in a central vein with modest inflammation and decreased fatty alteration. Fluorouracil (20 mg/kg) was used as the standard of care in group IV, and typical sinusoids were seen. According to the findings, group II was given 25 mg/kg of DMBA promotes necrosis, while our test compound fucoidan and the standard drug did not produce necrosis. (Fig.8)

Kidney histopathology report

In the histological study of kidney tissue, group-I control cells showed a normal cortex and medulla region. Group II treated with 25 mg/kg of DMBA-induced glomeruli showed glomeruli are surrounded by intact tubules; interstitial inflammation and necrosis were observed. Group III, treated with the test compound fucoidan (250 mg/kg), observed normal conditions without any inflammation or fibrosis. Group IV, treated with the standard control of fluorouracil (20 mg/kg), observed normal without any necrosis. The results are illustrated in (Fig. 9).

Histopathology report of mammary tissues

Histology study of mammary tissues of Group-I control showed breast tissues composed of terminal duct lobule units and fatty tissues. Group II, treated with 25 mg/kg of DMBA, induced high mitotic division, necrosis, and reactive lymphatic follicular hyperplasia. Group III, treated with fucoidan test extract (250 mg/kg), produced no necrosis and showed lobules of breast tissue lined by ductal myoepithelial cells. Group IV, treated with the standard drug fluorouracil (20 mg/kg), showed breast tissues composed of terminal duct lobules with normal cells. (Fig 10)

Discussion

Natural sources, such as medicinal plants and seaweeds, have been used to treat numerous diseases for hundreds of years. [15]. Assessment and evaluation of the hazardous properties of a natural substance extract or compound are usually the first step in screening natural substances for pharmacological properties. As a result, the current investigation was carried out in Sprague Dawley rat to assess and focus on the acute and sub- acute toxicity of the fucoidan extracted from *Turbinaria conoides.*

The acute toxicity study is utilized to check the harmful effects of a compound to the organism given as a single or short-term exposure [16]. Our study primarily analyzed the mice's mortality, behavioral changes, body weight, and other natural changes in their general well-being after the administration of fucoidan extract 500mg/kg single dosage was given and the test animals were checked with control group animals administered with vehicle group and the results showed intoxic effect in the test group.

In sub-acute toxicity, animals treated with 50mg/kg, 100mg/kg, and 200mg/kg resulted in no statistically significant difference in body weight, relative weight of vital organs, food and water intake compared with the control group was observed. Serum glucose and cholesterol showed a statistically significant reduction at all the doses compared to control, and the reduction was dose-dependent manner with reduced changes in biochemical or haematological parameters. Histopathological investigation revealed no significant toxic indications at the organ level in the fucoidan treated groups. Based on the results Fucoidan is considered safe from the findings of acute and sub-acute toxicity studies.

[17] Discovered similar action in fucoidan from *Sargassum wightii* Greville, which was confirmed by the FTIR investigation. In an acute toxicity assay, they administered 2000mg/kg of fucoidan to the animals and found no detrimental effects or mortality. Similarly, fucoidan extract at doses ranging from 100 mg/kg to 400 mg/kg was given orally for a 28-day sub-acute toxicity trial. Compared to the control group, there was no significant change in body weight, the relative weight of essential organs, food, or water consumption. Compared to the control vehicle group, all biochemical tests indicated a substantial statistical reduction in a dose-dependent manner.

[18] Have investigated the antioxidant, anti-inflammatory, and chemoprotective activity of the fucoidan extract from Laminaria japonicum in Wistar rats. Toxicity study of the fucoidan from *Laminaria japonicum* against diazinon (DZD) was analyzed through the acute and sub-acute studies, and significant results were obtained in the 100-200mg/kg of fucoidan extract compared to the DZD induced rats.

[5] Investigated the anticancer efficacy of sulfated polysaccharide fucoidans derived from *Laminaria japonica* through acute and sub-acute toxicity studies tin Wistar rats. According to the findings, 300mg/kg of fucoidan-treated rats showed no significant effects without any adverse effects in both acute and sub-acute studies.

