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HEPATIC AND HEMATOLOGICAL ACTIVITIES OF HYDRO-ALCOHOLIC EXTRACT OF *ERIOBOTRYA JAPONICA* FRUITS IN SWISS ALBINO MICE

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ABSTRACT

The hydro-alcoholic extract of fruits of *Eriobotrya japonica* was evaluated for its toxicity on hepatic and hematological parameters in swiss albino mice. Animals of either sex were treated with different doses of the extract for its acute toxicity tests. The doses taken were 500,1000,1500,2000 mg/kg. In this LD_{50} was calculated. After finding the LD_{50}, optimum dose for sub-acute toxicity studies were taken. The effect of the plant extract on hepatic and hematological parameters were taken into consideration. The doses taken were 250 and 500 mg/kg b.w. Histopathology of liver and spleen were also done. The results shows that in acute toxicity studies, it showed that LD_{50} was 500mg/kg b.w and in sub-acute studies it showed variable biochemical parameters with no toxicity on liver and spleen.

KEY WORDS: **Hydro-alcoholic extract, acute and sub-acute toxicity, *Eriobotrya japonica.***

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INTRODUCTION

The early civilization shows that herbal plants have been used in the treatment of various ailments all over the world including India. These plants have given rise to synthetic drugs as well as traditional herbal medicine. India has a rich wealth of medicinal plants, especially the Himalayan Belt and particularly Jammu and Kashmir has been quite useful. Thousands of herbal plants have been scientifically proved to be beneficial but toxicity evaluation of these plants have been quite useful. These plants have shown different pharmacological activities, various phytoconstituents like alkaloids, glycosides, tannins and many others have shown these activities. The various parts of these plants i.e the aerial parts like stem, leaves, fruits, flowers and the underground parts tubers, roots have proved to be useful [1-6]. Most of the pharma companies are carrying out extensive research on herbal medicines because they are having lesser side effects and wider acceptability. About 80% of the world population dependent on these plants. The toxicity evaluation is necessary in order to render the plant safe. Different toxicity tests-acute, subacute and chronic toxicity tests are usually carried out. Acute toxicity gives information about LD50 whereas subacute and chronic type gives data about the toxicity at low doses carried for a longer period of time [7-11]. *Eriobotrya japonica* commonly known as Loquat(Family Rosaceae) is an evergreen tree having many medicinal uses. The leaves are of great importance and have been used to treat nausea, vomiting, belching, hiccups and gastro-intestinal disorders. The flowering period of this plant is from April to June. Although it is native to China and Japan, it grows in many parts of the world including India. One variety found in Kashmir also. The reported bioactive compounds include flavonoids, triterpenic acids, carotenoids, volatile compounds which attribute to aroma, oleanolic acid and ursolic acid. The reported pharmacological activities include anti-oxidant,anti-mutagenic, anti-viral, hypolipidemic, anti-inflammatory and other activities [13-18].The present study focused on the fruits of *Eriobotrya japonica* on which very little work has been done.

Plant Material

The fruits were collected from the Shalimar area of the Kashmir valley in Srinagar district, in the months of April to June. The fruits were authenticated by Dr Naqshi, who worked as Plant Taxonomist in the Centre of Plant Taxonomy, Kashmir University. Later the sample of the fruits were kept in herbarium of Department of Taxonomy under voucher No 1012(KASH) Dated 15-09-2008 for future reference of the plant.The fruits were dried in a room having temperature 18-32^{o} C. After this, they were pulverized into a coarse powder by using a grinder.

Preparation of the extract.

The powdered material having weight of 500 gms was macerated with 50 % of ethanol for 48 hrs. After this, it was filtered using Whatmans filter paper. Again 50% ethanol was used for the second maceration and again filtered. Both the filtrates were combined and the solvent used. Ethanol was recovered. The extract was evaporated to dryness. The process was allowed to repeat a number of times. The yield of the extract was noted. The final extract was kept in a fridge at 4^{o} C for future experimental studies.

