Original Research Article **Pharmacology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

EVALUATION OF ACUTE AND SUB-ACUTE TOXICITY OF ETHANOLIC DRY FRUIT EXTRACT OF *CRATAEGUS OXYCANTHA L.* IN MALE WISTAR RATS

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ABSTRACT

Crataegus oxycantha popularly known as Hawthorn is widely distributed in northern hemisphere especially in Asia, Europe and parts of America. Howthorn consist of more than 200 species and several parts of the plant is traditionally used in the china and Europe. In India it is widely distributed in the temperate Himalayan regions and the different parts of plant is used for multiple health effects like hypolipidaemic, hypotensive and anti-atherosclerotic purposes due to its active phytochemical properties. This species C. *Oxycantha* has not been adequately studied and the histopathological changes for its toxicity and safe use have not been established. Thus The aim of the present study was to evaluate acute and sub-acute toxicity of the ethanolic dry Fruit powder extract of *Crataegus Oxycantha* (coc) on male wistar rats at different dose levels of 100, 200, 500, 1000, and 2000mg/kg b.w.) At a rate of 1.0ml /rat/day to different sets of animals for 30 days. Results from the present study have elucidated that treatment of *C.Oxycantha* exerts no significant signs of toxicity at any dose level when compared with the control group used in the study. Behavioural, Biochemical as well as haematological parameters were unaltered throughout the study. Histopathological sections of different organs have no signs of tissue damage or necrosis. The results of the study have suggested there was no obvious toxicity observed with the treatment of *C.Oxycantha* fruit powder extract and this preclinical toxicological evaluation strongly supports our further studies on various cardiovascular abnormalities.

KEYWORDS: ***Crataegus oxycantha*, acute and sub-acute toxicity, haematological parameters, histopathology**

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INTRODUCTION

Plant kingdom is consider now a day's one of the most important sources of the active substances with therapeutic potential to cure a variety of diseases in humans (Gill et al., 2010; 2011). Toxicological evaluation of these crude extracts from the plants may needs to be completely screened for any potential agents of toxicity. The Plant *Crataegus oxyacantha,* commonly known as Hawthorn (Family Rosaceae) is a thorny tree that thrives in hedgerows and fields in the temperate regions of Europe and the British Isles and Asia. Its name originates from the Greek word kratos meaning strength which means the nature of the wood. Other names include white thorn and hog berry. It blooms in May producing luscious red fruits and hence receives one of its most popular names, May-blossom.[1] Hawthorn tea is most widely known for its medicinal uses and primarily for its cardiovascular applications. Hawthorn is rich in triterpenic acids like oleanolic acid and ursolic acid ; polyphenols like, catechin, epicatechin, procyanidin B2, procyanidin B5, procyanidin C1, hyperoside, isoquercitrin and chlorogenic acid.[2] The herb hawthorn have flavonoid pigments and procyanidin pigments in its flowers, berries and leaves, which is said to lower blood pressure and cholesterol.[3] The berries, leaves, and flowers of the hawthorn plant have been used for medicinal purposes.[6] Current claims suggested that hawthorn could be used as an alternative therapy for various cardiovascular diseases, such as angina, hypertension, hyperlipidaemia, arrhythmia, and New York Heart Association (NYHA) functional class II congestive heart failure.[7,8] Nowadays, it is gaining importance for its potential cardiovascular enhancing and protective properties.[9] and numerous laboratory tests and clinical trials have demonstrated hawthorn's efficacy in the treatment or prevention of cardiovascular diseases and the most substantial evidence for clinical benefits of hawthorn is its use in chronic congestive heart failure (CHF).[10] A meta-analysis of randomized, placebo-controlled trials of hawthorn extract in combination with standard CHF therapy suggested several beneficial cardiovascular effects of hawthorn as compared to placebo.[11] Similarly, a 2008 Cochrane review, wherein all primary literature pertaining to the health effects of hawthorn on humans was assessed, found a significant benefit in symptom control and physiologic outcomes from hawthorn extract as an adjunctive treatment for chronic heart failure.[12] Besides, the antioxidant, positive inotropic, anti-inflammatory, and anti cardiac remodelling effects and other cardiovascular protective effect of the hawthorn active ingredients were demonstrated in various *invivo* and *invitro* experiments. *Crataegus* has a number of pharmacological properties, but the specific mechanism is not clear. Despite the extensive use of the plant, *Crataegus oxycantha L.*, acute and sub-acute toxicity has not been studied extensively particularly the tissue morphological changes up on administration of different doses. Motivated by this, we investigated at present to report the acute and sub-acute toxicity of the *Crataegus oxycantha L.* ethanolic fruit extract on biochemical indices of liver and kidney functions as well as some haematological, Histopathological parameters in albino wistar male rats, which will be a guidance for our future studies of its safe administration on different systems particularly the molecular level mechanism of action in the cardiovascular system and its complications.Laboratory animals are sensitive to toxic substances present in the plants. Hence, the administration of the extracts in increasing amounts enables the evaluation of the acute and sub-acute toxicity limits. Therefore, we investigated with five different dose of *Crataegus oxycantha*, taking into account other factors such as age, weight, species, diet and environmental conditions and we followed the OECD guidelines for acute and sub-acute toxicity studies.[13]