[19] Have also studied the anticancer property of fucoidan through invitro and invivo studies. They found the high anticancer activity of fucoidan in the in-vitro condition compared to the invivo oral administration. They also concluded that administrating a higher concentration of fucoidan and chemotherapeutic drugs through oral or intravenously can result in better anticancer activity in *invivo* conditions. Tokita *et al.* 2010 have also found significant results against MDA-MB-231 and MCF-7 breast cancer while administrating fucoidan extract in combination with (Cisplastin) CDDP and (Tamoxifen) TAXOL. A significant result was observed in morphological changes of the nucleus, and the number of positive cells increased in a time-dependent manner.

[20] Examined the anticancer properties of fucoidan in a variety of cancer cell lines, concluding that the anticancer activity of fucoidan was due to the activation of cell cycle arrest, apoptosis, and immune activation mechanisms such as activation of inflammation, stem cell mobilization, and oxidative stress mechanisms.

[21] Have found the skin cancer-inducing property of DMBA and the anticancer property

of stigmasterol present in *Azadirachta indica* found to have an anticancer property in it.

Similar results were obtained by [22] in treating 5% fucoidan extracted from brown seaweed *Laminaria* was treated in Sprague Dawley rats. In an invivo anticancer study similar to ours on administering the 7,12-dimethylbenz(a) anthracene intragastrically (DMBA) breast cancer inducing carcinogen, they reported that lesion formation was only observed in the DMBA induced Sprague Dawley rats. At the same time, the other groups showed delayed tumor formation.

In our current study Sprague Dawley rats were induced with 25mg/kg of DMBA was treated with 250mg/kg of fucoidan. The obtained results showed significant anticancer potential of the fucoidan compared to the standard fluorouracil treated with 20mg/kg.

[23] Have reported the potential of fucoidan extracted from brown algae to reduce the tumor formation induced in Sprague- Dawley rats by 7, 12-dimethyl benz[a]anthracene (DMBA) experimental mammary carcinogenesis in rats. Xue *et al.* 2017 have found that fucoidan.

Reduced the incidence of tumors in test animals induced with DMBA and increased the level of CD4, CD8 T cells, and natural killer cells. Thus the immunomodulatory protective effect of fucoidan induced with mammary carcinoma was observed in fucoidan- treated animals.

[24] Also reported similar activity *Porphyra dentate* red macroalgae ethanol extract showing the anticancer activity against breast cancer.

Our hematological tests showed reduced blood glucose level on fucoidan treated test group, which is interrelated with previous study report observed with the hypoglycemic potential of fucoidan extracted from *Sargassum wightii* [25]

Hematology testing is an important parameter and a very sensitive parameter used to detect adverse toxic effects. It acts as the index value that gives the overall physiological and pathological condition. However, our test sample fucoidan does not show any abnormality. Histopathology testing revealed normal organ architecture; from the result of our study and in correlation with previous reports, it was found that fucoidan is safe and non-toxic to normal cells while it can act on breast cancer cells.

CONCLUSION

The fucoidan extract of *Turbinaria conoides* at a concentration of 500 mg/kg body weight revealed no harmful effects during acute toxicity testing, thus demonstrating that the fucoidan extract is safe and does not have any toxic effects. Given that there was no mortality recorded during a 14-day period, fucoidan extract likely provides some therapeutic benefits. Similar to acute toxicity, repeated doses of mid- and high doses administered over the course of 28 days demonstrated to be both safe and non-toxic to the test animals. The sub-acute toxicity research on fucoidan, which was isolated from *Turbinaria conoides*, is based on biochemical, hematological, and histopathological data. The findings make it abundantly evident that fucoidan extract can be used as an efficient anticancer treatment for breast cancer.

