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Pharmacological screening of antitumor potential on Cycas beddomei Dyer.: An endangered species of Cycadaceae family

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***Abstract*---**The present work is focused on *In vitro* and *In vivo* antitumor properties of *Cycas beddomei Dyer.* Petroleum ether and ethanol extracts of *Cycas beddomei Dyer.* was screened for cytotoxic potential using MTT assay and antitumor activity using Ehrlich Ascites Carcinoma (EAC) mice model. Two doses (200 & 400 mg/kg body weight) of both the extracts were treated on the EAC-bearing mice and compared with standard Doxorubicin. Further, hematological and biochemical parameters were also evaluated. Extracts of *Cycas beddomei Dyer.* is not showing any toxicity and mortality on treatment with 2,000 mg/kg bw. The ethanol extract was observed to be more selective on Liver cancer cells are more sensitive (16.82±4.35 µM) compared to standard Doxorubicin (1.52±1.76 µM) followed by Lung cancer (A 549; 19.87±3.16), Prostate cancer (DU 145; 51.96±5.37 µM), and Colon cancer (HT 29; 64.81±5.92 µM). The plant was safe at 2,000 mg/kg weight and compare to pet ether, ethanol extract decreased the tumor size and viable cell count in EAC-bearing mice. The hematological, biochemical, and histological parameters were also restored with the treatment. The extracts of *Cycas beddomei Dyer.* exhibited substantial cytotoxic and antitumor activity in a dose-dependent manner on the selected model.

***Keywords*---**Cycas beddomei Dyer., phytochemicals, MTT assay, biochemical, histological studies.

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Introduction

The abnormal modern lifestyle, including unhealthy food habits, pollution, smoking, etc., creates an unfavorable environment for healthy cells, contributing to the dysregulation of cell growth and becoming immortal cells. This phenomenon is called cancer, and it can invade other tissue and generate tumors. (Sung et al., 2020) There are more than 277 cancer types with various genetic diversity in the world. From the statistical data on prevalence, forms, tendencies, and death rate of cancer, it is evident that lung cancer is the leading type, followed by breast cancer. (Mathur et al.,2020). In 2021, more than nineteen million cancer cases and more than ten million deaths were recorded, excluding non-melanoma skin cancers. (Seved et al.,2017). These statistics will be increased substantially in succeeding decades. The genetic variations and the complex pathophysiology of cancer make it difficult to target with the current medication. (Mandal, Ananya, 2019) The severe side effects of the cancer chemotherapeutic agents affect the patient’s life quality. There is a need to address these problems with better therapeutic output. (Farooq sultana et al.,2021)

Plant-based medicine is proved effective in treating various ailments, including cancers, for chronic usage. For example, Vincristine, Vinblastine, paclitaxel, Camptothecine, and their semi-synthetic derivatives are in multiple forms to target various cancers at various stages of cancer disease. Plant-based medicine is attaining a reputation across the globe for its better safety profile. The current research is steering towards natural products-based drug discovery, connecting it with combinatorial chemistry and pharmacology. (Seca et al.,2018) (Abu-Darwish et al.,2018) (Dokuparthi et al.,2021)

In the current work, *Cycas beddomei Dyer.* of Cycadaceae family is selected for screening its cytotoxic profile both *In vitro* and *In vivo*. The Cycad plants grow like palm trees with short trunks. The Cycad plants have been known to humankind over centuries and grow in tropical regions. (Mankga et al.,2020) In folklore medicine, the seeds and other parts of the plants are used for microbial infections, wound healing, pain, fevers, rheumatoid arthritis, muscular spasm, sores, boils, etc. Pharmacologically the plant is proven to have antibacterial, antifungal, analgesic, anti-arthritic, and antiulcer activity. (Alekhya Cheruku et al.,2016) (Alekhya et al.,2016) (Cheruku Alekhya et al.,2012)

Materials and Methods

Plant material

Cycas beddomei Dyer. was collected from the forest areas of Tirupathi and authenticated by Dr. K. Madhavachetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati, and voucher specimen was (Pt 027) preserved in the herbarium.

Animals

Swiss albino mice (male; weighing around 30-35gm) were used for the study, selected randomly, and acclimatized in cages (polypropylene). Rat pellet diet, water, standard temperature (24±2˚C), humidity (30-70%), and daylight cycle (12:12) were maintained during this period. Prior approval from the institutional animal ethics committee (IAEC) (1447/PO/Re/S/11/CPCSEA-37/A) was taken.