Figure 1
Crataegus oxycantha plant

Figure 2
Crataegus oxycantha berries

MATERIALS & METHODS

Collection of the plant material

Crataegus oxycantha berries was collected from Chamba district, Himachal Pradesh, India during the month of March to May, 2014. The plant was identified and authenticated by Taxonomist Dr.K.G Bhat, Retired Professor, Department of Botany, Poorna Prajna college,Udupi, Karnataka,India. Ethical Clearance has been obtained for this study from the Institutional Animal Ethics committee, SRM Medical college Hospital and research centre (IAEC Approval No.084/835/IAEC-2014), SRM University, India.

Preparation of Ethanolic plant extract for in vivo studies

This study was performed in the department of Pharmacology, SRM Medical college Hospital and Research centre, Tamilnadu. The 1 Kg dried fruit powder of *Crataegus oxycantha* was successively extracted using soxhlet apparatus in 5:1 ratio of ethanol and dried fruit powder. The resultant crude extract was evaporated to dryness using a rotary evaporator at 70°C. The yield obtained was 80 gm., stored at ˍ4°C in the fridge and used in the entire study period the residue was suspended in distilled water and administered orally by using oral feeding gavage to experimental rats.

Selection of animals

For the purpose of acute and sub-acute toxicity studies, adult male wistar albino rats weighing out 150 to 200 g were purchased from National Institute of Nutrition (NIN), Hyderabad, Telangana, India.

Maintenance of experimental rats

The rats were kept in properly numbered polypropylene cages (421x290x190mm) with stainless steel top grill having facilities for pelleted food. The animals were maintained in automated 12 hours light and dark cycle at 26^{o}C ± 2^{o} C in an air exchange room and all animals were quarantined and acclimatized to laboratory conditions for 15days prior to commencement of the experiment. The animals were fed with standard pellet diet supplied by Sai Enterprise, Chennai, Tamilnadu, India. All animals provided with RO water *ad libitum*. Animal experiments were performed according to the ethical guidelines suggested by the institutional animal ethics committee (IAEC). Autoclaved clean paddy husk used as bedding material and changed thrice a week.

Acute toxicity studies of the ethanolic dry fruit powder extract

Acute toxicity studies are conducted according to the guidelines of OECD, 420. Thirty six male wistar albino rats weighing 150-200g were used for the acute toxicity study. They were randomly distributed into one control group and five treated groups, containing six animals per group and were on standard normal diet provided with water *ad libitum*. All the animals are dewormed and they were allowed to acclimatize for two weeks in the micro and macro environment before the experiment. The treated group received the extract orally varying doses (100,200,500,1000, 2000mg/ Kg b.w) at a rate of 1.0ml /rat/day to different sets of animals for 2days.Control group animals given distilled water served as control. They were continuously observed for 42hours to detect any changes in behavioural or autonomic responses, spontaneous activity, irritability, corneal reflex, defecation and urination, mobility, aggressiveness, sensitivity to sound and pain and respiratory movements. Any mortality during the experimentation period was also recorded and there was no deaths occurred. The aim of the acute studies is to determine the LD50 values. The mortality rate was assessed and expressed as LD50. It was calculated by using the following equation (Turner and Hebborn, 1965)the results show the *C. Oxycantha* did not produce death even at a high dose(2000mg/kg b.wt) and so it is found to be safe at all the given doses.

$$LD50 = \text{the apparent least dose lethal to all in a group} - \Sigma(a \times b)/N$$

N= number of animals in each group, a= dose difference, b= mean mortality, Σ= sigma.