List of abbreviations

SD - Sprague Dawley rat

ANOVA- Analysis of variance

EDTA - Ethylenediamine tetraacetic acid
DMBA - (7, 12-Dimethylbenz (a) anthracene)
ALT - Alanine aminotransferase
AST - Aspartate aminotransferase
HB - Haemoglobulin
PCV -Packed cell volume
RBC - Red Blood cell count
WBC -White Blood cell count

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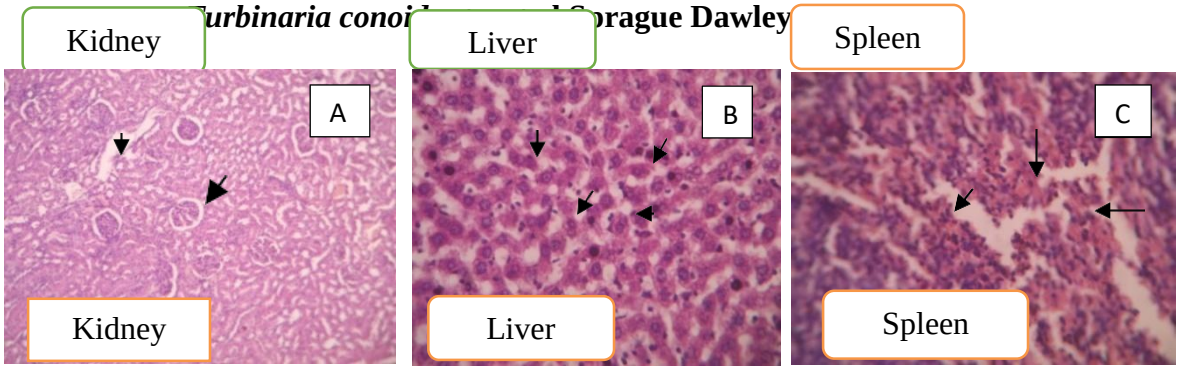
Figure 1: Histopathology images of high dose 200mg/kg fucoidan extract from *Turbinaria conoides* treated Sprague Dawley rats group

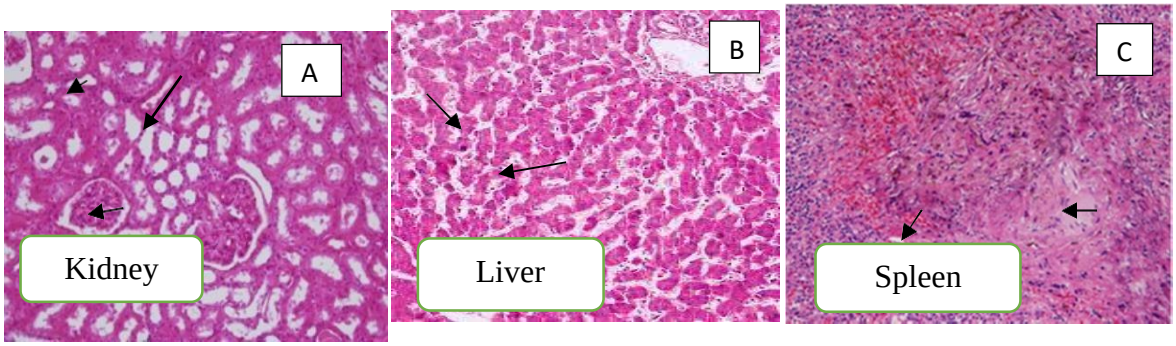
(A) Kidney (B) Liver (C) Spleen
(A)Kidney - papillary ducts , glomerular capsule, (B) Liver- hepatic cells, central veins
(C) Spleen- venus sinus, Neutrophils, lymphocytes

Figure 2: Histopathology images of mid dose 100mg/kg fucoidan extract from *Turbinaria conoi* Sprague Dawley



(A)Kidney - Papillary ducts , glomerular capsule, (B) Liver- hepatic cells, central veins (C) Spleen- venus sinus, Neutrophils, lymphocytes

Figure 3: Histopathology images of Low dose 50mg/kg fucoidan extract from *Turbinaria conoides* treated Sprague Dawley rats group



(A) Kidney (B) Liver (C) Spleen
(A) Kidney - papillary ducts, glomerular capsule, (B) Liver- hepatic cells, central veins
(C) Spleen- venus sinus, Neutrophils, lymphocytes