Drugs and Chemicals

Wagners reagent, Zinc, Magnesium, Methanol, Tri-sodium citrate, Acacia gum, Acetic anhydride, Citric acid, Concentrated hydrochloric acid, Concentrated sulphuric acid Diethyl ether Ferric chloride, Ehanol, Olive oil, Chloroform, , Formaldehyde, Glacial acetic acid, Albumin Kit, Alkaline Phosphatase kit, Bilirubin Kit, SGPT Kit, SGOT Kit, Total Protein Kit. All these chemicals and enzymatic kits were obtained from Merck Laboratories, Accurex Biomedical Pvt Ltd, Sisco Research Laboratories, Central Drug House Ltd New Delhi Qualigen fine chemicals, Crest Biosystems Goa India, Bengal chemicals & Pharmaceutical Ltd,

Mumbai Ashirwad Industries from Punjab provided the mice feed in the form of pellets.

Phytochemical Screening

The hyhro-alcoholic extract of the fruits of *Eriobotrya japonica* obtained was subjected to qualitative tests.These tests were performed for identification of different phytoconstituents like saponins, glycosides, , flavonoids, carbohydrates, tannins, alkaloids, proteins and steroids, terpenes, phenolics according to the standard qualitative methods [19-21].

Pharmacological Study [12]

Animals and Exposure conditions

The animals used were swiss albino mice weighing about 20-25 gms.First of all, acute oral toxicity study was conducted, which give us the LD_{50} of the hydro-alcoholic extract. LD_{50} was found to be 500 mg/kg b.w. These animals were procured from IIIM (Indian Institute of Integrative Medicine) Jammu. They were housed in clean cages made of polypropylene. The temperature was maintained from 18 to 32^{o} C, relative humidity (70%) and 12 hrs dark/light cycle, these were considered as standard laboratory parameters. The animals were given a rodent pellet diet procured from Ashirwad Industries. Water was given *ad-libitum,* and strict hygienic conditions were maintained. The procedures related to the experiment were performed in accordance to CPCSEA guidelines after approval from the Institutional Animal and Ethics Committee (IAEC) of the Department of Pharmaceutical Sciences, University of Kashmir[No. F-IAEC (Pharm.Sc) APPROVAL/2008/4 Dated Oct 23rd,2008].

Hepatic and hematological activity

The swiss albino mice were given the hydroalcoholic extract of *Eriobotrya japonica* fruits (EBJF) once daily. The mice were acclimatized for a period of seven days before the initiation of experiment. The animals of either sex (20-25 g body weight) were divided into three groups of six mice each. The treatment of the hydro-alcoholic extract was given according to the following protocol.

Group I Normal Control (2% aqueous gum acacia)
Group II EBJF (250 mg/kg b.w)
Group III EBJF (500 mg/kg b.w)

The whole experiment was continued for 14 days. The mice of Normal Control group received only 2% gum acacia. At the end of 14 days,mice were fasted overnight and blood from these animals was collected by cardiac puncture. The whole blood samples were kept for haemoglobin and white blood corpuscles estimation. After this, the blood was kept to clot for one to two hours and serum was separated by centrifuging. The serum obtained was evaluated for different biochemical parameters The results of these estimations were subjected to ANOVA followed by students t test, $p > 0.05$ was taken as non significant, $p < 0.05$ – significant, $p < 0.01$- highly significant and $p<0.001$ as very highly significant. After this, the animals were sacrificed. The various organs-liver and spleen were taken out, from these mice and preserved in 10% formalin. Histopathological studies were conducted on these organs. Biochemical estimations evaluated were

a) Liver Function Tests

i. Serum Bilirubin Levels
ii. Serum Glutamate Oxaloacetate Transaminase (SGOT)
iii. Serum Glutamate Pyruvate Transaminase (SGPT)
iv. Serum Total Proteins
v. Serum Albumin
vi. Serum Alkaline Phosphatase

b) Blood Function Tests

i. Hemoglobin Value
ii. WBC Count

ESTIMATION OF BILIRUBIN [22,23]

