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Original Article

Myelo-protective activity of aqueous and methanolic leaf extracts of vitex doniana in cyclophosphamide-induced myelo-suppression in wistar rats.

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ABSTRACT

Myelo-suppression is the most common dose-limiting side effect of chemotherapy during the complex anti-cancer treatment and together, with its complications can also be the most lethal. The present study was designed to investigate the Myelo-protective activity of the aqueous and methanolic leaf extracts of Vitex doniana (V. doniana) in cyclophosphamide (CP) -induced anaemic models. Fifty (50) wistar rats were divided into 5 groups of 10 rats per group labeled A to E. Group A received saline (5mL/kg) orally and served as baseline control whereas group B received CP (3mg/kg) intra-peritoneally (IP) alone, serving as positive control. Group C received only the aqueous extract (200mg/kg) orally, while group D received aqueous extract (200mg/kg) orally and CP (3mg/kg) IP. Group E received methanolic extracts (300mg/kg) orally and CP (3mg/kg) IP daily. On the Day 8, CP treatment was stopped and blood samples were collected from all the rats for full blood count (FBC). Blood and bone marrow samples were also collected on Day 15 and 22 respectively, from the rats for FBC and cell counts respectively. The results at Day 8 showed significant decrease ($P<0.05$) in the parameters studied in-group B, compared with the other groups. Groups D and E showed non-significant increase while group C had significant increase compared to group A. There was also a non-significant increase in-group D compared with E. The recovery phase (day 15), showed that CP-induced anaemia was not restored in-group B, with significant decreases ($P<0.05$) in haematological parameters compared to groups C, D and E. Groups D and E also showed slight decrease in the parameters compared to day 8. Day 22, however showed an increase in groups D and E whereas group B recorded further decrease in the parameters compared to day 15. Significantly increased neutrophil count ($P<0.05$) was generally observed in groups D and E compared with group B. The bone marrow cellularity (BMC) in-group B decreased significantly ($P<0.001$) when compared with baseline control whereas group D BMC increased significantly ($P<0.001$) compared with groups B and E. The results show that leaf extracts of V. doniana could reduce toxicity induced by CP, with the aqueous extract showing much greater myelo-protective ability. This may indicate its usefulness in the possible treatment of anaemia in cancer therapy, even in humans.

1. Introduction

In the year 2000, malignant tumors were responsible for 12 percent of the nearly 56 million deaths worldwide from all causes. Cancer has emerged as a major public health problem in developing countries, matching its effects in industrialized nations and in many countries, more than a quarter of deaths are attributable to cancer [1] . Mortality has always been high among patients with malignant diseases, but even today, despite all the complex anti-cancer therapy, the mortality rate stands at about 50% within 5 years in cases requiring systemic treatment [1].

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Myelo-suppression is the most common dose-limiting side effect of chemotherapy during the complex anti-cancer treatment and together, with its complications can also be the most lethal. Antineoplastic therapy-associated hematopoietic toxicity (myelo-suppression) will often result in neutropenia, thrombocytopenia, and anaemia. The patients may be at an increased risk of infection or bleeding, or may experience symptoms from anaemia.The uses of many chemotherapy drugs lead to some degree of myelo-suppression [2]. Myelo-suppression is characterized by the decrease in bone marrow cellularity, frequency and content of stem and progenitor cells. Granulocyte-macrophage progenitors (CFU-GM) are the most important suppressed group among haematopoietic cells resulting in neutropenia [2].

Chemotherapy-induced haematopoietic toxicity is a multi-factorial challenge that influences the treatment of oncology patients; therefore, it is essential to introduce means to provide myelo-protective effects [3]. The colony-stimulating factors and interleukins induce cell viability by inhibiting apoptosis [4, 5, 6].
Traditional herbal medicines are naturally-occurring, plant-derived substances with minimal or no industrial processing that have been used to treat diseases within local or regional healing practices [7]. Government, international agencies and corporations are increasingly investing in traditional herbal medicine research [7].

