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Myeloprotective potential of leaves extract fractions of *Cnidoscolus aconitifolius* in myelo suppression

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Abstract

Chemotherapy has been associated with myelosuppression. Myeloprotection is the main remedy to myelosuppression. Nature has been endowed with natural molecules rich in therapeutic components and one of such nature is *Cnidoscolus aconitifolius* (CA) which has been reported to be rich in antioxidants. The aim of the study was to investigate myelo-protective potential of CA in myelo-suppressed animal model. Albino rats (n = 32) were divided into 8 groups of 4 rats each labelled A-H out of which 7 groups (B-H) were induced intraperitoneally for myelosuppression with cyclophosphamide (CP) and only 6 groups (C-H) were treated with CA extracts for four weeks. Group A served as normal control, groups B-H was induced for myelosuppression with cyclophosphamide. Group B served as myelo-suppressed control, groups C, D and E, received 100mg/kg body weight (bw) methanol fractions of CA while groups F, G and H received 200mg/kg body weight methanol fractions of CA. Two milliliters of blood were collected into ethylenediaminetetraacetic acid containers for total white blood cell count and absolute granulocyte count on days 3, 12 and 21 using mindray auto analyser BC-360. On day 30, bone marrow (BM) was harvested from one rat per group for BM examination and cellularity. Following administration of (CP), myelosuppression set in as observed in significant decreased ($P < 0.05$) in total white blood cell (TWBC) count and absolute granulocyte count. Extract fractions of CA caused significant increase ($P < 0.05$) in TWBC count and absolute granulocyte count in all the treated groups on days 12 and 21 compared with baseline. Bone marrow cellularity of all the treated groups revealed normal myeloid erythroid ratio indicating normal marrow cellularity. The findings in this study suggested myelo-protection by *Cnidoscolus aconitifolius* against cyclophosphamide induced myelosuppression.

Keywords: Myelosuppression; Myeloprotection; *Cnidoscolus aconitifolius*; Cyclophosphamide; Cellularity

1. Introduction

Myeloprotection refers to protection of the haematopoietic system. Myeloprotection can be achieved by some agents termed myelo-preservation agents because of their ability to combat the haematologic events that are commonly observed with the toxic chemotherapeutics (Kaplan, 2020). Myelo-preservation refers to preserving bone marrow or haematopoietic stem and progenitor cells (HSPC) whereby stem cells are protected from chemotherapy damages (Weiss, *et al,* 2019). This can reduce chemotherapy related toxicity, making chemotherapy safer and more tolerable and also reduce the need for rescue interventions that address the effects of myelosuppression such as growth factors or blood and platelet transfusions. Myeloprotection is also noticed when immune cells proliferate without any marked toxic effects (Kim *et al,* 2017). Chemotherapy-induced myelosuppression is as a result of interruption of normal

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haematopoiesis (Javarapa *et al*, 2018). Chemotherapy in other words greatly destroys haematopoietic stem cells thereby resulting to anaemia, neutropenia and thrombocytopenia (Taylor *et al*, 2017). Improvement in anaemia and leucopenia in other instances indicate myeloprotection in myelo-suppressed rats (Ufelle, *et al* 2016). The myelo-protective activity is observed by judging the changes in haematological parameters of cyclophosphamide (CP) induced anaemia model (Ufelle, *et al* 2018). Myeloprotection therefore offers remission to myelosuppression which is a life threatening condition. Haematopoiesis begins with a single cell type known as haematopoietic stem cell which resides in the bone marrow and has capacity to transform to various blood cell lines. These cells differentiate to form erythrocytes, leukocytes, and thrombocytes. Myelosuppression is undoubtedly among the numerous side effects of chemotherapy through damage to haematopoietic stem cells (Saad and Kathem, 2020). As a result of myelosuppression, patients may experience anaemia, neutropenia and thrombocytopenia. Deaths that occur after cytotoxic treatment usually happen either due to infections occasioned by drug induced leucopenia or from bleeding as a result of thrombocytopenia. All patients receiving chemotherapy and radiation experience some form of immunosuppression for a short duration (Banjamin, 2020). Myelosuppression occurs during cytotoxic therapy in patients undergoing cancer treatment.