Acute oral toxicity studies

The oral acute toxicity study of *Cycas beddomei Dyer.* Extracts were evaluated according to Organization for Economic Co-operation and Development (OECD) guideline 423 on Swiss albino mice, where the limit test dose of 4000 mg/kg body weight was used. All the animals were kept overnight fasting before every experiment with free access to water. The animals were divided into four groups, each comprising five animals. The 1st group served as a negative control, while the 2nd, 3rd, and 4th were considered tested groups that received extracts at a dose of 300 mg/kg, 2000 mg/kg, and 4000 mg/kg body weight orally. Before dose administration, the body weight of each animal was determined, and the dose was calculated according to the body weight. The animals were observed for any toxic effects for the first four hours after the treatment period. Further animals were investigated for three days for parameters such as body weight, urination, food intake, water intake, respiration, convulsion, tremor, temperature, constipation, eye, skin colors, etc. (Kifayatullah et al.,2015)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The cytotoxic potential of extracts of *Cycas beddomei Dyer.* was determined by MTT assay using A 549 (lung), DU 145 (prostate), HT 29 (colon), HepG2 (liver) cells which were obtained from American Type Culture Collection (ATCC), 10801 University Boulevard Manassas, VA 20110, USA. The purple formazan produced by reducing MTT salt with mitochondrial enzymes represents the viability of cells. The intensity measured spectrophotometrically can be proportionate to the quantity of the living cells and can be expressed as IC50 values. The cells are incubated in a 96 well plate with the test samples at standard conditions (37°C, 5% CO_2, 72 hours) with subsequent MTT (20µl, 2mg/ml, phosphate-buffered saline) treatment followed by 3hours incubation under identical conditions. The Colored Formazan was extracted with DMSO (100µL), and the intensity was measured using a spectrophotometer (540 nm) in triplicates, and the values were compared with standard (Doxorubicin) and blank. (OECD guidelines 2001)

Tumor cells

The transplantable murine tumor cells were obtained from Amala Cancer Research Centre, Thrissur, Kerala, India. The tumor is maintained in the ascitic form by transplanting 1 million tumor cells to the animals (intraperitoneally) every week. (Dagli et al., 1992) Ascitic fluid was collected from the animal, and a concentration of 20 million cells per ml was maintained using sterile ice-cold normal saline solution.

In vivo anticancer evaluation

The animals were divided into seven groups (five animals each). A suspension of the carcinoma cells (20 million cells per ml) was prepared, and 0.1 ml was injected to all the groups, excluding the control. (Rinku Mathappan et al., 2019)

Group 1: Normal control
Group 2: EAC control group
Group 3: EAC + Doxorubicin (0.4mg/kg bw. i.p.)
Group 4: EAC + Pet ether extract (200mg/kg bw. oral)
Group 5: EAC + Pet ether extract (400mg/kg bw. oral)
Group 6: EAC + Ethanol extract (200mg/kg bw. oral)
Group 7: EAC + Ethanol extract (400mg/kg bw. oral)

On the next day, all the groups received respective treatments for two weeks. An intragastric catheter was used to administer the extracts orally. After 14 days of treatment followed by one-day fasting, the animals were sacrificed. The blood & serum parameters, tumor volume, packed cell volume, viable and non-viable cell count, mean survival time, and the percentage increase in life span were measured. (Bala et al., 2010) (Brandao et al., 2013)

Estimation of blood & serum parameters

Blood was collected by cardiac puncture and parameters such as RBC count, WBC count (differential), and hemoglobin content. The blood was centrifuged for 20 minutes at 5,000rpm to obtain serum to measure the serum parameters such as SGOT& SGPT levels were also determined to determine the hepatic function. (Flavia et al.,2006) (Santosh Kumar et al.,2007)

Estimation of tumor and packed cell volume

The ascitic fluid was collected from the peritoneal cavity and separated into two equal parts. The first part is centrifuged (1000rpm, 10 minutes) to measure packed cell volume (PCV) using a graduated centrifuge tube.