Sub-acute toxicological Evaluation

Experimental setup

To find out the effective dosage of *C.Oxycantha,* sub-acute toxicity studies were carried out by the Biswas method. The Extract was suspended in water and administered orally at varying doses (100, 200, 500, 1000, and 2000mg/kg b.w.) at a rate of 1.0ml /rat/day to different sets of animal for 28days. Studies conducted by following OECD 407 guidelines (Ozolua et al., 2010).[17]Which is shown in Table no 1.

Weekly body weight

The body weight of each rat was assessed using a sensitive balance during the acclimatization period, once before commencement of dosing, once weekly during the dosing period and once on the day of sacrifice which is depicted in Table no 3.

Mortality and clinical signs

During the four-week dosing period, all the animals were observed daily for clinical signs, morbidity and mortality patterns once before dosing, immediately after dosing and up to 4 hour after dosing.[14] On 30th day, 1.5ml blood collected from all the animals and centrifuged for 30 sec at 2000 rpm to separate serum for biochemical analysis. On 31st day the rats were sacrificed under thiopentone over the dose of anaesthesia and the liver, kidney and hearts were excised immediately and thoroughly washed in cold saline. All the tissues were weighed, recorded and preserved in 10% formalin for histopathology evaluation.

Relative organ weight

On 31st day, the animals were sacrificed under thoipentone anaesthesia. Different organs namely the heart, liver, and kidneys were carefully dissected out and weighed in grams. The relative organ weight of each animal was then calculated as follows,

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g) X 100}}{\text{Body weight of rat on sacrifice day (g)}}$$

Haematological assay

The collected blood samples were estimated for haematological parameters like haemoglobin, HCT, RBC, WBC, ESR, MCV,MCH, MCHC, PLT, Clotting time , PT,APTT.[15, 16] Blood samples were collected in 10% EDTA/ saline of pH7.2.

Biochemical parameters assayed

Biochemical parameters such as SGOT (Serum glutamate Oxaloacetate transaminase), SGPT (Serum glutamate pyruvate transaminase), ALP (Alkaline phosphatase) urea, uric acid, creatinine, total protein, Total bilirubin, Na^+, K^+,Cl^- , Albumin, Globulin, Bicarbonates in serum were assayed in the university central lab facility.Since liver, heart and kidney are organs of metabolism and excretion, potentially toxic agents are likely to affect them. So portions of these organs were fixed in buffered 10% formalin and 5 micrometers thick paraffin sections were made and stained with haematoxylin and eosin for microscopic examination.

Statistical analysis

The results of the biochemical estimations were reported as mean ± SD of six animals in each group. Total variations, present in a set of data were estimated by one way Analysis Of Variance (ANOVA) followed by the analysis of level of significance between different groups based on ANOVA using AGRES statistical package (Version 3.1). Difference among means were analysed by least significant difference (LSD) at 5% level ($p<0.05$).

RESULTS

The results of the toxicological evaluation of COC plant extract is shown in Table 2- 7. In this study the oral administration of ethanolic extract of COC did not produce any death or signs of acute toxicity up to dose of 2000 mg/kg b.w during the period of observation. In the acute toxicity study, rats were treated with different concentration of *C.Oxycantha* fruit extract from the range of 100mg/kg b.wt to 2000mg/kg b.wt which did not produce signs of toxicity, behavioural changes, and mortality in the test groups as compared to the controls when observed during the acute toxicity experimental period. These results showed that a single oral dose of the extract showed no mortality of these rats even under higher dosage levels indicating the high margin of safety of this extracts shown in table 2. Table 3 and 4 summarizes the effect of COC on body weights and organ weights which was not shown any significant increase. Later we observed the haematological parameters which is depicted in Table 5 and 6 shows significant increase of RBC count and haematocrit values and the other parameter like clotting time, PT and APTT were significantly increased among all the groups with no reduction in any other haematological parameters with the use of higher doses. And there were no significant changes of biochemical enzyme levels of ALT, AST, total bilirubin, creatinine, urea and other biochemical indicators with any given doses compared to the control group. These results are clearly indicates the safe use of *Crataegus oxycantha.*

Table 1
Experimental Design

Group	Experimental design
I	Control rats administered with normal saline
II	Plant extract treated rats (100mg/kg b.wt)
III	Plant extract treated rats (200 mg/kg b.wt)
IV	Plant extract treated rats (500mg/kg b.wt)
V	Plant extract treated rats (1000 mg/kg b.wt)
VI	Plant extract treated rats (2000 mg/kg b.wt)

Table 2
LD50 Evaluation by arithmetic method by Turner

Group	No. of Rats	No. of Animals dead	Dose difference(a)	Mean mortality(b)	Probit (a×b)
Control-IN. Saline	6	0	…….	……	……
Group-II100mg/kg	6	0	100	…….	…….
GroupIII200mg/kg	6	0	100	…….	……
GroupIV500mg/kg	6	0	400	…….	…….
GroupV1000mg/kg	6	0	500	…….	……
GroupVI2000mg/g	6	0	1000	…….	…….