In Nigeria, traditional and herbal healing systems play an important role in health care delivery, and about 70-80% of the population depends on herbal treatments for most of their illnesses [8]. *Vitex doniana (V. doniana)* is widespread deciduous forest tree largely found in coastal woodlands and savannah, but also in wetter areas at lower altitudes. It is found in deciduous woodlands (especially Brachystegia), secondary forests and dry forests. There are numerous medicinal uses for this tree. Earlier workers have reported the use of the fruits and leaves for medicinal purposes [9, 10]. V. doniana is used to improve fertility and to treat leprosy, dysentery jaundice, control of post partum bleeding after birth and anaemia [11,12]. It is also used to treat gastroenteritis, diarrhea and gonorrhea. The use of V. doniana suggests that it may possess anti-microbial activity [13]. There is limited information on the myelo-protective activity of leaf extract of V. doniana. Due to the paucity in information and its numerous medicinal properties and uses, it becomes necessary to investigate the myelo-protective activity of the leaf extract of V. doniana in myelotoxicity induced by cytostatic agents.

The general objective of this study is to investigate the myelo-protective activities of the methanolic and aqueous extracts of V. doniana in cyclophosphamide-induced myelo-suppression in wistar rats. This could offer a possible way to cushion the effect of anti-cancer drug therapy on the myeloid cells and hence prevent myelo-suppression during the duration of therapy and beyond.

2. Materials and Methods

2.1.Collection of plant materials

The plant materials were obtained and authenticated by the Botany department; University of Nigeria Nsukka and a voucher specimen was kept in the herbarium for future reference.

2.2.Animal housing

Fifty (50) Wister rats were purchased and housed in the animal house of the College of Medicine, University of Nigeria Enugu Campus. They were allowed to acclimatize for two weeks and fed with commercially available rat feed and have access to water ad *libitum*.

2.3.Preparation of extract

2.3.1.Aqueous Extraction

Three hundred grams [300g] of the ground, shade-dried, powdered leaves of *Vitex doniana (V. doniana)* were soaked in 200mL of distilled water at room temperature for 24 hours and the mixture sieved through muslim cloth and later filtered through Whatman No. 1 filter paper. The filtrate was concentrated in an incubator at 60oC. The concentrate was stored at 4°C until needed.

2.3.2.Methanol Extraction

Three hundred grams (300g) of the ground, shade-dried, powdered leaves of V. doniana were soaked in 2.5Litres of methanol for 48 hours with two- hourly vigorous shaking. The mixture was filtered through Whatman No. 1 filter paper and evaporated to dryness on a rotary evaporator (Model 349/2 Carting Ltd).

2.3.3.Phytochemical Analysis

Phytochemical analysis of the leaf extract of V. doniana was carried out in the Department of Pharmacognosy, University of Nigeria, Nsukka, Nigeria.

2.4.Experimental Design

The fifty [50] Wister rats were divided into 5 groups of 10 rats per group, labeled A to E. Group A received 5mL/kg 0f saline orally and served as baseline control. Group B received 3mg/kg of cyclophosphamide (CP) intra-peritoneally (IP) and served as positive control. Group C received only aqueous extract [200mg/kg] orally; Group D received aqueous extract [200mg/kg] orally and CP [3mg/kg] IP, Group E received methanol extract [300mg/kg] orally and CP [3mg/kg] IP for one week [protective phase]. CP treatment was stopped on Day 8 and Blood samples were collected from all the rats on day 8 for full blood count (FBC). Blood samples were also collected from all the rats whereas bone marrow samples were collected from 3 rats in each group on Day 15 and day 22 for FBC and cell counts respectively.

24.1.Sample Collection and Processing

Blood samples were collected from all the rats via the retro-bulber plexus of the medial canthus vein into EDTA anticoagulated

bottles for full blood count (FBC). Bone marrow samples were also collected for cell count by the method of Dacie and Lewis, [14].

2.4.2.Analytic Methods

Full blood count (FBC) and bone marrow cell counts were done using standard operative procedures as described by Dacie and Lewis [14].