Many plants have been shown to possess some myelo-protective and haematopoietic properties and example of such plant is *phoenix dactylifera* whose seed extract fractions progressively increased haematological parameters of myelo-suppressed wistar rats (Ufelle *et al*, 2016). Also extracts of *gongronema latifolium* exhibited myelo-protective and haematopoietic effects in cyclophosphamide induced myelosuppression in wistar rats (Ufelle *et al*, 2017). Traditionally, medicinal plants have been applied in medicinal preparation and creation of new therapeutic substances. Among such plants is *Cnidoscolus aconitifolius* (Mill). I. M. Johnst. Some Nigerian herbalists have employed *Cnidoscolus aconitifolius* as part of their herbal preparation to treat cancer (Ikpefan *et al* 2019). Report has it that *Cnidoscolus aconitifolius* (CA) possesses haematinic effects, erythrocyte membrane stabilization in protein energy malnutrition (Moura *et al*, 2019). Also reported is that CA possesses anti-inflammatory properties (Souto *et al*, 2011). *Cnidoscolus aconitifolius* is used in several herbal formations for the treatment of cancer, kidney infection, and weight control (Sanchez-Hernandez *et al*, 2017).

Since cyclophosphamide has been associated with myelosuppression, this present study is aimed at determining the myelo-protective potential of *Cnidoscolus aconitifolius* in cyclophosphamide induced myelo-suppressed animal model

2. Material and methods

2.1. Collection and Identification of plant materials

The leaves of the plant (*Cnidoscolus aconitifolius*) were harvested from my Garden at Judges' quarters in Makurdi town in Benue State, the North Central Region of Nigeria. It was taken to the herbarium of the Plant Science and Biotechnology Department of the University of Nigeria Nsuka (UNN) for authentication. The plant was authenticated with voucher number 194 by Dr. Abu. The leaves were air-dried, ground to powder and stored in airtight containers prior to use.

2.2. Acute toxicity text: (median lethal dose, LD_{50})

This was conducted on rats using Lork's method (1983). The LD_{50} was conducted in two stages. In stage one, 3 groups of 3 rats each were treated with intraperitoneal injection of 10, 100 and 1000mg/kg bw of extracts of *Cnidoscolus aconitufolius* respectively. The animals were observed for 24 hours for their behavior and mortality. At the end of 24 hours, no mortality occurred and this led to proceeding to phase 2. This phase involved three animals which were distributed into three groups of one animal each. The animals were intraperitoneally injected with higher doses (1600, 2900 and 5000 mg/kg) body weight of test substances (crude aqueous and crude methanol extracts of *Cnidoscolus aconitifolius*). The animals were also observed for behavior as well as mortality within 24hrs. At the end of 24hrs, mortality was observed on the group administered with the highest dose of 5000mg/kg body weight while no mortality occurred in groups administered with 1600mg/kg and 2900mg/kg body weight for both aqueous and methanol groups. The LD_{50} was calculated as the geometric mean of the highest non-lethal dose and the lowest lethal dose.

2.3. Methanol Extraction

One thousand grams (1000g) of the powdered samples was macerated with 1000ml of 70% methanol (model E Scale, model number ES0, China) and allowed to stand for 72 hours at room temperature with occasional shaking. It was then filtered using watman number 1 filter paper. The filtrate was concentrated with rotary evaporator, model (BUCHI; model number R100, England) and evaporated in a water bath (model number; B11, China) at 50^{o}C until dried (Kokate *et al*, 2009). The dry mass yielded 65.05g and then refrigerated in an air tight container at 4^{o}C till required for further experiments and analysis.

2.4. Fractionation and Isolation

Fractionation and identification of chemical constituents from crude extracts of the leaves of *Cnidoscolus aconitifolius* was carried out. The dried methanol crude extract was suspended in a measured amount of distilled water in order to make suspension in a separator funnel and extracted using different organic solvents like N-hexane, Ethylacetate and N-butanol respectively. Firstly, about equal volume of N-hexane was added to the methanol suspension and shaken carefully very well, and allowed to stand until two clear layers are formed. The hexane layer (upper one) was separated and the process repeated at least three times. Then, ethyl acetate was subsequently added to the aqueous layer and get ethyl acetate fraction. Finally, n-butanol was added to the aqueous layer, and obtain n-butanol fraction, leaving behind the residual aqueous fraction. All the solvents from each fraction were evaporated. The yield was weighed and calculated (Dilder, 2017). The dried fractions were taken through Tin Layer Chromatography and subsequently column chromatography depending on the standard method to obtain purer compounds.