Bilirubin reacts with diazotized sulphanilic acid in acidic medium to form pink colored azobilirubin with absorbance directly proportional to bilirubin concentration. Direct Bilirubin, being water soluble directly reacts in acidic medium. However indirect or unconjugated Billirubin is solubilised using a surfactant and then it reacts similar to direct Bilirubin. Reagent composition has two reagents. Reagent 1 is Total Bilirubin Reagent which contains Surfactant, HCl, Sulphanilic

Acid.Reagent 2 is Direct Bilirubin Reagent which contains Sulphanilic Acid and HCl and Reagent 3 contains Sodium Nitrite Reagent.
Assay parameters include Sample Volume 25/50(µl)-, Reagent Volume500/1000(µl)-, Incubation time - 5 mins, Incubation temperature-37^{o}C. Assay procedure includes Working Reagent-500 µl, Distilled Water-25 µl for blank, Working Reagent-500 µl, Standard / calibrator-25 µl for standard, and Working Reagent-500 µl, Test solution-25 µl for test. Mixed well, incubated for 5 minutes at 37^{o}C for Total Bilirubin and direct Bilirubin. Read Absorbance at 546/630 nm against reagent blank.

Calculation with calibrator / standard

$$\text{Total Bilirubin (mg/dl)} = \frac{\text{Abs. of Test}}{\text{Abs. of Standard}} \times \text{Concentration of Standard (mg/dl)}$$

ESTIMATION OF SGPT [22,24]

L-Alanine and 2-Oxoglutarate in presence of ALT (Alanine aminotransferase) gives Pyruvate + L- Glutamate.Pyruvate and NADH in presence of LDH (Lactate Dehydrogenase) gives L-Lactate + NAD. SGPT reagent contains L-Alanine, NADH (Yeast), LDH, 2 – Oxoglutarate, Tris Buffer (ph 7.5±0.1 at 25^{o}C).Assay parameters include Wavelength- 340 nm Sample Volume50/100(µl) Reagent Volume500/1000(µl) Reaction Temperature37^{0}C. Assay procedure includes Working Reagent and Test. Determined the mean absorbance change/min (A/min) for every reading and found the mean value. The general formula for converting absorbance change into international Units (IU) of activity is

ESTIMATION OF SGOT [22,24]

SGOT converts L-Aspartate and Ketogutarate to Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with 2,4 Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a pyruvate standard. The activity of SGOT is read off this calibration curve. Reagent contains L1 contains Substrate Reagent , L2 contains DNPH Reagent L3 contains NaOH Reagent (4N) S: Pyruvate Standard (2mM) Procedure includes Wavelength - 505 nm ,Temperature - 37^{0} C & R.T. and Light Path- 1 cm Calibration curve was first plotted using Enzyme Activity (U/ml) Substrate Reagent (L1) Pyruvate Standard (S) Distilled Water DNPH Reagent (L2) Mixed well and allowed to stand at R.T. for 20 minutes. Working Na0H Reagent (L3). Mixed well and allowed to stand at R.T. for 10min. Measured the absorbances of the tubes 2 – 5 against tube 1 (Blank). Plotted a graph of the absorbances of tubes 2 – 5 on the 'Y' axis versus the corresponding Enzyme activity on the 'X' axis. Assay procedure included clean dry test tubes labeled as Blank (B) & Test (T): Substrate Reagent (L1) Incubate at 37^{0}C for 3 minutes Sample Mixed well and incubated at 37^{0}C for 60 minutes DNPH Reagent (L2) Mixed well and allowed to stand at R.T. for 20 minutes Distilled Water Working NaOH Reagent (L3) Mixed well and allowed to stand at R.T for 10 min. Measured the absorbance of the test (T) against Blank (Blank) and read the activity of the test from the calibration curve plotted earlier. One sample blank is sufficient for each assay series. If Enzyme activity exceeds 190 U/ml dilute the sample with distilled water and repeat the assay. Multiply the value with the proper dilution factor. System parameters included Wavelenth-505 nm,Sample Vol 0.10 ml, Reagent Vol 6.00 ml.

