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Protective Effect of the Leaf Extracts of *Combretum racemosum* P. Beauv (Combretaceae) on Cyclophospahamide Induced Pancytopaenia and Liver Injury in Male Rats

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Abstract: Chemotherapy-induced haematopoietic toxicity is a multifactorial challenge that influences the treatment of oncology patients and consequently requires intervention. The protective effect of the crude Methanol leaf Extracts of *Combretum Racemosum* (MECR) was investigated in cyclophospahamide induced pancytopaenia and liver injury in male rats. Acute toxicity studies and preliminary phytochemical screening was performed using standard methods. A total of 25 male albino rats were divided into 5 groups (n = 5). Group E served as normal control group and received normal saline (5 mL kg^{-1}). Group D received cyclophosphamide (3 mg kg^{-1}) intra-peritoneally and served as the cyclophosphamide (positive) control. Group A received 100 mg kg^{-1} of the MECR (p.o). Group B and C received 200 and 400 mg kg^{-1} of MECR, respectively (p.o). Cyclophosphamide was also administered daily to rats in groups A-C, respectively at a dose of 3 mg kg^{-1} intraperitoneally for a period of 14 days. On the 15th day, blood was collected from all the groups by retroorbital puncture for evaluation of blood parameters (complete blood count) and liver marker enzymes. Acute toxicity studies revealed an oral LD_{50} value >5,500 mg kg^{-1} in rats. Preliminary qualitative phytochemical screening showed the presence of alkaloids, saponins, flavonoids, terpenoids, glycosides, resins, carbohydrates and steroids. Administration of cyclophosphamide to rats resulted in myelo-suppression and liver injury. Cyclophosphamide also produced liver injury in the cyclophosphamide control group as evident from the increase in the serum level of hepatospecific markers of liver injury. Concomittant administration of MECR and cyclophosphamide resulted in the mitigation of cyclophosphamide induced bone marrow and liver toxicity. The leaf extract of *Combretum racemosum* possess liver and bone marrow protective properties in cyclophosphamide induced cytotoxicity and justifies the use of this plant in folk medicine in South East Nigeria as a tonic.

Key words: *Combretum racemosum*, cyclophosphamide, anemia, hepatospecific markers, complete blood count, Nigeria

INTRODUCTION

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. Cyclophosphamide is a cytotoxic chemotherapeutic agent well established in the treatment of chronic lymphocytic leukemias, lymphomas and solid tumors as well as an immunomodulatory agent (Aschan *et al*., 1999). The parent compound is inactive *in vitro* and *in vivo* and exerts its biologic activity through metabolites, mainly phosphoramide mustard by hepatic microsomal enzymes (Huang *et al*., 2000). Chemotherapy-induced haematopoietic toxicity is a multifactorial challenge that influences the treatment of oncology patients therefore, it is essential to introduce means to provide myelo-protective effects (Nichols *et al*., 1994). Anaemia is a common finding in cancer. Anaemia is also a common finding particularly in developing countries where pharmacoeconomic considerations and poor financial status make it difficult for a majority of the population to afford quality drugs (multivitamins) and good food.

Moreover, the management of cancer with immunosuppressive drugs (cyclophosphamide) still complicates the picture of anaemia. Therefore, there is

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need to evaluate medicinal plants for possible hemopoietic or myelo-protective properties as these plants have been found to be very effective and with fewer side effects because they are harmonious with the body system.

Herbal medications are used widely in developing countries for the treatment of various diseases and ailments. This is because they are seen as alternatives to orthodox medicines in terms of costs and perceived side effects (Okochi *et al*., 2003). One of the main problems found in the field of medicinal plants is the lack of pharmacological, toxicological and clinical evidence. In many cases, this practice is commonly associated with their traditional use. Only a small fraction among thousands of medicinal plants used in the world have been rigorously tested in controlled studies; therefore the evidence of toxicity risks and verification of eficacy is even lower (De Smet, 2002). The use of herbal medicines by the traditional practitioners for treatment of diseases remains the main stay of health care system and is gaining increasing popularity, especially among the rural populace in the developing countries. Its rising popularity could be attributed to its advantages of being efficacious and also a cheap source of medical care. *Combretum racemosum* has been used in folk medicine for many years as an antidiarrhoeal and antiulcer herb. The leaves are used for the treatment of anaemia, dysentery, cholera, menorrhagia, helminthic infestations, depression and as a tonic. The antiulcer potency of *C. racemosum* has been validated scientifically (Okwuosa *et al*., 2006).