2.5. Thin Layer Chromatography and Column Chromatography

The sample was prepared by adsorbing 20g of the extract of *Cnidoscolus aconitifolius* with 60g of silica gel (60-120 mesh). The mixture was air dried and meticulously layered on top of the packed silica gel in the column (14cm length) using a glass funnel. The extracts in the column was eluted with 100ml of methanol at the rate of 1ml per minute (Still et al, 1978). The eluates was concentrated and labeled, taking record of the percentage yield of the fraction. The factions were subjected to (GC-MS)

2.6. Gas chromatography- mass spectrometry (GC-MS sample preparation)

The sample was dissolved in a solvent, ratio of 1:10 v/v of methanol, then filtered with micro filter Nylon 0.45µm and transferred to the GC-MS machine (Model no.-GC7890B 5977A MSD) (manufacturer – Agilent technologies USA) in to a sample vial of about 2µl of the sample. It was then injected at 250^{θ}C in the injection port and splitters at a rate of 5:1 before reaching the Gas Chromatographic column which was conditioned at an Oven set initially at 110^{θ}C hold for 2mins at rate of 10^{θ}C/min raised to 200^{θ}C hold at 0 min, then at rate of 5^{θ}C/min to 280^{θ}C and finally hold for 9min. The sample was volatilized and separated into various components of ions of mass to charge ratio m/z, then transferred to the mass selective Detector. The resulting mass spectrum of the component were identified and qualified standard reference Library in the data analysis software which gives the compound names in respects to its quality comparison in percentage.

2.7. Phytochemical analysis

Phytochemical analysis of leaves extracts of *Cnidoscolus aconitifolius* was carried out in the department of chemistry, Ahmadu Bello University, Zaria with the method described by Harborne, (1984), sofowora (1993), and. Treas and Evans (2002). In general tests for the presence or absence of phytochemical compounds, using the above methods involve the addition of an appropriate standard chemical agent to the extract in test-tube.

2.8. Ethical Clearance

Ethical clearance was obtained from Ahmadu Bello University Zaria Animal Ethics Committee. The standard rules and regulations of use of animals for research purposes were strictly adhered to as was approved by the committee

2.9. Animals

Male and female albino rats weighing 100-200g aged 2-3 months were purchased and housed in the animal house of School of Veterinary Medicine, Ahmadu Bello University Zaria. They were maintained at a controlled light condition of 12-h height/ dark cycle and temperature (30±1.0^{o}C). They were acclimatized for two weeks and fed with normal rat feed (Ultima feeds Nig.,Ltd, Nigeria) and water *ad libitum* and throughout the study period.

2.10. Induction of myelosuppression

Bone marrow and blood toxicity was induced by injecting the rats with cyclophosphamide 40mg/kg body weight (bw) intra peritoneally (IP) once a day for two consecutive days in the first week, the rats with total white blood cell (TWBC) count less than 3 .5 x 10^{9}/l were selected for the study. Further myelo-suppression with cyclophosphamide 10mg/kg bw once a week for the subsequent three weeks was given to the myelo-suppressed groups.

2.11. Treatment groups

Albino rats (n = 32) were divided into 8 groups of 4 rats each labelled A-H out of which 7 groups (B-H) were induced for myelosuppression with cyclophosphamide and only 6 groups (C-H) were treated with CA extract fractions for four

weeks. Group A served as normal control, groups B-H was induced for myelosuppression with cyclophosphamide. Group B served as cyclophosphamide control, groups C, D and E, received 100mg/kg bw methanol extract fractions of CA while groups F, G and H received 200mg/kg bw methanol extract fractions of CA.

2.12. Sample Collection/Assays

Two milliliters (2ml) of blood sample from each rat was collected from retro-orbital plexus (through the ocular vie), both myelo-suppressed and non myelo-suppressed groups on days 3rd (which serves as the baseline), 12th and 21st into ethylenediaminetetraacetic acid (EDTA) anticoagulant containers. Complete blood count was carried out using mindray auto analyser BC-360 following manufacturer's instruction.

Bone marrow was collected from both myelo-suppressed and non myelo-suppressed groups on day 30, smeared on microscope slide and allowed to air dry, fixed in methanol and stained with leishman;s stain for bone marrow cellularity and examination. Myeloid : Erythroid (M:E) ratio was done by enumerating the myeloid lineage and erythroid lineage in the bone marrow, their proportions to each other was presented as a ratio.