Fig.4 *Invivo* Anticancer activity- Hematological Assay

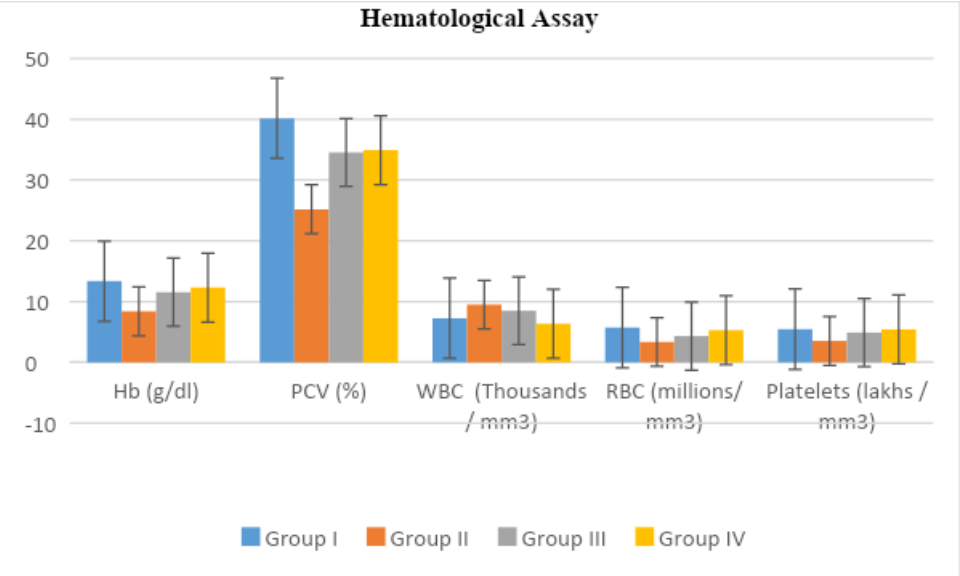


Fig.5 *Invivo* Anticancer activity- Serum Liver Function Markers

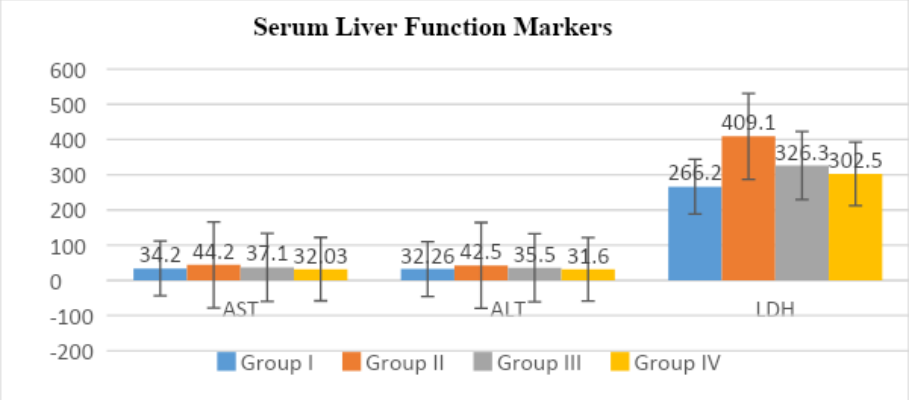