Estimation of viable and non-viable cell count

The second part of ascitic fluid was further subjected to the separation of the cells, and the viability was measured using 0.4% trypan blue solution. The stained cells indicate the non-viability of the cells. It is calculated by using the formula:

Cell count = (No. of cells × Dilution factor) / (Area × Thickness of liquid film)

Determination of mean survival time and percentage increase in the lifespan

The mortality will be monitored by the recording percentage increase in life span (% ILS) and mean survival time (MST) as follows:

% ILS = [(Mean survival time of treated group/ Mean survival time of control group)-1] × 100

Histopathological studies

The tumor specimens were separated from the mice and preserved in formalin (adjusted with 10% Phosphate buffer). Before examining the specimens, they were rinsed with ethyl alcohol followed by xylene and suspended in paraffin wax. 5µm thin specimens were prepared; eosin and hematoxylin were added to stain the specimens. All the specimens were observed under a binocular microscope and recorded.

Statistical analysis

Data will be analyzed using one-way Analysis of Variance (ANOVA) and expressed as mean ± S.E.M. Statistical significance was fixed $p < 0.05$.

Results and Discussions

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The ability of the extracts on the growth and viability of the cancer cell lines was estimated by incubating with various concentrations of the extracts. The effect of the extract was compared with the standard Doxorubicin, and after incubating with *Cycas beddomei Dyer.* extracts, for 72hr at 37°C and 5% CO_2, ethanol extract exhibited potent cytotoxic property against the selected cancer cell lines (Table 1). Pet ether extract is showing minimum cytotoxicity against Lung cancer (A 549; IC_{50}=51.35±6.18 µM), Prostate cancer (DU-145; IC_{50}= 42.22±4.35 µM), Liver cancer (HepG2; IC_{50}= 25.92±2.64 µM), and no significant activity against Colon cancer (HT 29; IC_{50}=>100µM). Whereas, for ethanol extract, it is found that the Liver cancer (HepG2) cells are more sensitive (16.82±4.35 µM) among others when compared to the standard Doxorubicin (1.52±1.76 µM) followed by Lung cancer (A 549; 19.87±3.16), Prostate cancer (DU 145; 51.96±5.37 µM), and Colon cancer (HT 29; 64.81±5.92 µM).

Acute toxicity studies

Cycas beddomei Dyer. was showing no toxic symptoms and was found safe even at 2000 mg/kg concentration. Hence, as working doses, the present study selected 1/10th and 1/5th of 2000 mg/kg, i.e., 200 mg/kg and 400 mg/kg.

In vivo anticancer evaluation

Tumor growth

After the plant treatment with the extracts of *Cycas beddomei Dyer.*to the animal, the ethanol extract showed a significant reduction in texture deformity and cancer cell count in a dose-dependent manner ($p<0.05$). At a higher concentration (400mg/kg bw), the ethanol extract reduced the number tumor cells (1.26±0.65ml), which is equivalent to the standard Doxorubicin (0.21±0.02ml).

The high-dose treated groups of EAC-bearing mice significantly reduced the packed cell volume to 0.67±0.34 ml from 3.21±0.34 ml. Viable cell count (cells×10^6 /mL) was minimized with Doxorubicin (0.45±0.11) and ethanol extract (1.05±0.47), significant with the values of the EAC-bearing mice group (9.03±0.02). The reduced non-viable cell count was increased as high as 3.97±0.08 cells×10^6/mL in the Doxorubicin followed by ethanol-treated group 3.05±0.18 cells×10^6/mL.

Survival parameters

Ethanol extract treated groups have increased the median survival time and ILS percentage as 200mg/kg bw (29.16±2.35; 64.19±1.32%) and at 400mg/kg bw (34.37±2.55;84.67±1.25%). The petroleum ether extract at 400mg/kg bw also showed mean survival time as 29.64±2.87 days and % ILS as 45.34±1.44. In comparison, the standard Doxorubicin exhibited 43.75±0.04;101.64%, respectively (Table 2 & Figure 1).

Haematological parameters

The haematological parameters of the EAC-bearing mice were improved, and the RBC count and Haemoglobin content significantly decreased, and the WBC count increased. The treatment with the standard Doxorubicin and the extract improved ($p<0.05$) the haematological parameters. Ethanol extract refurbished the RBC count and Haemoglobin content as 3.04±1.37&7.85±1.35 at 200mg/kg bw and 3.76±0.84 &9.34±1.11 at 400mg/kg bw. WBC count was brought to normal by the plant treatment5.94±0.32 &5.15±1.07 at low and high doses with improved differential WBC parameters and pet ether haematological parameters as mentioned in Table 3 & Figure 2.

