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Antitumor activity of methanolic extract of *Cyclea peltata*

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Abstract

Phytocompound based drugs are soon gaining popularity as alternates for treatment of chronic and serious illness. *Cyclea peltata* has largely been classified as medicinal plant and used to treat various illness. Soxhlet based extraction of the plant was carried out using various solvents and subjected to chromatographic analysis to understand the variety of phytoconstituents present in the crude extract of the plant. Invitro and invivo testing for anticancer activity of the methanolic extract of the plant revealed potent significance among the haematological and cytotoxic parameters. The results, thus, indicate efficacy for anticancer activity although the main constituent of the crude extract is yet to be established.

Keywords: *Cyclea peltata*, cell lines, phyconstitutent, methanol, anticancer.

Introduction

Over the past few decades cancer has been the major cause of death worldwide caused by loss of cell control by a number of external (chemicals, radiations, tobacco etc) and internal factors (mutations, weakened immunity, hormones etc). A major portion of the current medical research is involved with the development of new novel drugs for cancer treatment [1]. Many drugs used in cancer therapy are marked by their wide range of side effects. The interest in natural compounds, fuelled partly by the adverse effects of current chemotherapy as well as the ongoing search for better ways to deal with the disease, is increasing within the scientific community. Scientists are now in the process of developing newer drugs by using the natural basic skeleton of isolated phytocompounds that target the unique mechanism of cancer cells. The traditional medicinal system of India like Ayurvedha and Unani has survived more than 3000 years, mainly by depending on plant drugs. The ancient texts like Rigveda (4500-1600 BC), Atharvaveda mention the use of several plants as medicines. The books in ayurvedic medicine such as Charaka Samhita and Susrutha Samhita refer to the use of more than 700 herbs.

Cyclea peltata (lam) Hook.F and Thomas is a slender twining shrub, frequently climbing on tall trees. It grows throughout India and Srilanka and is commonly called 'Patha' or 'Rajpatha'. Due to the high medicinal value of this plant, 'National Medicinal Plant Species in high trade sourced from tropical forests [2]. The kani and kurichiya tribal people of Kannur district in Kerala has been cultivating this plant for generations, for it's medicinal properties [3]. The present study aims to evaluate the anticancer property of methanolic extract obtained from the roots of *Cyclea peltata* (MECP) on Daltons Ascites Lymphoma (DAL) in Swiss Albino mice.

Materials And Methods

Plant material and Extraction

The plant was collected from Kayamkulam, Kerala, India. Under laboratory conditions it was cleaned, shade dried, powdered in a mechanical grinder, extracted with methanol by Soxhlet extraction and concentrated by using vaccum rotator evaporator.

Preliminary phytochemical screening

Preliminary phytochemical screening of the powdered sample was carried out to determine various parameters such as moisture content, total ash value, acid insoluble ash value, water soluble ash value, alcohol soluble and water soluble extractive values. Qualitative chemical tests were carried out to screen the presence of various phytoconstituents like alkaloids, steroids, flavonoids, terpanoids, phenolic compounds and tannins present in MECP.

Chromatographic analysis

Chromatographic analysis of MECP was carried out using thermo GC-Trace Ultra Ver: 5.0 GC-MS (Model Thermo MS DSQ II gas chromatograph). A fused-DB35-MS Capillary standard Non-polar Column Dimension (30mts, ID: 0.25 mm, FILM: 0.25µm)was used. The GC temperature program was as follows: initial temperature was 100 C, held for 1 min, increased to 130 C at a rate of 2 C/min, then to 200 C at a rate of 3 C/min, and finally to 280 C at a rate of6 C/min and held for 10 min. The split ratio was 1:12,injection temperature was 250 C, transfer line temperature was 270 C, and the mass spectrometer was operated at 70 eV in run time 29 min.

Invitro cytotoxicity Study

Experimental Animals

Adult Swiss albino mice (20±5 gm) was purchased from Animal Breeding station, Veterinary College, Mannuthy, Kerala, India. The animals were maintained under standard environmental conditions in polypropylene boxes and fed with standard pellet feed and water ad libitum. The study was carried out after obtaining permission from Institutional animal ethics committee and CPCSEA regulations were adhered to during the study.