2.13. Statistical Analysis

The results were interpreted as Mean ± Standard Error of Mean (SEM) in triplicate for each experiment. One way analysis of variance (ANOVA) was used to determine the difference among the groups using statistical package for social science (SPSS) version 17.0. Probability values ($P \leq 0.05$) were chosen to be statistically significant

3. Results

The effects of CP induced myelosuppression was investigated and compared with similar parameters of normal non myelo-suppressed rats as shown in tables 1 and 2. Following induction of myelosuppression in rats, leucopenia was apparent as indicated by the significant reduction ($P \leq 0.05$) of TWBC and absolute granulocyte count in the myelo suppressed groups. Table 1 and 2 also shows TWBC count and absolute granulocyte count of myelo-suppressed rats following treatment with 100mg/kg and 200mg/kg body weight extract fractions of *Cnidoscolus aconitifolius* respectively. There was restoration of TWBC and absolute granulocyte after 2-3 weeks of treatment with the extract fractions.

Table 1 TWBC count of cyclophosphamide induced myelo-suppressed rats following treatment with methanol extract fractions of *Cnidoscolus aconitifolius* from day 3 to day 21

S/N	GROUPS	DAY3 ($\times 10^9$/L)	DAY12 ($\times 10^9$/L)	DAY 21 ($\times 10^9$/L)
1	A: Normal control	5.60 ± 0.06	5.76 ± 0.03	5.73 ± 0.07
2	B: Negative Control	2.50 ± 0.06*	3.56 ± 0.07*a	2.00 ± 0.06*ab
3	C: Suppressed: 100mg/kg bw MF1	3.10 ± 0.06*	7.40 ± 0.06*a	5.30 ± 0.06*ab
4	D: Suppressed: 100mg/kg bw MF2	2.90 ± 0.06*	5.70 ± 0.06a	5.10 ± 0.06*ab
5	E: Suppressed: 100mg/kg bw MF3	1.00 ± 0.06*	7.20 ± 0.06*a	4.30 ± 0.06*ab
6	F: Suppressed: 200mg/kg bw MF1	2.70 ± 0.06*	8.30 ± 0.06*a	4.00 ± 0.06*ab
7	G: Suppressed: 200mg/kg bw MF2	1.40 ± 0.12*	6.30 ± 0.06*a	5.50 ± 0.06ab
8	H: Suppressed: 200mg/kg bw MF3	3.30 ± 0.12*	8.60 ± 0.06*a	5.90 ± 0.06ab

Values presented as mean ± SEM. * = significant relative to group A at $P < 0.05$, a = significant relative to value by day 3 (baseline) at $P < 0.05$, b = significant relative to value by day 12 at $P < 0.05$, Key; bw (body weight), MF (methanol fraction) SEM (standard error of mean).

Table 2 Absolute granulocyte count of cyclophosphamide induced myelo-suppressed rats following treatment with methanol extract fractions of *Cnidoscolus aconitifolius* from day 3 to day 21

S/N	GROUPS	DAY 3 ($\times10^9$/L)	DAY 12 ($\times10^9$/L)	DAY 21 ($\times10^9$/L)
1	A; Normal control	1.90 ± 0.06	2.00 ± 0.06	1.86 ± 0.03
2	B; Negative. Control	0.93 ± 0.09*	1.16 ± 0.07	0.50 ± 0.06^{*ab}
3	C: Suppressed: 100mg/kg bw MF1	0.30 ± 0.06*	1.30 ± 0.06^{*a}	1.20 ± 0.06^{*a}
4	D:Suppressed: 100mg/kg bw MF2	0.60 ± 0.06*	2.80 ± 0.15^{*a}	2.20 ± 0.06^{ab}
5	E: Suppressed: 100mg/kg bw MF3	0.70 ± 0.06*	2.10 ± 0.06^{a}	1.10 ± 0.06^{*ab}
6	F: Suppressed: 200mg/kg bw MF1	0.90 ± 0.06*	2.60 ± 0.06^{*a}	1.20 ± 0.06^{*ab}
7	G: Suppressed: 200mg/kg bw MF2	0.60 ± 0.06*	3.10 ± 0.06^{*a}	2.90 ± 0.06^{*a}
8	H: Suppressed: 200mg/kg bw MF3	0.30 ± 0.06*	2.20 ± 0.06^{a}	1.60 ± 0.06^{ab}

Values presented as mean ± SEM. * = significant relative to group 1 at $P < 0.05$, ^{a} = significant relative to value by day 3 (baseline) at $P < 0.05$, ^{b} = significant relative to value by day 12 at $P < 0.05$, **Key;** bw (body weight), MF (methanol fraction), SEM (standard error of mean).