Table 3
Changes in the body weight of rats during the treatment with different doses of Crataegus Oxycantha

Dose(mg/Kg)	Day 7	Day 14	Day 21	Day 28
Group-I(Control)	160±0.8	162±0.8	170±1.4	183±1.4
GroupII(100mg/kgb.wt)	158±0.6	160±1.6	165±0.4	170±0.6
GroupIII(200mg/kgb.wt)	161±0.1	163±0.5	165±0.2	172±0.1
GroupIV(500mg/kgb.wt)	165±1.1	165±0.6	168±0.6	173±0.6

GroupV(1000mg/kgb.wt)	163±0.2	165±1.7	172±0.1	181±0.3
GroupVI(2000mg/kgb.wt)	165±0.1	170±0.5	175±0.3	192±0.2

N= 6 animals in each group and values are expressed as mean± SEM.
Data were analysed by one- way ANOVA followed by Tukey multiple comparison test.
P <0.001 which is statistically significant. No significant difference in values between groups.

Table 4
Effect of ethanolic extract of Crataegus Oxycantha on change in organ weight of control and experimental rats

Organ Weight	Group –I	Group- II	Group –III	Group- IV	Group -V	Group-VI
Liver	4.02±0.12	4.05±0.34	4.10±0.65	4.18±0.34	4.01±0.78	3.97±0.64
Kidney	1.32±0.19	1.33±0.12	1.37±0.16	1.36±0.65	1.30±0.66	1.33±0.23
Heart	1.00±0.06	1.03±0.02	1.05±0.12	1.00±0.16	0.89±0.05	0.91±0.13

N= 6 animals in each group; values are expressed as Mean±SEM.
Data were analysed by one-way ANOVA by tukey multiple comparison test.

Table 5
Effect of ethanolic extract of Crataegus Oxycantha on Haemtological parameters in control and experimental rats

Parameters	Group I	Group I	Group III	Group IV	Group V	Group VI
Hb(g/dl)	16.31±0.21	15.61±0.12	15.90±0.12	14.39±0.13	13.85±0.37	14.04±0.42
RBC's ($\times10^6$)	7.45±0.12	8.24±0.07	7.35±0.10	8.30±0.09	8.28±0.57	8.26±0.08
WBC($\times10^3$)	10.37±0.12	10.47±0.09	11.38±0.12	10.45±0.15	11.31±0.07	10.30±0.07
Hct(%)	47.34±0.08	45.35±0.11	51.26±0.11	48.2±00.04	46.35±0.13	51.26±0.08
MCV(fL)	58.17±0.04	56.32±0.06	58.30±0.07	61.38±0.09	59.18±0.06	60.26±0.06
MCH(pg)	17.32±0.12	16.34±0.07	17.61±0.27	18.26±0.08	17.48±0.11	18.26±0.06
MCHC(pg/dl)	29.28±0.07	30.32±0.12	29.29±0.07	28.33±0.08	31.25±0.76	30.30±0.10
PLT($\times10^3$)	345.49±0.15	330.32±0.09	345.28±0.10	375.97±0.18	458.16±1.06	457.10±2.48

N= 6 animals in each group and values are expressed as mean± SEM.Data were analysed by one- way ANOVA followed by Tukey multiple comparison test. P value of the all the doses was statistically significant (P<0.05) when compare with control group and there was no significant difference between the groups.

Table 6
Effect of ethanolic extract of Crataegus Oxycantha on clotting time, PT and APTT in control and experimental rats

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI
Clottingtime(sec)	50.70±0.24	55.54±0.19	67.56±0.08	82.26±0.05	70.33±0.38	65.30±0.10
PT(sec)	13.37±0.08	14.59±0.11	13.32±0.08	17.13±0.16	18.29±0.05	16.09±0.42
APTT(sec)	27.38±0.13	30.39±0.16	27.46±0.21	35.17±0.06	37.13±0.02	35.10±0.14

N= 6 animals in each group and values are expressed as mean± SEM.Data were analysed by one- way ANOVA followed by Tukey multiple comparison test. P value of the all the doses Clotting time, PT and APTT were statistically significant (P<0.05) when compare with control group and there was no significant difference between the groups.