Combretum racemosum is confined to tropical Africa and represented in Nigeria by a climbing shrub. In this study, the protective effect of *Combretum racemosum* in cyclophosphamide induced bone marrow suppression and liver injury was evaluated in rats.

MATERIALS AND METHODS

Chemicals: Absolute Methanol (Fischer Scientific Ltd.), cyclophosphamide (Biochem Pharmaceutical Industries Ltd.), sodium chloride (May and Baker, Nigeria Ltd.). All other reagents are of the analytical reagent grade.

Animals: A total of 25 male albino rats (130-180 g) were obtained from the Animal House of the College of Medicine, University of Nigeria Teaching Hospital, Enugu. These rats were kept in clean guazed cages at the Animal House of the College of Medicine, University of Nigeria, Enugu Campus under standard conditions of temperature (28±3°C) and a 12:12 h Light/Dark cycle. All animals were handled in this study according to international guidelines for handling experimental animals (World Medical Association/American Physiological Society, 2002).

Plant collection and taxonomy: The aerial parts of *Combretum racemosum* were collected from their natural habitat in Agulu town, Anambra state Nigeria. The plant was authenticated by a taxonomist at the Department of Botany, University of Nigeria, Nssuka. A voucher specimen was deposited at the Herbarium for future reference (UNH/47b). The aerial parts were dried under the shade to a constant weight and powdered using an electric a mill grater.

Extraction of plant material

Methanol extraction: The powdered leaves (1000 g) were macerated in 3.5 L of 80% methanol for 48 h with intermittent agitation. At the end of 48 h, the extract was strained through muslin and then filtered through a Whatman No.1 filter paper. The filtrate was evaporated to dryness on a rotary evaporator (Model 349/2 Corning Ltd., England). The residue was stored in the refridgerator (4±2°C) until required. The yield of the methanol extract was 12.3% (w/w). The residue (20 g) was dissolved in normal saline and made up to 100 mL with the solvent (200 mg mL^{-1}) and from this appropriate dilutions were made for the study. This was labeled the Methanol Extract of *Combretum Racemosum* (MECR).

Phytohemical test: Phytochemicals present in the extract were identified by qualitative chemical tests (Trease and Evans, 2002).

Acute toxicity test (Median Lethal Dose, LD_{50}): This was performed on rats and the Lorke (1983) procedure of LD_{50} determination was used.

Experimental design: A total of 25 male albino rats (130-180 g) were divided into5 groups (n = 5). Group E served as normal control group and were given normal saline (5 mL kg^{-1}). Group D received cyclophosphamide (3 mL kg^{-1}) intra-peritoneally and served as the cyclophosphamide (Positive) control. Group A received 100 mL kg^{-1} of the MECR. Group B and C received 200 and 400 mL kg^{-1} of MECR, respectively. Extract administration was by the oral route through an orogastric cannula. Cyclophospphamide was also administered daily to rats in groups A-C, respectively at a dose of 3 mg kg^{-1} intraperitoneally for a period of 14 days. On the 15th day, blood was collected from all the groups by retroorbital puncture under ether anaesthesia in vials containing EDTA (Ethylenediamine Tetraacetic Acid) for evaluation of blood parameters (CBC) and the rest was put into plain tubes for liver marker enzyme determination.

Liver marker enzyme determination

Alanine and aspartate transaminase: Alanine and Aspartate transaminases were analyzed using the end

point techniques of Reitman and Frankel (1957) with protocols described in Randox kit (Randox Laboratries Ltd., United Kingdom).

Alkaline phosphatase: Alkaline phosphatase was estimated using the method of Roy (1970) (Teco Diagnostics, USA).