2.5.Statistical Analysis

Statistical analysis of the data was carried out through the Statistical Package for Social Science (SPSS) computer software version 11 using ANOVA and student's t-test at 95% confidence limit with P-value of (<0.05) being considered as significant. Results were expressed as mean ± standard deviation (mean ± SD).

3.Results

Table 1 shows the mean ± standard deviation (mean ± SD) of the haematological parameters on the Day 8 of the study. The results revealed that cyclophosphamide (CP) control (group B) showed significant decrease in haematological parameters when compared to baseline control (group A). Group B also showed significant decreases (P<0.05) in the parameters compared with groups C, D and E. A non-significant increase was observed in groups D and E while group C (300mg/kg aqueous extract only) showed statistically significant increase (P<0.05) in haematological parameters compared to the baseline control (group A).

Table 1. The Mean ± SD of the haematological parameters on the Day 8 of the experiment (Protective phase).

Parameters	Group A Baseline control	Group B 3mg/kg Cyclophosphamide (CP) alone.	Group C-200mg/kg Aqueous extract	Group D-200mg/kg Aqueous extract and CP.	Group E-300mg/kg Methanol extract and CP.
Haemoglobin (g/dL)	11.5 ± 0.42	9.70 ± 0.62	13.3 ± 0.55	11.75 ± 0.5	10.83 ± 0.35
Haematocrit (L/L)	0.34 ± 0.83	0.29 ± 1.2	0.39 ± 1.5	0.33 ± 1.3	0.31 ± 0.55
Total WBC (× 10^9/L)	5.80 ± 0.35	2.35 ± 0.40	4.50 ± 0.46	3.80 ± 0.44	2.43 ± 0.25
Platelet (× 10^9/L)	130 ± 5	122 ± 10	137 ± 2	128 ± 4	142 ± 7
Neutrophil (%)	60 ± 3	29 ± 3	65 ± 2	58 ± 2	53 ± 1
Lymphocyte (%)	37 ± 2	68 ± 2	32 ± 3	39 ± 3	44 ± 2
Monocyte (%)	2 ± 1	1 ± 0.5	2 ± 0.56	2 ± 0.31	2 ± 1
Eosinophil (%)	1 ± 0.5	2 ± 1	1 ± 0.23	1 ± 0.33	1 ± 0.5

Table 2. The Mean ± SD of haematological parameters on the Day 15 of the experiment (7 days post-withdrawal of Cyclophosphamide treatment) (Recovery phase).

Parameters	Group A Baseline control	Group B 3mg/kg Cyclophosphamide (CP) alone.	Group C-200mg/kg Aqueous extract	Group D-200mg/kg Aqueous extract and CP.	Group E-300mg/kg Methanol extract and CP.
Haemoglobin (g/dL)	11.5 ± 0.42	5.8 ± 0.54	13.8 ± 1.5	11.3 ± 0.5	10 ± 0.37
Haematocrit (L/L)	0.34 ± 0.83	0.16 ± 2	0.40 ± 2	0.33 ± 1	0.31 ± 0.82
Total WBC (× 10^9/L)	5.80 ± 0.35	1.5 ± 0.38	6.2 ± 0.5	5.4 ± 0.75	5.1 ± 0.5
Platelet (× 10^9/L)	130 ± 5	75 ± 10	134 ± 3	125 ± 3.5	128 ± 6
Neutrophil (%)	60 ± 3	20 ± 5	134 ± 3	57 ± 3	55 ± 2
Lymphocyte (%)	37 ± 2	78 ± 3	65 ± 2	40 ± 2	43 ± 3
Monocyte (%)	2 ± 1	1 ± 0.2	2 ± 0.56	1 ± 0.3	1 ± 0.3
Eosinophil (%)	1 ± 0.5	1 ± 0.5	1 ± 0.23	2 ± 1	1 ± 0.6

Table 3. The Mean ± SD of heamatological parameters on the Day 22 of the experiment (14 days post-withdrawal of Cyclophosphamide treatment).