Table 3 Phytochemical composition of methanol extracts of Cnidoscolus aconitifolius

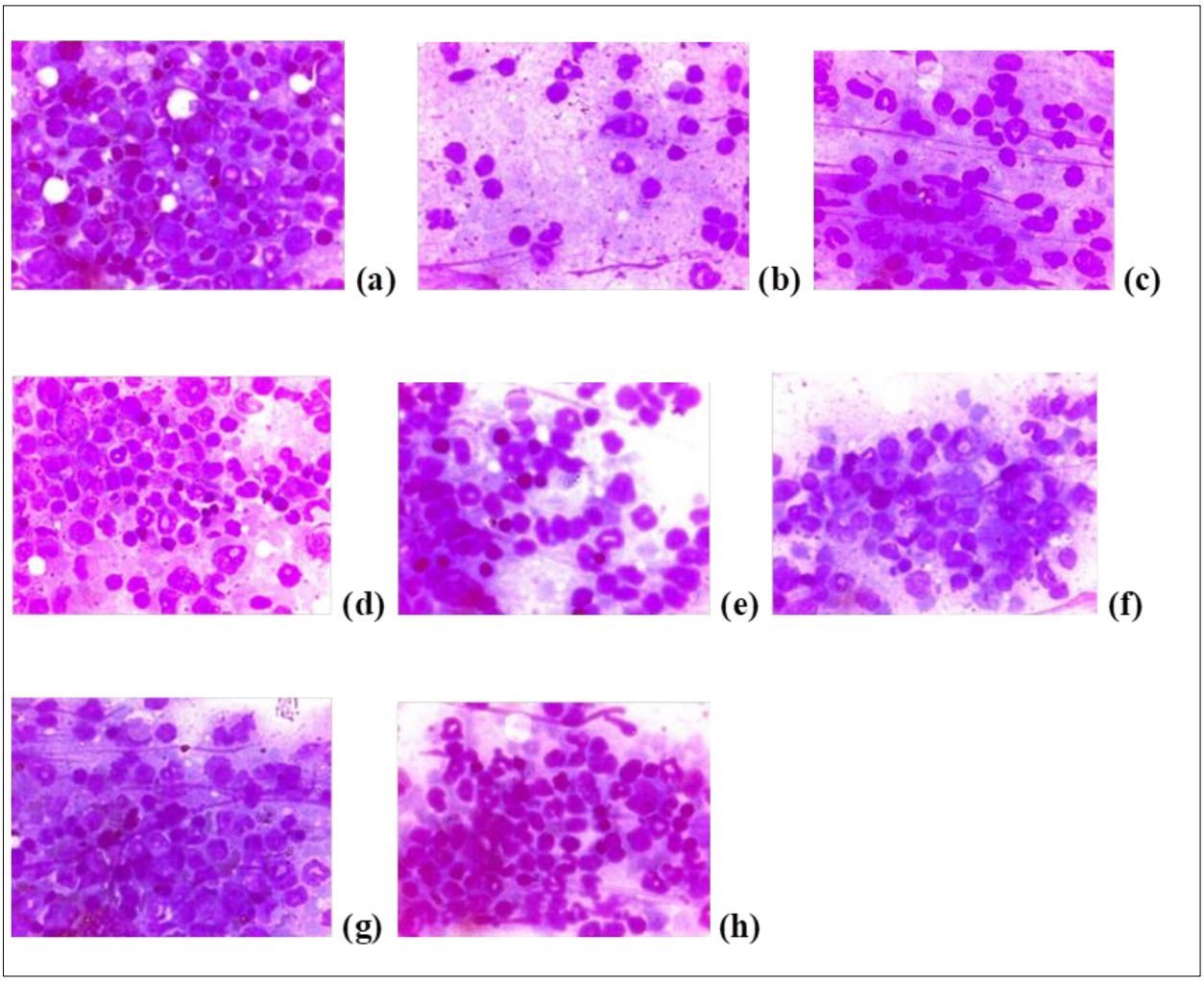
Phytochemical compound	Remark
flavonoid	+
saponin	+
phenolics	+
Tannin	-
Alkaloid	+
Glycoside	+

Key; present (+), absent (-)

Table 4 Myeloid erythroid ratio of the normal group, cyclophosphamide myelo-suppressed non treated group and cyclophosphamide myelo-suppressed treated groups.