Table 7
Effect of ethanolic extract of Crataegus Oxycantha on serum biochemical markers in control and experimental rats

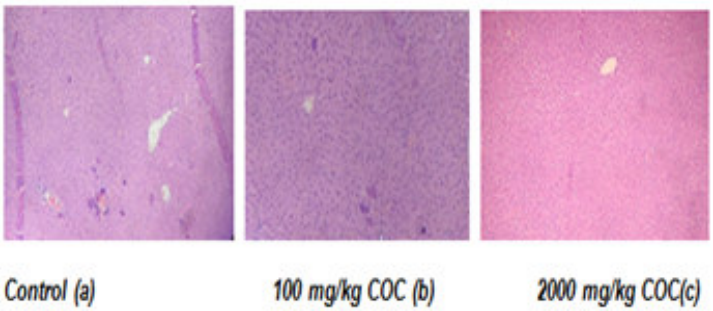
Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI
ALP(U/L)	85.32±0.14	86.25±0.21	85.34±0.09	110.30±0.10	100.48±2.59	110.52±3.72
AST(U/L)	88.02±0.39	86.16±0.61	88.24±0.33	95.89±0.42	87.81±0.26	92.99±1.51
ALT(U/L)	36.37±0.09	38.16±0.19	35.76±0.22	38.76±0.26	39.64±0.40	40.35±0.45
TotalProteins(g/dl)	7.48±0.11	7.41±012	7.48±0.11	6.71±0.18	7.26±0.23	7.30±0.06
TotalBilirubin(mg/dl)	0.4±0.0231	0.48±0.03	0.486±0.18	0.484±0.018	0.483±0.021	0.493±0.020
Urea(mg/dl)	33.25±0.10	32.45±0.09	33.54±0.10	35.11±0.06	31.34±0.09	34.28±0.12
Creatinine(mg/dl)	0.35±0.01	0.25±0.07	0.35±0.01	0.03±0.01	0.29±0.03	0.40±0.04
Albumin(g/dl)	3.28±0.11	3.85±0.25	3.30±0.10	4.25±0.08	5.26±0.03	5.28±0.08
Globulin(g/dl)	2.08±0.02	2.38±0.13	2.47±0.16	3.40±0.16	3.38±0.12	3.36±0.10
Na^+(mmol/L)	133.55±0.11	135.57±0.04	133.55±0.11	135.54±0.10	132.51±0.07	133.25±0.01
K^+(mmol/L)	4.43±0.13	5.30±0.08	4.44±0.14	4.28±0.14	5.45±0.11	4.59±0.21
Cl^-(mmol/L)	98.37±0.07	100.88±0.22	98.44±0.12	100.16±0.06	98.32±0.06	100.69±0.19
CO2(mmol/L)	22.47±0.09	23.30±0.09	22.42±0.11	24.35±0.09	24.33±0.11	25.22±0.09

N= 6 animals in each group and values are expressed as mean± SEM.

Data were analysed by one- way ANOVA followed by Tukey multiple comparison test. P value of the all the doses for liver enzymes, urea, creatinine, bilirubin and electrolytes were statistically significant (P<0.05) when compare with control group and there was no significant difference between the groups in any parameter.

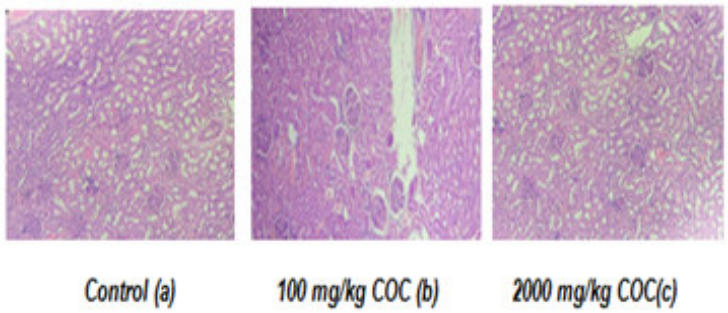
Histopathology observations of Liver, Kidney, Heart