Parameters	Group A Baseline control	Group B 3mg/kg Cyclophosphamide (CP) alone.	Group C-200mg/kg Aqueous extract	Group D-200mg/kg Aqueous extract and CP.	Group E-300mg/kg Methanol extract and CP.
Haemoglobin (g/dL)	11.5 ± 0.42	5.6 ± 0.98	13.9 ± 2	11.8 ± 0.33	10.5 ± 0.85
Haematocrit (L/L)	0.34 ± 0.83	0.16 ± 1.5	0.42 ± 1	0.34 ± 0.5	0.31 ± 2
Total WBC (× 10^9/L)	5.8 ± 0.35	1.43 ± 0.5	6.8 ± 1.5	6.1 ± 0.82	5.5 ± 0.4
Platelet (× 10^9/L)	130 ± 5	68 ± 2	135 ± 2	133 ± 3	130 ± 2
Neutrophil (%)	60 ± 3	19 ± 1	67 ± 2.5	62 ± 2	58 ± 3
Lymphocyte (%)	37 ± 2	79 ± 3	29 ± 0.82	35 ± 1	39 ± 2
Monocyte (%)	2 ± 1	1 ± 0.21	2 ± 0.3	2 ± 0.13	1 ± 0.5
Eosinophil (%)	1 ± 0.5	1 ± 0.41	2 ± 0.26	1 ± 0.24	2 ± 0.6

Table 4. The Mean ± SD of Bone Marrow Cellularity on day 15 and 22 of the experiment.

Parameters	Group A Baseline control	Group B 3mg/kg Cyclophosphamide (CP) alone.	Group C-200mg/kg Aqueous extract	Group D-200mg/kg Aqueous extract and CP.	Group E-300mg/kg Methanol extract and CP.
Day 15 (x106/femur)	13.50 ± 1.3	4.10 ± 0.95	13.80 ± 1.5	10.20 ± 1.1	6.50 ± 0.86
Haematocrit (L/L)	-	5.50 ± 1.2	15.30 ± 0.77	11.50 ± 0.32	7.10 ± 1.4

The mean ± SD of the haematological parameters on the Day 15 of the experiment (7 days post-withdrawal of CP treatment) are shown on table 2. In the recovery phase, CP-induced anaemia was not restored to normal in group B. Groups C, D and E revealed significant increases in haematological parameters compared to group B, but there was also decreased levels in groups D and E compared to day 8 of the experiment. There was general significant decrease ($P<0.001$) in the neutrophil count of group B, compared with groups D, E and A (the baseline control).

Table 3 shows the mean ± SD of heamatological parameters on the Day 22 (7 days post withdrawal of CP treatment). The results showed an increase in the parameters studied in groups D and E and further decrease in group B compared to day 15. Group C also showed an increase in the parameters whereas the comparison between groups D and E recorded a slight increase in group D compared to group E.

Table 4 shows the effect of Vitex doniana (V. doniana) on the bone marrow cellularity (BMC) and showed a statistically significant decrease in the BMC in groups B and E compared to the other groups. There was also significant decrease in groups D and E compared to group A (Baseline control), whereas group D had significantly increased BMC compared with group E. Phytochemical analysis result of the leaf extract of V. doniana showed that flavonoid, saponins, tannins, proteins, reducing sugars and glycosides were abundantly present. Alkaloids were shown to be moderately present whereas resins were present.