Maintenance of DAL cells

DAL cells were procured from Amala Cancer Institute, Thrissur, Kerala, India. The DAL cells were propagated in the peritoneal cavity of mice by injecting 1x10 ^{6} cells/mouse. Ascitic fluid was collected from DAL tumor bearing mice after 10-12 days using 18 guage needles from peritoneal cavity. The ascitic fluid was washed thrice in phosphate buffer saline (PBS) and the cell pellet was resuspended in PBS. The tumor cell count was done using trypan blue dye exclusion method in haemocytometer. The cell suspension was diluted to get 1x10^{6}/0.1ml.

Experimental Design

Short term cytotoxicity studies were done on DAL cells by Trypan blue exclusion method. Cells were aspirated from the peritonial cavity of tumor bearing mice and washed in PBS twice and counted using a haemocytometer. 2x10^{5} cells/ml were taken for cell cytotoxicity studies. Different concentrations of the compound was added to the cells and then made up to 1 ml with PBS. Cells were incubated for 3 hours at 37 degree Celsius. After incubation, the cell death was evaluated using Trypan Blue exclusion method. To the cell suspension, 3 drops of Trypan Blue (0.5% in PBS) was added and the cells were loaded immediately on to a haemocytometer. The number of dead cells was counted and the percentage of dead cells was calculated. Viable cells exclude the dye while non viable cells take up the dye and appear blue in colour. The percentage growth inhibition was calculated and CTC50 value is generated from the dose-response curves for each cell line (Table 1).

In Vivo Study

Experimental mice were divided into 5 groups (n=6). 1st group was kept as normal control. All the animals in each group except Group I received DAL cells (1x10^{6} cells/ mouse i.p). This was taken as day 0. Group I served as normal control and Group II served as DAL control. After 24 hrs of DAL inoculation Group IV and Group V received methanolic extract of *Cyclea peltata* at a dose of 100 and 200mg/kg/day for 14 consecutive days orally. Group III received reference drug 5 fluorouracil (10 mg/kg oral). 24 hours after last dose 5 animals of each group were sacrificed to study the tumor growth parameters (Mean survival time, viable cell count, non viable cell count, tumor volume, tumor PCV and body weight analysis). Blood was drawn from tail for evaluating haemotological parameters. The rest of the mice in each group were kept with food and water ad libitum to check percentage increase in life span of the tumor host. Mouse developing huge amount of ascitic and becoming morbid were killed by deep anesthesia and the time was taken as survival time, and peritoneal fluid was aspirated and measured.

The designation of the animal groups and treatment details were as follows:

Group I	Normal control
Group II	DAL control
Group III	DAL +5-FU (10 mg/kg)
Group IV	DAL + MECP 100mg /kg
Group V	DAL+MECP 200mg/kg

Tumor growth parameters

All the mathematical functions used for this study are included in Table 1

Table 1: Mathematical formulae for analysing cytotoxicity and tumour growth parameters

Study undertaken	Formulae used if any
Cytotoxicity studies	$\%\ \text{Growth Inhibition} = 100 - \frac{\text{Total cell} - \text{Dead cells}}{\text{Total cells}} * 100$
Body weight analysis	$\%\ \text{Decrease in body weight} = \frac{\text{Decrease in body weight}}{\text{Initial body weight}} * 100$
Mean Survival Time	$\text{Mean Survival Time (in the same population)} = \frac{\text{Time of First death} + \text{Last death}}{2}$
Life Span	$\%\ \text{Life Span} = [\frac{\text{Mean survival of treated group}}{\text{Mean survival of control group}} - 1] * 100$
Tumour Volume	Measured directly in a graduated sterile tube
Cell count	$\text{Cell Count} = \frac{\text{No. of cells} * \text{Dilution Factor}}{\text{Area} * \text{Thickness of Liquid Film}}$
Tumour packed cell volume	Measured as percentage

Body weight analysis

All the mice were weighed for every five days, after tumor inoculation. Average gain in body weight was determined and recorded. The percentage decrease in body weight were calculated by the formula.

Mean Survival Time (MST)

After induction, everyday, check all the groups for mortality and record how many days the mouse is survived. The mean survival time (MST) and percentage increase in lifespan (ILS %) was calculated by using the formula.