Biochemical parameters

The abnormal biochemical profile of the EAS-bearing mice was revived significantly ($p<0.05$) when compared with the disease control (Table 4). Animals treated with Doxorubicin and *Cycas beddomei Dyer.* for the period of 14 days have got re-established biochemical profile. SGOT, SGPT, SALP, and Bilirubin levels were recovered to normal levels. Treating the EAC-bearing mice with the extracts at a dose of 400mg/kg bw exhibited significant development in the biochemical parameters. Especially, treatment with ethanol extract has improved the SGOT levels (58.97±0.12 IU/L), SGPT levels (45.94±0.11IU/L), SALP levels (81.17±0.25IU/L), and Bilirubin levels (1.93±0.37 mg/dL) of serum. The total protein content was restored to 7.15±0.37 mg/dL from 5.37±1.09 mg/dL(Disease control) with a dose of 400mg/kg bw.

Histopathological studies

The physiological alterations in the tissue specimens of the EAC-bearing mice treated with various plant extract concentrations and standard drug were studied using microscopic examinations. In liver cells, it is observed that the tumor cells were associated with dead tissue and angiogenesis. The tumor area was comparatively darker than the normal tissue, with inflammatory infiltrates. The regular architecture of the hepatic tissue was distorted. The treatment of the plant extracts at the high dose restored the regular anatomical features of the tissue in a dose-dependent manner with improved texture (Figure 3). Whereas, Doxorubicin re-established the homeostasis with minimum effect on the sinusoids and tissue texture.

When compared to the control group, disease control group shows infiltrating neoplasm composed of cells arranged in nests and lobules. Individual cells have pleomorphism and hyperchromatic round to oval nuclei with moderate eosinophilic cytoplasm. The surrounding area shows lymphocytic infiltration. Foci of necrosis and karyorrhectic debris are also seen. The liver section from the Standard group animals shows altered lobular architecture with pan lobular inflammation. Individual hepatocytes show binucleation and reactive atypia. Portal triad shows periportal inflammation. The central vein shows congestion and dilatation. Sinusoids show dilatation. When the animals treated with low dose of Pet Ether Extract shows altered lobular architecture with interface hepatitis. Individual Hepatocytes show binucleation, cytoplasmic vacuolation, and focal reactive atypia. It also shows focal areas of necrosis noted. Portal triad shows mild periportal inflammation. Central vein and Sinusoids show dilatation. But with high dose of pet ether extract, only mild interface hepatitis and periportal inflammation was observed. Ethanol extract reverted the pathological features at portal triad and ballooning and Cytoplasmic vacuolation at individual hepatocytes in a dose dependant manner.

Conclusion

Briefly, the results of the present investigations inferred potent *in vitro* cytotoxicity property of *Cycas beddomei Dyer.* and significant *In vivo* antitumor property. *Cycas beddomei Dyer.* was found cytotoxic to the cancer cell lines that grow rapidly. The plant is also found to target multiple cell lines and Liver cancer (HepG2) cells are found to be more sensitive, followed by Lung cancer (A 549), Prostate cancer (DU 145), and Colon cancer (HT 29) when compared with Doxorubicin. *Cycas beddomei Dyer.* extracts were observed to show no toxic symptoms on the animals at a concentration of 2,000 mg/kg bw. From the *In vivo* study results, it is found that ethanol extract exhibits comparatively better antitumor property than petroleum ether extract. Ethanol extract reduced the tumor size and viable cell count in a dose-dependent manner. The mean survival time and life span of the animals were also improved with the *Cycas beddomei Dyer.* treatments. The EAC-bearing mice's hematological, biochemical, and histological parameters were also revived with the treatment in a dose dependant manner and more significantly with the ethanol extract.

This study suggests the ethanol extract of *Cycas beddomei Dyer.* exhibits significant antitumor activity when compared to pet ether extract in the selected models. Various secondary metabolites include alkaloids, carbohydrates, phenols, steroids, terpenoids, glycosides, saponins, especially tannins and flavonoids of *Cycas beddomei Dyer.* May directly involved in the exhibited antitumor activity.