4.Discussion

The present study was designed to investigate the myelo-protective activity of the leaf extract of V. doniana in cyclophosphamide-induced anaemia model. V. doniana is used to improve fertility and to treat leprosy, dysentery jaundice, control of post-partum bleeding after birth and anaemia (don Maydell, 1986; Ladeji, et al, 2005). It is also used to treat gastroenteritis, diarrhea and gonorrhea and the juice may be squeezed into the eye to treat eye problems. The use of V. doniana suggests that it may possess anti-microbial activity (Kilani, 2006). The phytochemical analysis of V. doniana revealed the presence of flavonoids, alkaloids, saponins, tannins, resins, proteins, reducing sugars and glycoside. The myelo-protective activity is observed by judging the changes in haematological parameters of CP-induced anaemia model. Cyclophosphamide belongs to the nitrogen mustard subclass of alkylating agents under cytotoxic drugs used in the

treatment of lymphomas, some forms of leukaemia and some solid tumors [Shanafelt, et al, 2007]. The present study showed a significant decrease in the haemoglobin (Hb) level in group B compared to the other groups on the day 8 of the experiment, with the Hb concentration being registered by the group C (Aqueous extract alone). Group C showed a general increase in the parameters compared to the other groups, followed by groups A and D. this showed that the myeloid cell were actually protected from the suppressive effects of cyclophosphamide in the first 8 days of the experiment, with greater protective effect coming from the aqueous extract. On the 15th day of the experiment, there was further significant decrease ($P<0.05$) in the haematological parameters in the group B (CP alone), whereas groups D and E showed non-significant decreases compared to day 8. Group C, however, showed non-significant increase. Comparison of groups D and E on day 15 (7 days post-withdrawal of CP) showed increases in all the parameters in group D compared with group E, except for platelet count and lymphocytes. There was also non-significant decrease in the Hb and haematocrit levels in group D compared to the baseline control in the recovery phase (day 15), showing that aqueous extract of V. doniana facilitated the recovery of the myeloid system 7 days after treatment with CP.

Day 22, however, recorded further decrease in the parameters studied in the CP-treated rats in group B, whereas there were increases in groups C and D, with a negligible increase in group E compared to day 8 of the experiment. Group C had the highest levels in all the studied parameters except for lymphocyte and monocyte, compared with other groups. The increase in the haematological parameters in the treated group D and the continuous decrease in the CP-treated group (B), shows that there is possible protection from the effect of CP. Group D showed an initial non-significant decrease in day 15 compared to day 8, which can be the result of the residual effect of the immunosuppressive effect of CP despite the protection from the extract. This was followed by an increase in day 22 showing that the immunosuppressive effect of CP has been overcome by the myeloid cells, leading to regeneration and subsequent increase in the haematological parameters of the animals, compared to day 15. There was generally greater protection conferred by the aqueous extract compared to the methanolic extract even with the greater concentration of the methanolic extract administered. This could be the result of absorption of the aqueous extract having a water-based solvent which will enhance absorption, especially from the gastrointestinal tract (GIT), being administered orally.

The bone marrow results on day 15 showed significant decrease in groups B, D and E and non-significant increase in group C compared with the baseline control. Comparison between groups D and E showed significant increase in group D compared with group E. Day 22, however recorded greater bone marrow cellularity with significant increases in group C compared to day 15. There was significant decrease ($P<0.05$) in group D and a significant increase ($P<0.05$) in group C compared to the baseline control (group A). Groups B and E also recorded highly significant decrease ($P<0.001$) compared to group A. comparison between groups D and E showed significant ($P<0.05$) increase in bone marrow cellularity in group D compare with group E. Increased cellularity in the group D shows the greater myelo-protective capability of the aqueous extract over the methanolic extract, since myelo-suppression is shown by decreased bone marrow cellularity.

The continuous increase in the haematological parameters in group C (aqueous extract 200mg/kg) can be attributed to the contents of the leaves as shown by the phytochemical studies. These include flavonoid, reducing sugar, glycosides, saponins, proteins and alkaloids, and they might have contributed to the myelo-protective activity as mainly observed in the aqueous extract.

5.Conclusion

The study has shown that the leaf extracts of Vitex doniana (V. doniana) actually possesses myelo-protective properties with the greater protection coming from the aqueous extract. This may be very useful in cancer therapy and will go a long way to prevent the myelo-suppression (anaemia) exhibited by cancer drugs such as cyclophosphamide (CP), which can be lethal as shown by the positive control group, if not properly addressed.

6.References

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