S/NO	GROUP	MYELOID	ERYTHROID
1	A: Normal control	3	1
2	B: Negative. control	1	4
3	N: Suppressed: 100mg/kg bw MF1	3	1
4	O: Suppressed: 100mg/kg bw MF2	1	2
5	P: Suppressed: 100mg/kg bw MF3	1	2
6	T: Suppressed: 200mg/kg bw MF1	3	1
7	U: Suppressed: 200mg/kg bw MF2	1	1
8	V: Suppressed: 200mg/kg bw MF3	5	3

Key: bw=body weight, MF= methanol fraction.



(a): normal control group, (b): cyclophosphomide myelosuppressed non treated group (c), (d), (e): cyclophosphomide myelosuppressed treated group with 100mg/kg bw methanol extract fractios MF1, MF2, and MF3 repectively, (f), (g) (h): cyclophosphomide myelosuppressed treated group with 200mg/kg bw methanol extract fractios MF1, MF2, and MF3 repectively

Figure 1 Bone marrow pictures of the normal group, cyclophosphamide myelo-suppressed non treated group and cyclophosphamide myelo-suppressed treated groups

4. Discussion

Myelosuppression induced by cyclophosphamide from the above result (table 1) is associated with lower white blood cell count (Taylor *et al*, 2017) and absolute granulocyte cont. This could be as a result of the effect of cyclophosphamide on rapidly dividing cells like haemopoietic cells which in turn causes suppression of haemopoiesis (Javarapa *et al*, 2018). Following induction of myelosuppression with CP, TWBC count and absolute granulocyte count were significantly reduced ($P \leq 0.05$) on day 3 as shown in table 1 and table 2 which also revealed that group B which was induced for myelosuppression with CP without any treatment had progressive significant decrease ($P \leq 0.05$) in TWBC and absolute granulocyte count on days 12 and 21 compared with day 3 which was the baseline indicating non reversal of myelosuppression due to lack of treatment. It was observed that on day 12 which was after one week of administration of 100mg/kg and 200mg/kg methanolic leaves extract fractions of CA, there was high significant increase ($P \leq 0.05$) in TWBC and absolute lymphocyte count as revealed in table 1 and table 2. The value of TWBC count and absolute lymphocyte count was observed to be higher in the groups that were treated with 200mg/kg bw (F, G and H) than the groups that received 100mg/kg bw (C, D and E) respectively. Invariably, the extract showed dosage dependant in its function which is in conformity with the work of (Ufelle *et al*, 2016). Nevertheless, the value of TWBC count and absolute granulocyte count on day 21 though significantly higher ($P \leq 0.05$) compared with baseline (day 3) are lower than the values on day 12. The reason for the surge on day 12 could be as a result of the quick response by the bone marrow coupled with treatment with the extracts fractions to compensate for the sudden depletion of the marrow cells.

Fractionation of the methanol leaves extract of *Cnidoscolus aconitifolius* revealed three fractions indicated as MF1, MF2 and MF3.

Table 3 which displayed the phytochemical composition of *Cnidoscolus aconitifolius* revealed the presence of flavonoids. saponin, phenol, alkaloid and glycoside which has been reported to possess anti-inflammatory, anti-diabetic, antioxidant, anti-microbial and anti-bacterial properties (Souto *et al*, 2013)

Table 4 revealed normal myeloid erythroid ratio of the myelo-suppressed treated group compared with the myelo-suppressed non treated group.

Figure 1 which presented the bone marrow pictures of the animals in the study group revealed that groups A (normal control group), C-H (treated groups) showed apparently normal erythroid sequence of maturation. Meanwhile group B which was myelo-suppressed and not treated showed hypo-cellular marrow.

Abbreviations

- (TWBC) total white blood cells,
- (bw) body weight,
- (MF), methanol fraction,
- (CA) *Cnidoscolus aconitifolius*,
- (CP) cyclophosphamide,
- (IP) intraperitoneal

5. Conclusion

The administration of leaves extract fractions of *Cnidoscolus aconitifolius* increased white blood cell count and absolute granulocyte count with higher effect observed with a dosage of 200mg/kg bw. This therefore showed that when *Cnidoscolus aconitifolius* extracts when administered following cytotoxic treatment, myelosuppression effect was reversed which is significant because if this extract is employed during cancer treatment, suspension or termination of treatment will not be invoked thereby allowing full treatment course hence successful chemotherapy without myelosuppression complications.

Compliance with ethical standards

Statement of ethical approval

Ethical clearance was obtained from Ahmadu Bello University Zaria Animal Ethics Committee. The standard rules and regulations of use of animals for research purposes were strictly adhered to as was approved by the committee

Disclosure of conflict of interest

The authors have declared no conflict of interests.

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