Determination of Tumor Volume

After 14 days treatment the mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube

Viable and Non-viable cell count

After 14 days treatment animals are slightly anaesthetized with diethyl ether. From the intraperitonial cavity of mice take 0.2 ml of cell suspension were mixed with 0.5 ml of 0.4% trypan blue, 0.3 ml of normal saline or PBS and kept aside for 5 min and not more than 15 min. From this one drop of solution was taken on a neubar chamber and a cover slip is placed. This is placed on Haemocytometer and the viable and non-viable cells were counted under 10X power. Viable cells doesn't take colour and these cells appear in white colour on blue background Non-viable cells (dead cells) take blue colour and give dark blue shading to the cells, cell count was calculated using formula.

Tumor packed cell volume

The ascitic fluid was collected into Wintrobe's tube and it was centrifuged at the rate of 3000 rpm for a period of one hour. The volume of packed cells read directly as percentage.

Haematological Parameters

After 14 days treatment the animals were fasted over night and blood sample collected from retrorbital plexus and used for the estimation of Hemoglobin (Hb) content, Red blood cell count (RBC), white blood cell count (WBC), packed cell volume (PCV) and differential count by standard procedures.

Statistical Analysis

All data are expressed as mean $\pm$ S.E.M. Statistical significance (p) calculated by ANOVA followed by Dunnett's test, $P<0.05$ was considered as statistically significant.

Results

Phytochemical analysis of the methanolic extract of *C. peltata* (MECP)

The percentage yield of Methanolic Extract of *Cyclea peltata* (MECP) was found to be 5.00 % w/w. Qualitative chemical analysis of MECP provided the information regarding various types of phytoconstituents like alkaloids, terpanoids, steroids, carbohydrate, saponin, flavanoids etc. Table 2, 3 and 4 showed the presence and absence of various phytoconstituents.

Table 2: Physicochemical parameters of Cyclea peltata

Cyclea peltata		
Physical Characters	Colour	Brown
	Odour	Pungent
	Taste	Bitter
	Nature	Smooth
Moisture Content	Loss on drying	85.5% w/w
Ash Values	Total ash value	11.22 w/w
	Acid insoluble ash	1.07 w/w
	Water soluble ash	1.06 w/w

Table 3: Yield of the extract obtained on various solvents used in the study

Solvent	Empty weight of Petri plate	Wt. of Petri Plate + Residue	Actual wt. of Residue	
			Extraction volume	% yield
Petroleum ether	52.3	52.67	0.37	3.7%
Chloroform	45.6	45.92	0.32	3.2%
Ethanol	53.1	53.43	0.33	3.3%
Methanol	56.0	56.5	0.5	5%
Water	46.7	46.9	0.2	2%

Table 4: Preliminary phytochemical examination of MECP

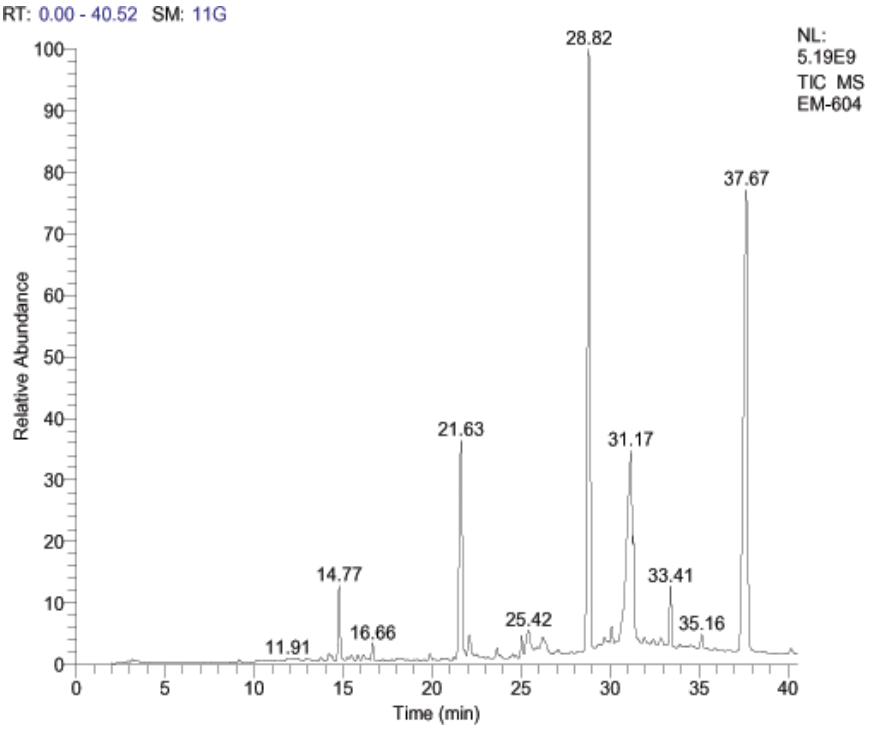
S. No.	Phytochemicals	MECP
1	Alkaloids	+
2	Carbohydrate	+
3	Steroid	+
4	Flavanoids	+
5	Phenols	_
6	Saponins	+
7	Aminoacid	+
8	Terpanoids	+
9	Tannin	+