ESTIMATION OF TOTAL PROTEINS[22,25]

Proteins, in an alkaline medium, binds with the cupric ions present in the biuret reagent to form a blue-violet coloured complex. The intensity of the colour formed is directly propotional to the amount of proteins present in the sample.

Reagents used are Biuret Reagent and Protein Standard (8 g/dl) Procedure includes Wavelength / Filter : 550 nm , Temperature : R. T. / 37^{o}C Light path : 1 cm Pipette into clean dry test tubes labeled as Blank (B), Standard (S), and Test (T): Biuret reagent (L1),Distilled Water,Protein Standard (S) and Sample Mixed well and incubated at 37^{o}C for 10 min. or at R.T. for 30 min. Measured the absorbance of the Standard (Abs. S), and Test Sample (Abs. T) against the Blank within 60 Min.

$$\text{Total Proteins in g / dl} = \frac{\text{Abs.T}}{\text{Abs. S}} \times 8$$

ESTIMATION OF ALBUMIN [22,26]

Albumin binds with the dye Bromocresol green in a buffered medium to form a green coloured complex. The intensity of the colour formed is directly propotional to the amount of albumin present in the sample. Reagents include BCG Reagent and Albumin Standard (4 g/dl) Procedure includes Wavelength - 630 nm , Temperature - R.T ,Light path - 1 cm. Pipetted into clean dry test tubes labeled as Blank (B), Standard (S), and Test (T): BCG reagent (L1), Distilled water, Albumin Standard (S) and Sample. Mixed well and incubated at R.T. for 5 min. Measured absorbance of the Standard (Abs. S), and a Test Sample (Abs. T) against the Blank.

$$\text{Albumin in g/dl} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 4$$

ESTIMATION OF ALKALINE PHOSPHATASE (ALP) [22,27]

ALP at an alkaline pH hydrolyses p-Nitrophenylphosphate to form p-Nitrophenol and phosphate. The rate of formation of p-Nitrophenol is measured as an increase in absorbance which is proportional to the ALP activity in the sample. Reagents contain Buffer Reagent and Substrate Reagent Procedure includes Wavelength - 405 nm, Temperature- 37^{0}C , Light path- 1 cm Pipetted into a clean dry test labeled as Test (T) : Working Reagent Incubate at the assay temperature for 1 minute and add Sample Mixed well and read the initial absorbance A_0 after 1 minute & repeat the absorbance reading after every 1, 2 & 3 minutes. Calculated the mean absorbance change per minute (A / min.) ALP Activity in U/L = A / min. x 2754

ESTIMATION OF HEMOGLOBIN [28]

Filled the graduated measuring tube up to the bottom graduation line (mark 2) with N/10 Hydrochloric Acid. Took a drop of blood. Sucked 20µl blood into the capillary pipette precisely up to the mark, wiped the pipette point and blew the blood into the measuring tube. A good mixture of the liquid was obtained by repeated suction and blowing. The mixture was dark brown after about one minute. Added water by means of the water pipette and mixed with the glass stirrer until the colour of the solution matches the colour of the test rods. Read the result by diffused day-light exactly three minutes after adding the blood to the Hydrochloric Acid. The Haemometer is equipped with two non-fading glass colour standards.

ESTIMATION OF WBC COUNT [28]

The blood is diluted with a suitable diluting fluid which destroys the red blood corpuscles and stains the nuclei of the white blood cells. The leucocytes are then counted in a haemocytometer and their number in undiluted blood calculated Apparatus and Reagents used are Haemocytometer (counting chamber), WBC pipette and Turk's fluid Placed a counting chamber with its “centred” cover slip near the microscope. Drew blood upto the mark 0.5, followed by Turk’s fluid to the mark 11. Mixed the contents of the bulb for 2 minutes.