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Conflict of Interest: None

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Tables

Table 1. IC_{50} values of extract and Doxorubicin in MTT assay

Treatment	*Cycas beddomei Dyer*		Doxorubicin (µM)
	Pet ether extract (µM)	Ethanol extract (µM)	
A 549 (Lung)	51.35±6.18	19.87±3.16	0.31±1.82
DU 145 (Prostate)	42.22±4.35	51.96±5.37	2.1±1.63
HT 29 (Colon)	>100	64.81±5.92	2.34±2.71
HepG2 (Liver)	25.92±2.64	16.82±4.35	1.52±1.76

Table 2. Effect of *Cycas beddomei Dyer.* on EAC-bearing mice

Treatment	Body weight (gm)	Tumor volume (ml)	Packed cell volume (ml)	Viable cell count (cells×10^6 /mL)	Non-viable cell count (cells×10^6 /mL)	Median survival time (days)	ILS %
Normal control	31.24 ±0.12	-	-	-	-	-	-
Disease control	35.37 ±0.31	4.96±0 .31	3.17±0.1 4	9.03±0.02	0.72±0.03	22.37±0.0 4	0
Pet ether	33.82	3.67±1	3.28±0.5	5.18±0.84	1.21±0.77	24.82±1.9	32

extract (200mg/kg bw)	±2.66	.11	5			2	.16
Pet ether extract (400mg/kg bw)	32.73±2.17	2.83±0.82	2.63±0.71	3.76±0.37	2.67±0.14	29.64±2.87*	55.34*
Ethanol extract (200mg/kg bw)	29.38±2.78	2.75±1.03	1.17±0.48	3.14±0.69	2.18±0.33*	29.16±2.35*	64.19*
Ethanol extract (400mg/kg bw)	29.17±2.94	1.26±0.65*	0.67±0.34*	1.05±0.47*	3.05±0.18*	34.37±2.55*	84.`06*
Positive control (Doxorubicin)	30.08±0.77	0.21±0.02*	0	0.45±0.11*	3.97±0.08*	43.75±0.04*	101.64*

Data represented as mean±SEM, n=5 animals, *p<0.05 is considered as statistically significant

Table 3. Effect of *Cycas beddomei Dyer*. on haematological parameters of EAC-bearing mice

Treatment	RBC (cells×10^6/mm^3)	WBC (cells×10^3/mm^3)	Hb content (%gm)	Differential count		
				Monocytes (%)	Lymphocytes (%)	Neutrophils (%)
Normal control	5.17±0.03	4.27±0.11	12.09±0.11	1.85±0.04	74.19±0.02	19.34±0.02
Disease control	2.83±0.1	6.44±0.13	6.24±0.07	1.12±0.14	30.17±0.02	75.11±0.4
Pet ether extract (200mg/kg bw)	2.78±0.19	6.32±0.18	6.81±1.44	1.22±1.03	41.12±0.25	68.82±1.38
Pet ether extract (400mg/kg bw)	3.12±1.06	6.17±0.71	7.57±1.63	1.29±1.14	53.19±0.17	53.57±1.17
Ethanol extract (200mg/kg bw)	3.04±1.37	5.94±0.32*	7.85±1.35*	1.31±0.82	59.32±0.61	61.65±1.37
Ethanol extract (400mg/kg bw)	3.76±0.84*	5.15±1.07*	9.34±1.11*	1.52±0.76*	62.48±0.77	34.62±1.36*
Positive control (Doxorubicin)	4.12±0.14*	3.84±0.22*	11.17±1.03*	1.7±0.26*	71.35±0.15*	21.92±0.09*

Data represented as mean±SEM, n=5 animals, p<0.05 is considered as statistically significant

Table 4. Effect of *Cycas beddomei Dyer.* on biochemical parameters of EAC-bearing mice

Treatment	SGOT (IU/L)	SGPT (IU/L)	SALP (IU/L)	Total protein (mg/dL)	Bilirubin (mg/dL)
Normal control	35.36±1.06	25.93±1.06	75.03±1.27	9.06±1.1	0.97±0.34
Disease control	80.69±1.09	69.11±1.17	117.82±1.2	5.37±1.09	3.62±1.06
Pet ether extract (200mg/kg bw)	73.15±0.12	58.74±0.24	112.72±0.36	5.87±0.51	3.16±0.25
Pet ether extract (400mg/kg bw)	69.83±0.09	52.16±0.92	98.34±0.28	6.45±0.57	2.81±0.16
Ethanol extract (200mg/kg bw)	70.22±0.17	55.76±0.08	102.43±0.07	6.12±0.29*	2.94±0.17
Ethanol extract (400mg/kg bw)	58.97±0.12*	45.94±0.11*	81.17±0.25*	7.15±0.37*	1.93±0.37*
Positive control (Doxorubicin)	39.17±1.27*	29.62±1.11*	79.64±1.23*	8.49±1.22*	1.03±1.1*