Fig.6 Graphical Representation Serum Renal Function Markers

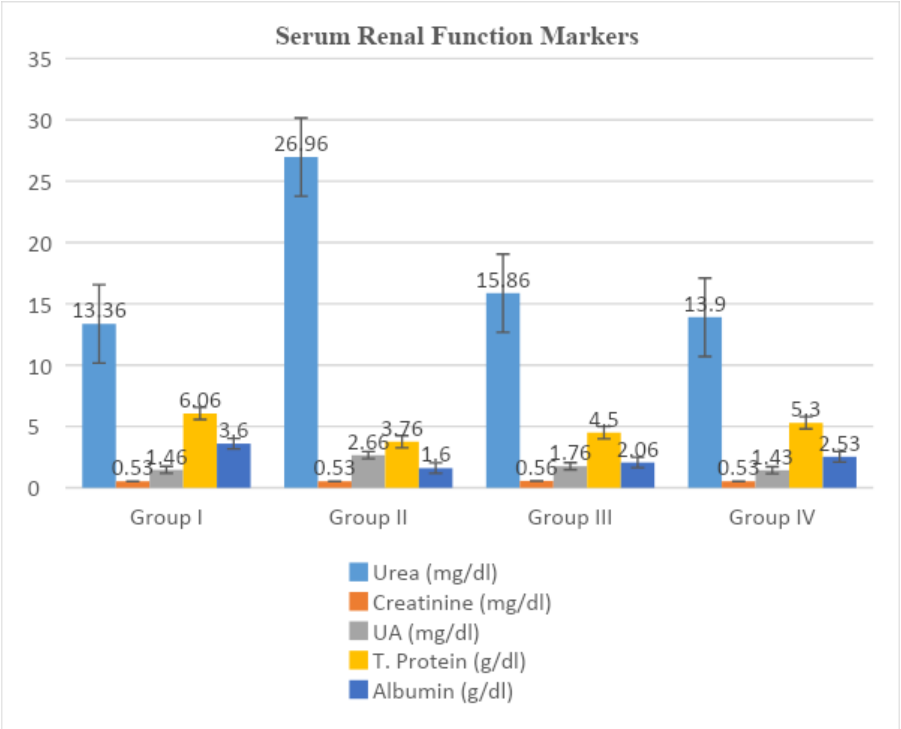


Fig.7 Graphical Representation of Serum Enzymatic Antioxidant Enzymes Assay.