Discarded the first 2-3 drops of the diluted blood and charged the counting chamber, taking the necessary precautions. Placed the 'charged' haemocytometer on the stage of the microscope and allowed the cells to settle for two minutes. Focussed the lines and the cells to get a general impression of the distribution of cells. Switched to high power and counted the cells in the four groups of 16 squares each. Dilution obtained: The volume of the bulb is 10 (11-1=10). From the tip of the pipette to the mark 1 .0, the stem contains only Turk's fluid which does not take part in the dilution of blood. Thus 10 volumes of the diluted blood (in the bulb) contains 0.5 volume of blood and 9.5 volumes of Turk's fluid, giving a dilution of half in ten or one in twenty i.e., 1: 20. The blood is thus diluted 20 times. The volume of one small square is 1/160 c mm (side= 1/4 mm, area =1/4 x 1/4= 1/16 sq mm, depth =l/10 mm, volume= l/4 x ¼ x 1/10= 160 cubic millimeters, Number of cells counted in 64 squares= 192, Number of cells in one square= 192/64=3, Volume of one square=1/160c mm (mm3),Thus, there are 3 cells in 1/160 c mm of diluted blood, The number of ce1ls in 1c mm blood was 160 x 3 = 480 As the dilution employed was 1: 20, the number of cells in 1 cubic mm of undiluted Blood is = 480 x 20=9600/ c mm of blood, Result expressed as: TLC=9600/ cubic mm (mm^3).

HISTOPATHOLOGICAL STUDIES [29,30]

After the preservation of liver and spleen in 10% formalin, the liver and spleen sections were processed. The various steps included the following.

1. Preparation of the Tissues.
2. Processing of Tissues
3. Embedding in paraffin
4. Preparation of sections
5. Staining.

1. In fixation, there is killing and hardening of tissue. The tissues are placed in a fixative. Blocks are made which are not more than 0.5 cms thick. 10% formalin is widely used fixative. After fixation of the tissue, it is washed from 3 to 24 hrs in running water before it is subjected to dehydration, clearing and embedding.
2. Processing of tissues. Every specimen is marked and given an identifying number or name. The tissue is embedded in paraffin or colloidon, nitrocellulose and carbowax. Dehydration is done by 80%,90% or 100% alcohol or acetone.
3. Embedding in paraffin is increased by the use of shallow tin pans. The pans are gently warmed with a Bunsen burner and filled with paraffin which is melted and filtered. When paraffin hardens, it contracts from the sides of the pan, the mass is lifted out and then cut into blocks having appropriate size.
4. Preparation of the sections. Various knives are used for microtomes of different types, 110 mm knife is usually used for cutting frozen sections. 120 mm and 185 mm knives are used for routine paraffin blocks.
5. Staining. Sections are picked up on albuminized slides and dried before staining. Paraffin section(5 microns thick) are stained with hematoxylin and eosin.

RESULTS

% age yield of the hydro-alcoholic extract = 30 %

Eriobotrya japonica fruits showed the presence of alkaloids, glycosides, flavonoids and carbohydrates

Acute studies

LD_{50} of the hydro-alcoholic extract was 500mg/kg b.w

Sub-acute studies

Table 1

Effect of hydro-alcoholic extract of Eriobotrya japonica fruits on liver enzymes and hematological parameters in swiss albino mice

Treatment	Dose Mg/kg b.w	Bilirubin Levels (mg/dl)	SGOT Levels IU/L	SGPT Levels IU/L	Total Protein Levels g/dl	Albumin Levels g/dl	Alkaline Phosphatase Levels U/L	Hemoglobin Levels g/dl	WBC Count Levels Thousand/cubic mm
Normal Control	0.2 ml of 2% gum acacia	0.58	60.03	46.24	6.81	3.45	72.16	7.40	1900
EBJF	250mg/kg b.w	0.58	60.91	45.38	6.85	3.61	82.86	8.03	1700
EBJF	500 mg/kg. b.w	0.36	52.67	26.54	6.97	3.74	60.47	8.16	1566.66