\+ Presence - Absence

Chromatographic studies of *C. peltata*

The resulting Gas Chromatography–Mass Spectrometry from MECP has totally 526 molecule. Range of molecular weights differs from 16.043 Kg/Mol till 658.492 Kg/Mol. Out of which 326 molecule has CAS(Chemical Abstract Services) number from Pubchem deposition and others are ambiguities nature in the extract. By avoiding repeated structures from the crude sample from GC-MS results, 192 total structures were derived which non repetitive. For the annotated ligand structures, Physical and Surface properties were calculated such as Molecular Weight, Molecular Formula and Surface area of the small molecule revealed around 50% of the molecules yield either anticancer or antiinflammatory properties (Figure 1).

Figure 1: Chromatogram indicating the compounds obtained on GC-MS analysis of MECP



In vitro cytotoxicity Study

In vitro cytotoxicity effect of MECP at concentrations 62.5, 125, 250, 500, 1000 mg/ml in DAL cell line using trypan blue dye exclusion assay method has been shown in Table I. The % of cell inhibition was 8.33, 22.48, 32.08, 55.46 and 79.48% respectively. CTC_{50} has been found to be 440 µg/ml (Table 5).

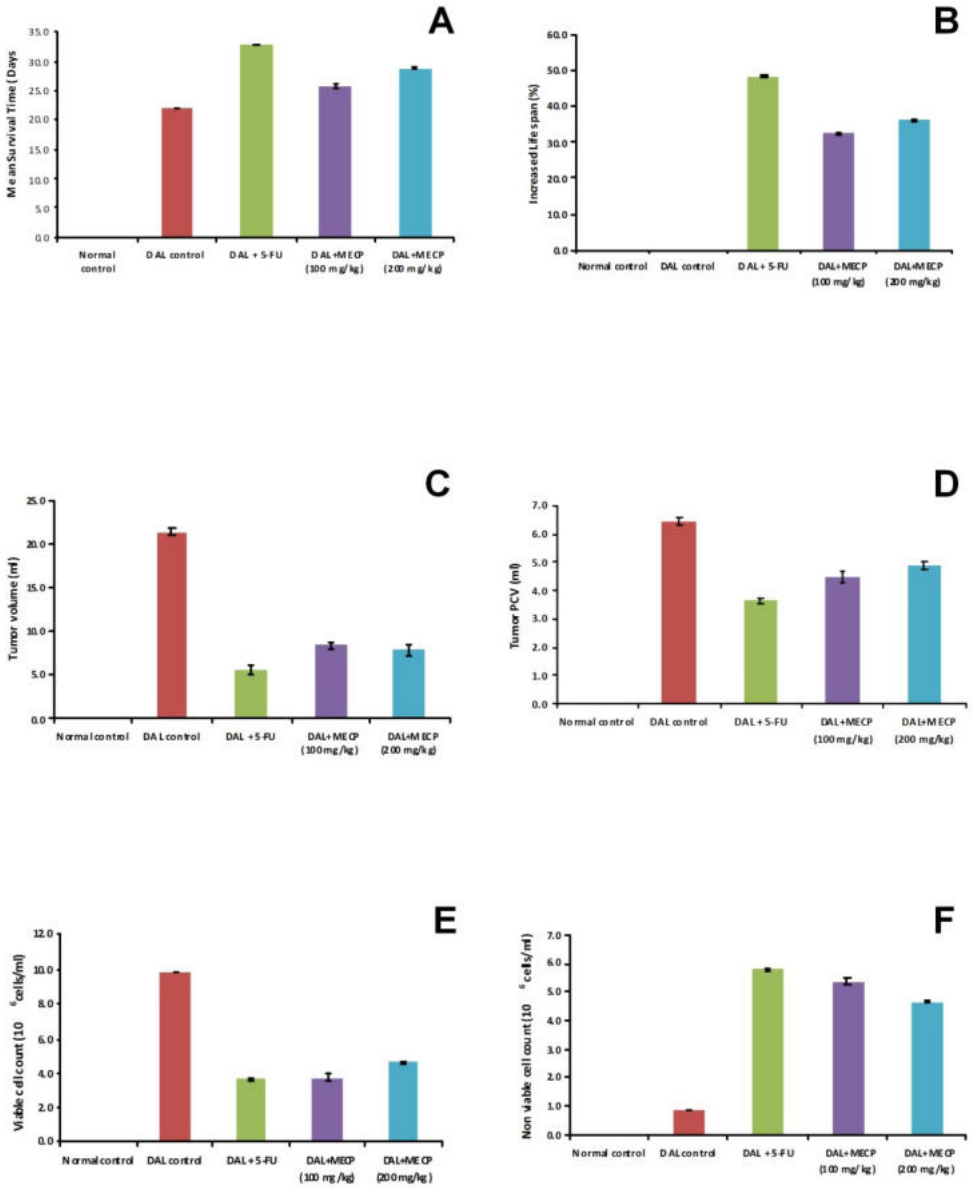
Table 5: Cytotoxic properties of MECP on DAL cells