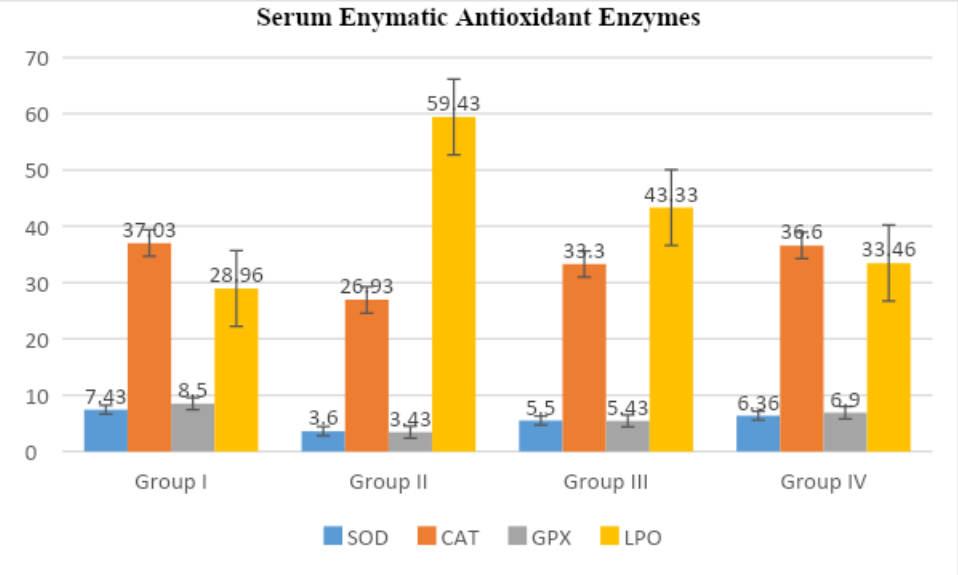
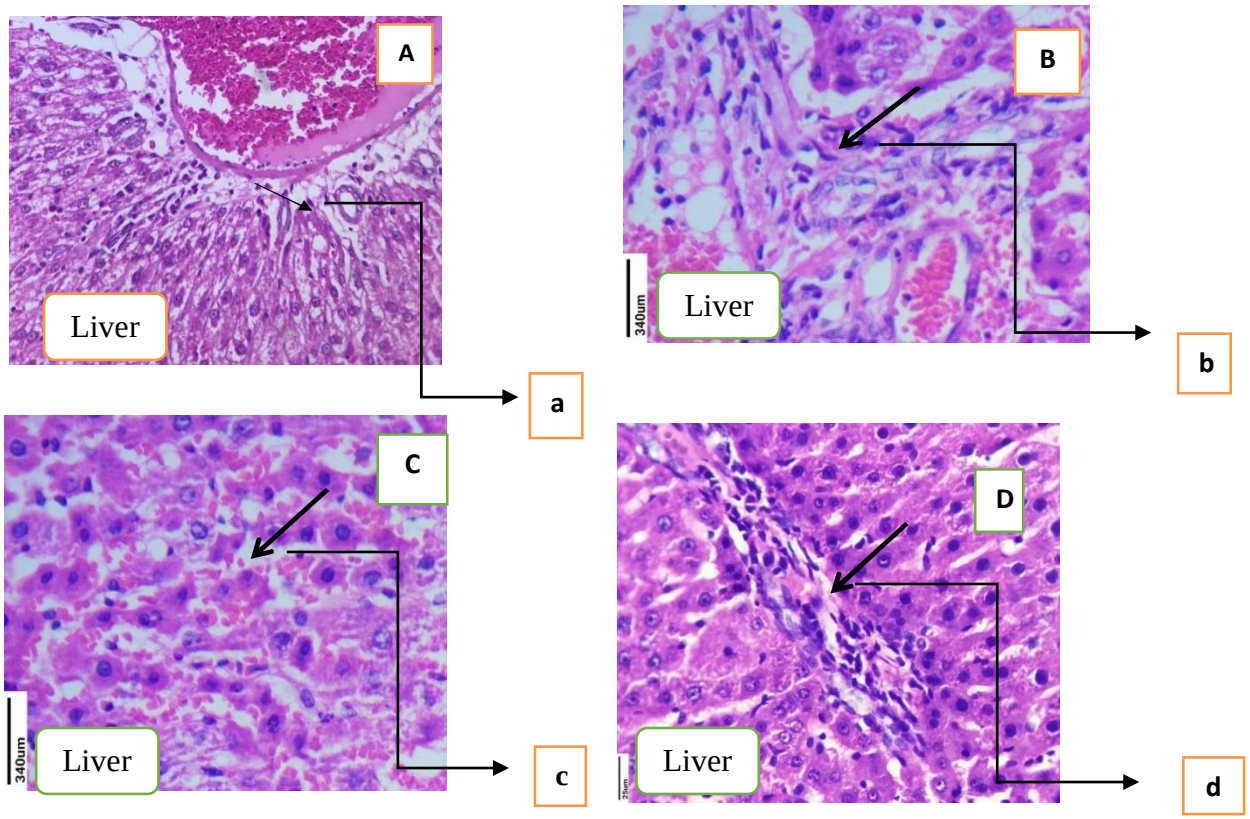