Complete blood count: Complete blood count was performed with an automated hematology system (Sysmex KX- 2N hematology analyzer, Sysmex Incorporation Kobe Japan).

Statistical analysis: Data were analyzed with the one way analysis of variance followed by Tukeys post-hoc compariconS using the statistical package SPSS Version 15. The results were expressed where appropriate as mean±SE of mean. Mean values of test groups were compared with those of control groups and regarded as significant at $p<0.05$.

RESULTS AND DISCUSSION

Result of acute toxicity studies revealed an oral LD_{50} value >5,500 mg kg^{-1} in rats. Preliminary qualitative phytochemical screening showed the presence of alkaloids, saponins, flavonoids, terpenoids, glycosides, resins, carbohydrates and steroids. Administration of cyclophosphamide to rats for 14 days resulted in myelo-suppression. The cyclophosphamide control had 3.56±1.03×10^{12} L^{-1}, 6.43±1.90 g dL^{-1}, 19.90±0.08%, 0.37±0.05%, 3.15±0.66×10^{9} L^{-1} and 217.25±64.66×10^{9} L^{-1} for Red Blood Cell (RBC), Haemoglobin (Hb), PCV, reticulocytes, Total White Blood Cell (TWBC) and platelets, respectively and these were significantly <8.76±0.46×10^{12} L^{-1}, 14.30±0.78 g dL^{-1}, 44.35±2.15%, 1.38±0.19%, 14.13±1.60×0^{9} L^{-1} and 654.50±132.16 ×10^{9} L^{-1} for RBC, Hb, PCV, Retics, TWBC and platelets, respectively recorded for the normal control group ($p<0.001$) (Table 1-3). Cyclophosphamide also produced liver injury in the cyclophosphamide control group as evident from the results of hepatospecific markers of liver injury which revealed mean values of 91.00±10.73 and 107.50±6.44 IU L^{-1} for ALT and ALP, respectively. These values were significantly >35.0±2.35 and 60.93±3.70 IU L^{-1} for ALTand ALP, respectively observed in the normal control group ($p<0.01$) (Table 1).

Concomittant administration of MECR and cyclophosphamide resulted in the mitigation of bone marrow and liver toxicity of cyclophosphamide. This protective effect was moderately dose dependent. The 200 mg kg^{-1} MECR showed non-significant mean differences in RBC, Hb, PCV and platelets when compared with the normal control ($p>0.05$).

The mean TWBC and reticulocyte counts of the 200 mg kg^{-1} MECR treated group were significantly lower than those of the normal control. However, the mean TWBC of the latter group was significanlty higher than that of the cyclophosphamide control group ($p>0.05$) (Table 3). Moreover, the 200 mg kg^{-1} MECR also protected against cyclophophamide induced liver injury

Table 1: Effect of crude methanol leaf extract of *Combretum racemosum* on hepatospecific markers of liver injury of Cyclophosphamide treated rats

Groups (A-E)	ALT IU L^{-1}	AST IU L^{-1}	ALP IU L^{-1}
100 mg kg^{-1} MECR+CYC	55.50±10.38[d]	29.50±5.55	97.50±19.07
200 mg kg^{-1} MECR+CYC	12.25±1.44[f]	7.50±1.50[b, e]	69.75±10.35
400 mg kg^{-1} MECR+CYC	28.75±7.41[f]	29.25±7.03	66.75±9.94
CYC control	91.00±10.73[b]	41.00±3.58	107.50±6.44[a]
Normal control	35.00±2.35	41.50±1.94	60.93±3.70
F-ratio	15.461	9.598	3.414
p-values	<0.001	<0.001	<0.05

[a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ with respect to the normal control; n = 5; [d]$p<0.05$; [e]$p<0.01$; [f]$p<0.001$ with respect to cyclphosphamide control, CYC = Cyclophosphamide

Table 2: Effect of crude methanol leaf extract of *Combretum racemosum* on some haematological indices of Cyclophosphamide treated rats