Data represented as mean±SEM, n=5 animals, $p<0.05$ is considered as statistically significant

Figures

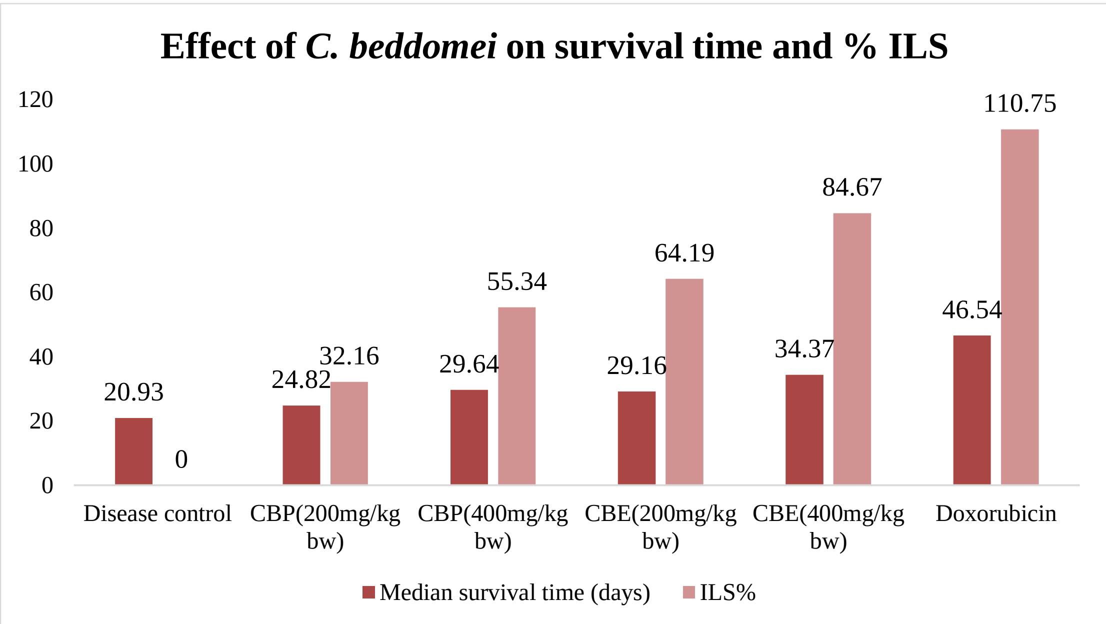

Figure 1. Effect *Cycas beddomei Dyer.* on survival time and % ILS