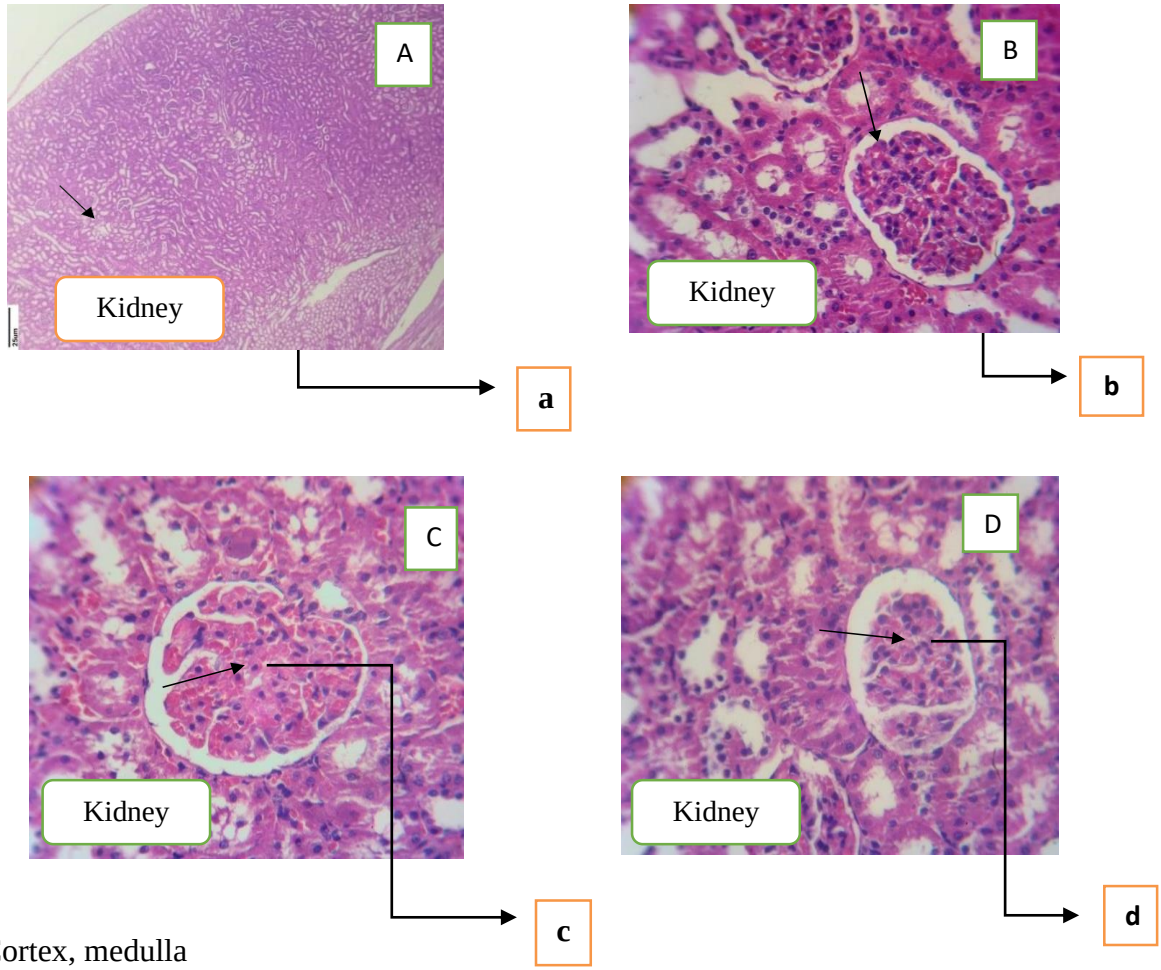
Figure 8: Histopathology report of Liver



a. Normal hepatocytes with normal central vein
b. Abnormal lobules, inflammation, necrosis, congestion and loss of some liver parenchymal cells
c. Normal central vein with mild inflammation
d. Normal central vein and normal sinusoids

(A) Control (B) 25 mg/kg DMBA induced (c) fucoidan extract from *Turbinaria conoides* 250 mg/kg Treated (d) 20 mg/kg Fluorouracil Standard control