Test drug	Test conc. (µg/ml)	Viable cell number	% growth inhibition	CTC 50 (µg/ml)
MECP	1000	16	79.48	440.00
	500	57	55.46	
	250	91	32.08	
	125	100	22.48	
	62.5	66	8.33	
Control	-	161	6.93	

Effect of MECP on tumor growth parameters

The effect of MECP on tumor growth is shown Figure 2. The mean survival time was significantly increased in MECP treated mice when compared to DAL control mice. The % increase in life span was found to be 20.45 and 36.36 in 100 and 200 mg/kg MECP treated mice. Treatment with 100 and 200 mg/kg of MECP significantly reduced the viable cell count, tumor volume and tumor packed cell volume in a dose dependent manner as compared to the DAL control group. Non viable cell count at different doses of MECP were significantly increased in a dose dependent manner.

Figure 2: Effect of MECP on tumor growth and tumor cell parameters

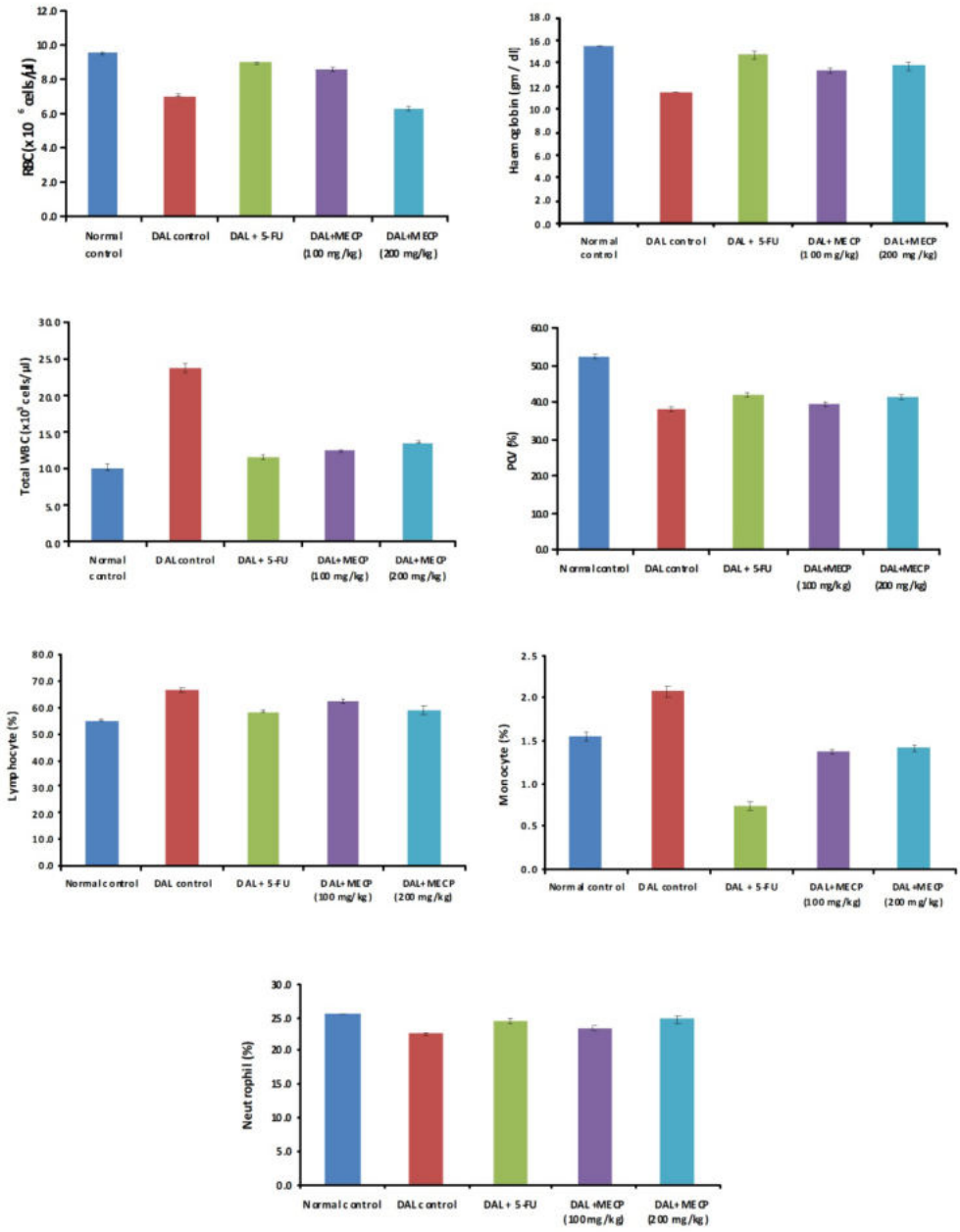


A - Mean Survival Time (Days); B - Increased Life span (%); C - Tumor volume (ml) ; D - Tumor PCV (ml); E - Viable cell count (10^6 cells/ml); F - Non viable cell count (10^6 cells / ml)

Effect of MECP on hematological parameters

The effect of MECP on haematological parameters of DAL induced mice were shown in Figure 3. Haemoglobin content and RBC count in DAL control group was decreased as compared to the normal control group. After treatment with 100 and 200 mg/kg of MECP Hemoglobin level significantly increased, whereas RBC levels were moderately increased to near normal levels. The WBC levels were significantly increased in DAL control when compared to normal. Treatment with 100mg/kg of MECP significantly decreased WBC count. PCV levels were lowered in DAL control group. Treatment with 100 mg/kg of MECP increased PCV value. In a differential count of WBC, neutrophils and monocytes increased whereas lymphocytes decreased in DAL model. Treatment with different concentrations of MECP restored these values to more or less normal.

Figure 3: Effect of MECP on haematological parameters



Discussion