Groups (A-E)	RBC (×10^{12} L^{-1})	Hb (g dL^{-1})	PCV (%)	MCHC (g dL^{-1})	MCV (fL)	MCH (pg)	Reticulocytes (%)
100 mg kg^{-1} MECR+CYC	5.01±1.00[a]	8.90±1.76	27.63±5.35	32.10±0.27	55.48±0.58[a]	17.80±0.04	0.49±0.07[c]
200 mg kg^{-1} MECR+CYC	5.92±0.63	10.30±1.18	32.63±3.35	31.45±0.54	55.68±0.19[b]	17.35±0.27	0.56±0.08[b]
400 mg kg^{-1} MECR+CYC	7.21±0.67[d]	12.60±1.15	39.56±3.16	31.80±0.39	55.38±2.05[a]	17.53±0.57	0.69±0.09[b]
CYC control	3.56±1.03[b]	6.43±1.90[a]	19.90±0.08[c]	32.58±0.27	55.38±0.68[a]	17.98±0.08[a]	0.39±0.05[c]
Normal control	8.76±0.46	14.30±0.78	44.35±2.15	32.23±0.24	48.65±1.65	16.38±0.28	1.38±0.19
F-ratio	6.418	4.741	5.090	1.264	6.025	3.996	13.514
p-values	<0.01	<0.05	<0.01	>0.05	<0.01	<0.05	<0.001

Table 3: Effect of Cyclophosphamide treatment on TWBC, WBC differentials and platelets

Groups (A-E)	TWBC (×10^{9} L^{-1})	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Platelets (×10^{9} L^{-1})
100 mg kg^{-1} MECR+CYC	6.28±0.80[c]	40.75±0.48[d]	56.00±0.82[d]	2.00±0.00	1.25±0.48	428.56±53.95
200 mg kg^{-1} MECR+CYC	8.03±0.45[b, d]	14.25±2.17[b, e]	79.50±3.30[a, d]	2.00±0.00	1.75±0.25	550.75±43.42
400 mg kg^{-1} MECR+CYC	11.33±0.99[f]	31.50±4.48	67.25±4.03	0.75±0.25	0.50±0.29	673.50±95.81[d]
CYC control	3.15±0.66[c]	30.25±1.11	67.50±0.65	1.25±0.63[a]	1.00±0.58	217.25±64.66[a]
Normal control	14.63±1.60	30.50±1.50	66.00±1.58	2.50±0.29	1.25±0.25	654.50±132.16
F-ratio	19.026	15.983	11.319	4.442	1.338	4.965
p-values	<0.001	<0.001	<0.001	<0.05	>0.05	<0.01

[a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ with respect to the normal control; n = 5; [d]$p<0.05$; [e]$p<0.01$; [f]$p<0.001$ with respect to cyclphosphamide control, CYC = Cyclophosphamide

by virtue of the non-significant differences in the mean levels of hepatospecific markers of liver damage ($p > 0.05$) (Table 1). Furthermore, the 400 mg kg^{-1} MECR abolished the cytotoxic effects of cyclophosphamide as evident from the non-significant mean differences in RBC, Hb, PCV, TWBC and platelet counts as well as the ALT and ALP levels when this treatment group was compared with the normal control group ($p > 0.05$) (Table 1-3). The 400 mg kg^{-1} MECR also showed significantly higher mean RBC count, TWBC and Platelet counts when compared with the cyclophosphamide control ($p < 0.001$) (Table 2 and 3).