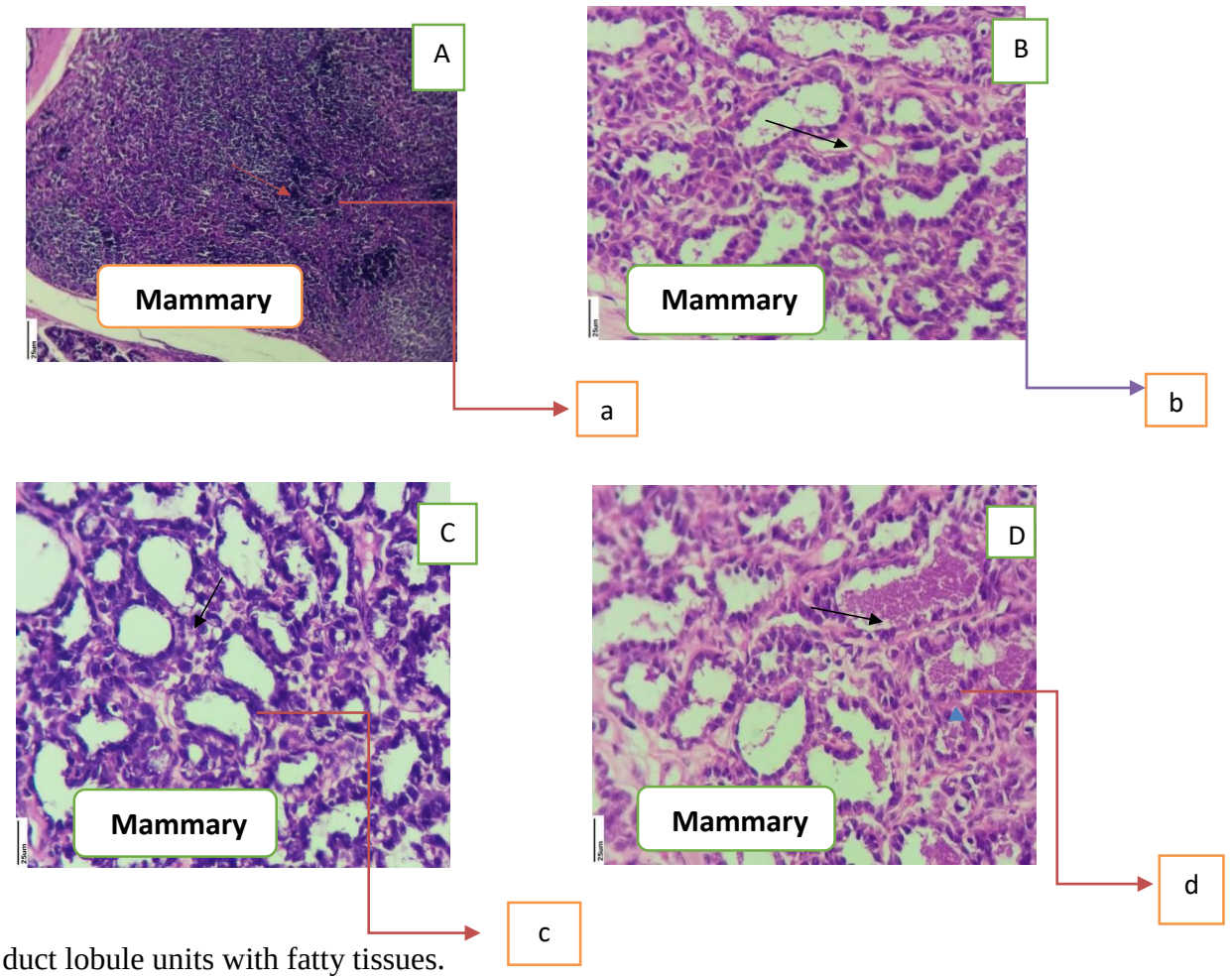
Figure: 9 Histopathology study of Kidney



a. Normal Cortex, medulla
b. Intact tubules surrounded –glomeruli, interstitial inflammation with necorsis
c. Normal cells and mild interstitial inflammation.
d. Normal cells and mild inflammation

(A) Control (B) 25 mg/kg DMBA induced (c) fucoidan extract from *Turbinaria conoides* 250 mg/kg Treated (d) 20 mg/kgFluorouracil Standard control

Figure: 10 Histopathology study of mammary gland tissue



a. Normal duct lobule units with fatty tissues.
b. High mitotic activity with the necrosis and reactive lymphatic follicular hyperplasia.
c. Hyperchromasia
d. Terminal duct lobule with some neoplastic cells.

(A) Control (b) 25 mg/kg DMBA induced (c) 250 mg/kg fucoidan extracted from *Turbinaria conoides* (d) 20 mg/kg Fluorouracil Standard control.