Figure 1

Effect of hydro-alcoholic extract of Eriobotrya japonica fruits on liver enzymes and hematological parameters in swiss albino mice

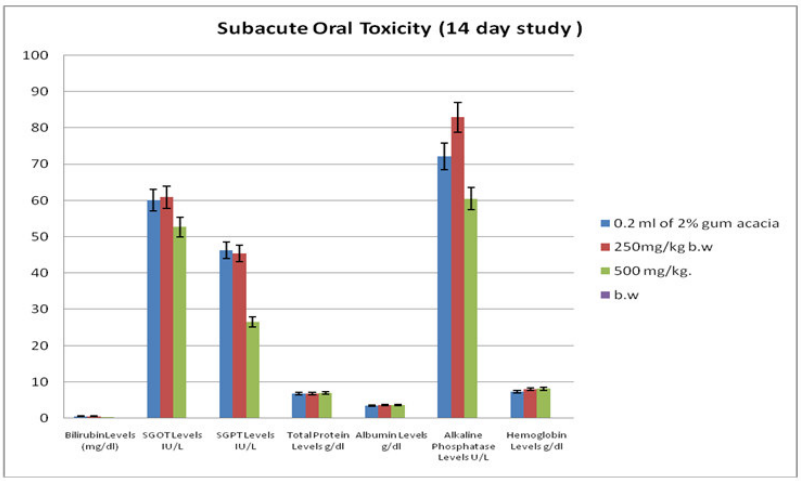
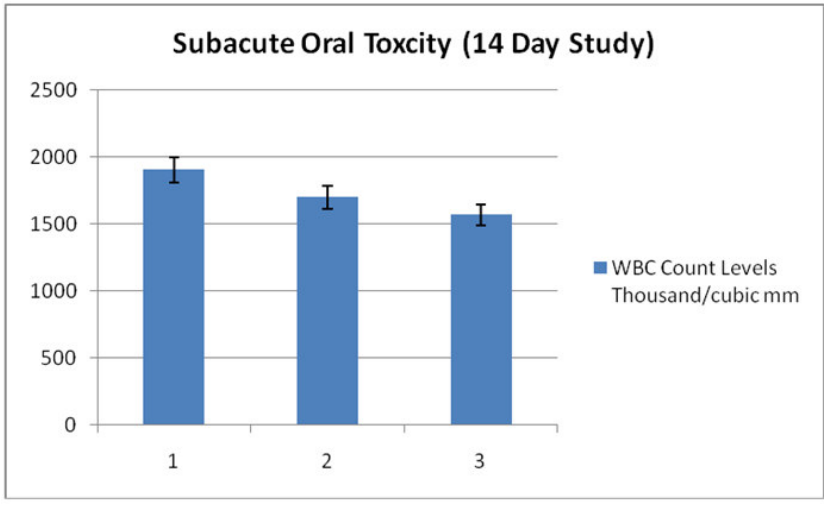


Figure 2

Effect of hydro-alcoholic extract of Eriobotrya japonica fruits on WBC Count in swiss albino mice



Results of biochemical estimations showed non significant changes in bilirubin,total protein levels, and albumin levels, whereas there was significant reduction occurred in SGOT,SGPT, alkaline phosphatase and WBC count.

Eriobotrya Japonica

fruit when given at two dose levels (250 & 500mg/kg) to mice of Group II & Group III respectively revealed the portal area with no abnormality in liver. Spleen of mice of Group I (Normal control) showed the normal spleen with red and white pulp areas in the splenic parenchyma. Extract of *Eriobotrya Japonica* fruit when given at two dose levels (250 & 500mg/kg b.w) to mice of Group II & Group III respectively also revealed the normal spleen.