The present study was carried out to evaluate the antitumor activity of MECP on DAL induced mice model. Inoculated mice treated with 100 and 200 mg/kg of MECP showed significant increase in life span. The reliable criteria for judging the value of any anticancer drug is the increase in life span [4]. There will be a steady increase in ascites fluid in DAL inoculated mice. Ascites fluid is the main nutritional medium for the tumor cells to grow [5]. Decrease in tumor volume in MECP treated mice proves the effective nature of the drug in destroying the tumor cells.

Haematological parameters are good indicators of the physiological status of animals [6]. Red blood cell is involved in the transport of oxygen and carbon dioxide in the body [7]. Thus, a reduced red blood cell count implies a reduction in the level of oxygen that would be carried to the tissues as well as the level of carbon

dioxide returned to the lungs. Anaemia in DAL inoculated mice is generally due to reduction in Hb and RBC count.
Previous reports stated that Packed Cell Volume (PCV) and haemoglobin are major indices for evaluating circulatory erythrocytes, and are significant in the diagnosis of anaemia [8]. Furthermore, reports that high Packed Cell Volume (PCV) reading indicated either an increase in number of Red Blood Cells (RBCs) or reduction in circulating plasma volume [9]. Similar results were obtained when MECP was administered. With regard to white blood cells and its differentials, administration of MECP reduced WBC count when compared to that of DAL control group.

Conclusion

From the above study, results indicate *C. peltata* as a good source with anticancer potential. Adequate profile of the significance of this plant with regard to phytoconstitution, invitro and invivo anticancer property helps to undertake further characterization and purification of a specific molecule that may present as a drug to cure cancer.

Authors' Contributions

Both authors have contributed equally to the manuscript and work

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