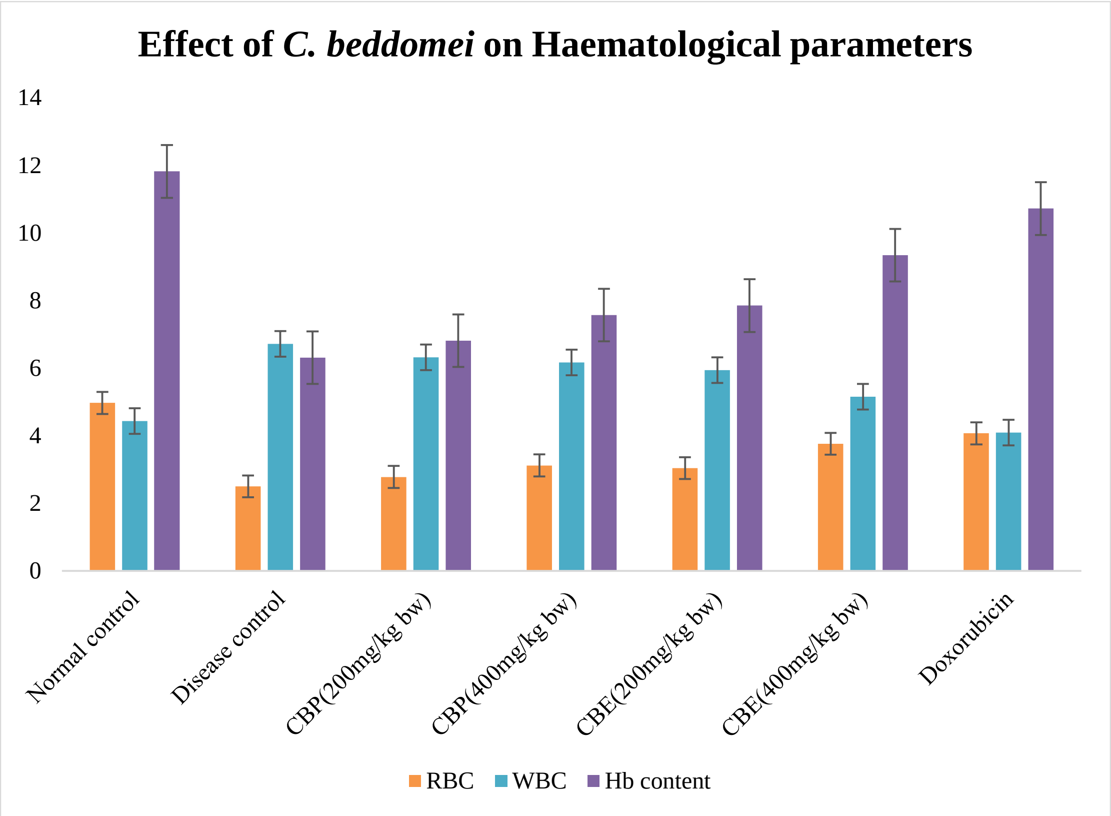

Figure 2. Effect of *Cycas beddomei Dyer.* on Haematological parameters

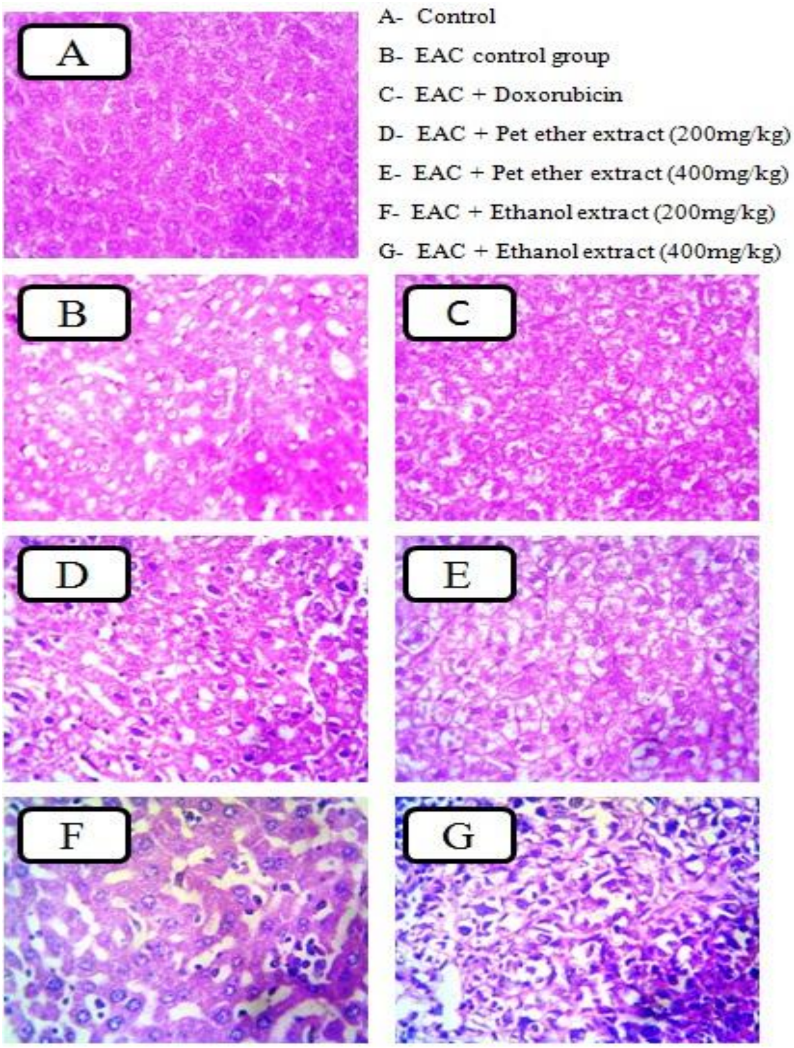

Figure 3. Histopathological study of Ehrlich Ascitis of Liver