DISCUSSION

The major problem to the use of traditional herbal plants is lack of clinical and scientific data in support of safety of the drug, because of improper information regarding the toxic effects of the herbal drug. These plants have some toxic substances in them and it is better to evaluate them according to some standard procedures and find their effects on different body organs like liver, kidneys, spleen and heart, in order to establish the safety of the plant. Liver is the main organ for detoxification. In the present study,liver function tests of the swiss albino mice were treated with subacute doses of the hydro-alcoholic extract of the fruits of *Eriobotrya japonica.* The results showed that the fruits contain alkaloids, glycosides, flavonoids and carbohydrates. While screening plants, laboratory rats and mice are mostly used because they are easily available and economical. First of all, acute toxicity studies were carried out i.e., LD_{50}.. The optimum dose of fruits of *Eriobotrya japonica* was 500 mg/kg b.w. Mice weighing between 20-25gms were used. They were procured from IIIM Jammu and kept in polypropylene cages under standard conditions of food, water, temperature . Maximum care was taken to ensure that the animals are in good health and free from any infectious disease. Male and female animals were separated from each other so that there was no interference in the evaluation of the biochemical studies. The temperature was kept at 15-25^{o}C and humidity of the room was at 70-75% The oral administration of the hydro-alcoholic extract showed the LD_{50} was 500mg/kg b.w. The present studies were carried out at the dose of 250 and 500 mg/kg b.w. The liver is the main organ of metabolism. Any hepatic injury due to metabolism of the toxic plant constituents and the failure of the liver to eliminate them shows that there is some abnormality in the working of liver.Albumin is the abundant plasma protein which maintains the osmotic pressure and carries transportation of endogenous and exogenous substances and the total proteins serve as reserves of proteins.Liver fails to synthesize albumin if its function is altered.Bilirubin shows the excretory function of the liver. SGPT and SGOT give information about the inflammation and necrosis of the liver.The concentration of alkaline phosphatase is also increased in liver diseases. Any deviation of the hematological parameters i.e hemoglobin value and WBC count also shows the efficacy and safety of the plant extract. Histopathological studies did not show any toxic effect of the hydro-alcoholic extract of the fruits of *Eriobotrya japonica* on liver and spleen of albino mice.[Fig 1-6] The importance of this plant in folk medicine as well as promoting pharmacological properties, make studies about its toxicity very important.

HISTOPATHOLOGY OF LIVER IN MICE (SUBACUTE TOXICITY STUDY 14- DAYS)

Figure 3
Group –I –Normal Control Liver of mice showing normal portal triad area. No abnormality seen. (H & E x 40X)

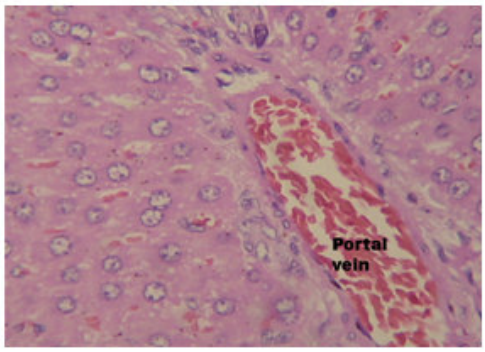


Figure 4
Group II –Eriobotrya japonica fruit (250mg/kg b.w) Liver of mice showing the portal area. No. abnormality seen. (H&E x 40X) HA = Hepatic Artery, BD= Bile Duct.