Combretum racemosum has a powerful folkloric reputation for the treatment of ulcers, diarrhea, sexually transmitted diseases, anaemia and debilitation, cholera and menorrhagia. The anti-ulcer effects of *C. racemosum* in various experimental ulcer models have been evaluated in rats (Okwuosa *et al*., 2006). The present study was designed to investigate the myeloprotective and hepatoprotective effects of the leaf extract of *Combretum racemosum* in cyclophosphamide-induced anaemia and liver injury model. Free radical reactions have been implicated in the pathology of many human diseases/disease conditions like arteriosclerosis, ischemic heart diseases, liver disease, diabetes mellitus, inflammation, neuro-degenerative diseases and immuno suppression. The reliable criteria for judging the haematopoietic activity of a plant is to observe haematological indices in anaemic animal model. Cyclophosphamide belongs to the nitrogen mustard subclass of alkylating agents under cytotoxic drugs. The main use of Cyclophosphamide (CP) is together with other chemotherapy agents in the treatment of lymphomas, some forms of leukemia and some solid tumors. Cyclophosphamide exhibits greatest cytotoxicity against cells actively replicating their DNA as unpairing of DNA strands at this stage makes the nucleotide residues more susceptible to alkylation. Several studies indicate that CP has a prooxidant character and generation of oxidative stress after CP administration leads to decrease in the activities of antioxidant enzymes and increase in lipid peroxidation in liver, lung and serum of mice and rats (Patel and Block, 1985; Venkatesan and Chandrakasan, 1995; Kaya *et al*., 1999; Lear *et al*., 1992; Mathew and Kuttan, 1997; Premkumar *et al*., 2001).

Drugs with multiple protective mechanisms including antioxidant activity, may be one way of minimizing tissue injury (Halliwell, 1991). A number of plants and plant isolates have been reported to protect free radical-induced damage in various experimental models (Scartezzini and Speroni, 2000). Phytochemical screening of C racemosum showed the presence of alkaloids, saponins, flavonoids, terpenoids, glycosides, resins, carbohydrates and steroids. A number of investigators have indicated that certain flavonoids, steroids and triterpenoids have protective effect on the liver and kidney due to their anti-oxidant properties (Defeudis *et al*., 2003; Takeoka and Dao, 2003). An important factor in the hepatoprotective activity of any drug is the ability of its constituents to inhibit the aromatase activity of cytochrome P-450, thereby favoring liver regeneration. On that basis, it is suggested that flavonoids in *Combretum racemosum* could be a factor contributing to its hepatoprotective ability through inhibition of cytochrome P-450 aromatase. ALT, AST and ALP are marker enzymes for assessment of liver function. Elevated levels of serum enzymes are indicative of cellular damages and loss of functional integrity of cell membrane in liver (Kumar *et al*., 2005). Damage to liver cells causes release of cellular enzymes into serum.

Hepatic activation of cyclophosphamide leading to the formation of toxic metabolites caused damage to the liver tissues as shown by increased ALT and ALP levels. Treatment with *Combretum racemosum* resulted in significant reduction in the level of these transaminase activities.

The ability of the extracts of *Combretum racemosum* to presrve the hepatic chords of the treated group when administered alone could be due to a membrane stabilizing effect. An important factor in the hepatoprotective activity of any drug is the ability of its constituents to inhibit the aromatase activity of cytochrome P-450, thereby favoring liver regeneration. On that basis, it is suggested that flavonoids in *Combretum racemosum* could be a factor contributing to its hepatoprotective ability through inhibition of cytochrome P-450 aromatase. Chemotherapy-induced anemia is one of the most common side effects experienced by cancer patients, occurring in approximately 70-90% of those undergoing treatment for the disease (Groopman and Itri, 1999). This is because chemotherapeutic drugs kill rapidly dividing cells in the body including cancer cells and normal cells which include red blood cells at the same time suppress bone marrow ability to produce new ones, resulting in decrease of blood Hb level.

It was observed that gavaging the animals with *Combretum racemosum* enhanced the Hb level, platelet count, TWBC, PCV and RBC count which was depleted by cyclophosphamide treatment in the cyclophosphamide control group. The myeloprotective effect of this plant extract may be due to free radical scavenging activity or direct interference with the formation of the active metabolite of cyclophosphamide (Phosphoramide) through inhibition of cytochrome P-450 enzyme system.

CONCLUSION

The leaf extracts of *Combretum racemosum* possess protective effect against cyclophosphamide induced liver and bone marrow toxicity and justifies the use of this extract in folklore as a tonic. This finding could offer the required intervention to ameliorate the effect of cyclophosphamide drug therapy on the myeloid cells and liver if addressed properly.

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