Figure 3
Liver



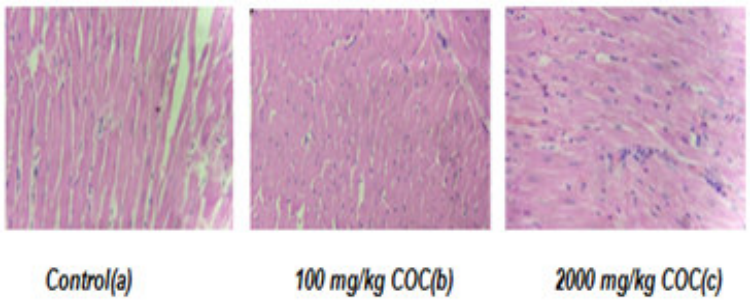
The liver showed lobules with hepatocytes radiating from the central vein to portal triad exhibiting the normal morphological feature in all the groups (fig 3 a,b,c)

Figure 4
Kidney



The kidney of control and treatment groups exhibiting glomeruli and tubules are seen which is unremarkable. Interstitium is normal indicating the absence of renal toxicity (fig 4 a,b,c)

Figure 5
Heart



Myocardial fibres which are appearing normal in control and treatment groups (fig 5 a,b,c)

The histology of Liver, Kidney,and Heart has shown normal morphology with no significant changes when compared with the control group (a) with the doses 100 mg/kg b.w (b) and 2000 mg/kg b.w (c).

DISSCUSION

The flavonoid content ologomeric proanthocyanidin possess good cardio tonic property and traditionally used in many countries for many purposes. The drug from flowers has anti-spasmodic, hypotensive, cardiotonic, diuretic and nervine-sedative properties. Hawthorn is most valuable remedy for cardiovascular system and considered to be one of the best cardiac tonic.[4] The oligomeric proanthocyanidins has strong vitamin C and flavonoids which is very much useful in many cardio vascular complications. It (citrin bioflavonoids) regulate the permeability of blood capillaries and promotes capillary stability when administered with vitamin C.[5] It is also utilized for their astringent qualities for relief of discomfort of sore throats, diarrhoea and dysentery. Antioxidants found in hawthorn may aid to prevent some of the damage from free radicals, mainly when it comes to heart disease.No physical changes were observed throughout the dosing period. All rats showed significant increase in body weight compared to their initial values. However there was no significant difference between the different treatment groups and the control, indicating that it did not have any adverse effects on the body weight, which is used to assess the response to the therapy of drug (Table 2). No mortality was observed during the whole experiment. During the dosing period and in the last day, the quantity of food and water intake by different dose groups was found to be comparable with control group. No abnormal deviations were observed. No significant changes were observed in the values of

different parameters studied when compared with controls and values obtained were within normal biological and laboratory limits. The weights of organs recorded did not show any significant differences in the treatment and the control group indicating that C.Oxycanthawas not toxic to heart, kidney, liver, spleen and brain (Table 3).There was no significant changes were observed in hemoglobin (Hb), red blood cell (RBC), white blood cell (WBC), Haematocrit, MCV ,MCH, MCHC, Plate let count PT and APTTin all the treated groups as compared to respective control groups (Table 4).Results of biochemical studies showed that there was no significant increase in the levels of the parameters at different doses ALP, AST, ALT in the different groups of animals treated with (100,200,500,1000,2000mg/kg b.wt) of the extract compared with control. This implies that the extract at the doses tested had no effects on the liver and kidney tissues (Table 7). Table 4 and 5 show the effect of the fruit extract on various haematological parameters and there was no significancant difference found when compare with control and experimental rats respectively. This result showed that the fruit extract at different levels tested did not produce considerable change in the levels of the different parameters tested and which shows the further safe use of the extract with its rich flavonoid contents. Histopathological examination of the liver, kidney and heart in the control and *C.Oxycanta* fruit extract fed groups showed no differences, indicating that feeding these fruit extract at these dose levels are safe and did not result in any adverse toxicological effect on these organs. (Figure 3-5).

ACKNOWLEDGEMENT

The authors are thankful to Prof A Sundaram Dean for giving the central laboratory facility to conduct the investigations, a special thanks to Dr. Jaison John Prof of Pathology for his support during the Preparation and interpretation of Histopathology of the tissues. We acknowledge the constant support by the SRM University management for providing central animal facility and other research facilities throughout the study period.

CONFLICT OF INTEREST

Conflict of interest declared none.

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