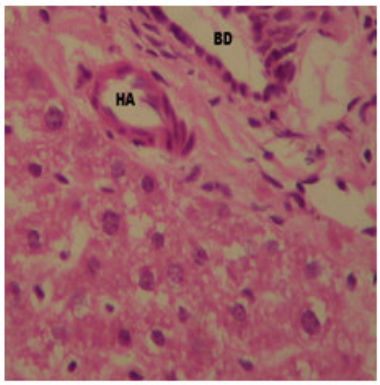
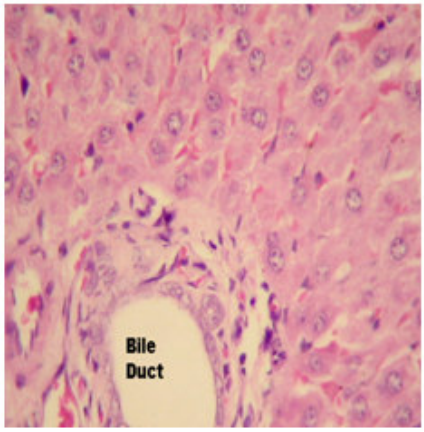


Figure 5
Group III : Eriobotrya japonica fruit (500 mg/ kg b.w) Liver of mice showing a large bile duct from portal area. No. abnormality seen (H&E x 40X) (H&E x 40X)



HISTOPATHOLOGY OF SPLEEN IN MICE (SUBACUTE TOXICITY STUDY 14- DAYS)

Figure 6

Group I –Normal Control Spleen of mice showing normal red and white pulp areas in the splenic parenchyma. WP= White Pulp, RP= Red Pulp. (H&E x 40X)

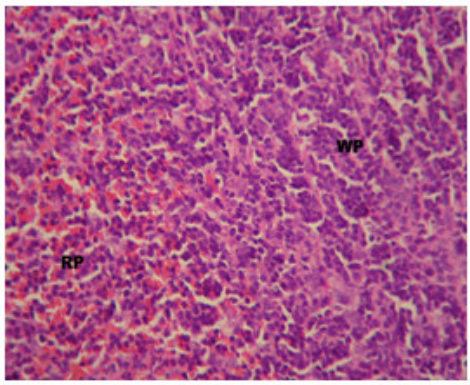


Figure 7

Group II Eriobotrya japonica fruit (250 mg/kg b.w) Spleen of mice showing the normal red and white pulp areas in the splenic parenchyma. (H&E x 40X) WP=White Pulp, RP = Red Pulp

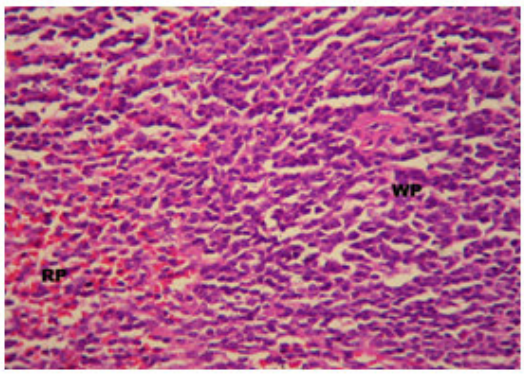
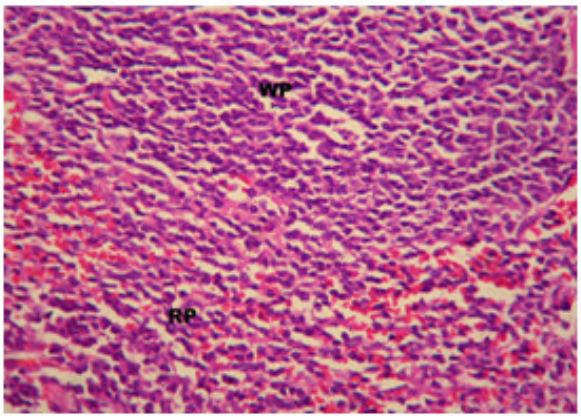


Figure 8

Group III Eriobotrya japonica fruit (500mg/kg b.w) Spleen of mice showing the normal red and white pulp areas in the splenic parenchyma (H&E x 40X) WP=White Pulp RP=Red Pulp



CONCLUSION

From the study, it can be concluded that the hydro-alcoholic extract of fruits of *Eriobotrya japonica* have beneficial effects on liver function tests. Further pharmacological and biochemical investigations will clearly elucidate the mechanism of action and will be helpful in projecting this plant as an therapeutic target in medical research.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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