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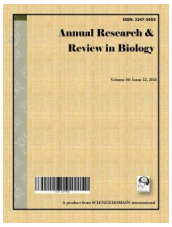

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African Polyherbal Formulation Possesses Chemopreventive and Chemotherapeutic Effects on Benzene- Induced Leukemia in Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AEO and FA designed and coordinated the study. Authors BEO and AFA performed the statistical analysis. Authors AEO, FA, AOA and LOI wrote the protocol while authors BEO and AEO wrote the first draft of the manuscript. Authors ARA, AOA and LOI managed the analyses of the study. Authors AFA, BEO and ARA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Chemotherapy and radiotherapy are effective cancer treatment options but they are accompanied by serious side effects and complications such as systemic cytotoxicity, chemo- or radio-resistance. Therefore, more effective therapies are sorely needed. This study investigated the chemopreventive and chemotherapeutic effects of a polyherbal formulation on benzene induced

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leukemia in Wistar rats. The polyherbal formulation is composed of fruits such as Orange, Lime, Lemon, Pineapple, Grape and Vegetables like; Pumpkin leave, Garden egg suspended in honey medium.

Methods: Leukemia was induced by injecting 0.2 ml of benzene solution intravenously through the tail of young rats at 48 h intervals for four times. Leukemia developed in 92% of rats some weeks after the last benzene injection and following up weekly observation for leukemia development in appropriate rat groups. Leukemia burden was assessed using indicator parameters such as total leukocyte, red blood cell count, hematocrit, and hemoglobin concentration. Exactly 0.48 ml of the polyherbal formulation was administered orally by gavage using oral cannular once daily post leukemia induction for four weeks.

Results: Leukemia induction reflected in significant reduction in hematocrit, hemoglobin concentration, red cell count and a marked and a significant increase in leukocyte count over the control ($p < 0.05$). There is significant difference between the chemopreventive group and chemotherapeutic (leukemia positive group treated with poly-herbal formulation) and the leukemia positive control group untreated.

Conclusion: This study reveals profound activity of the poly-herbal formulation at preventing and reversing experimentally developed leukemia in the rat groups, hence the ability of the polyherbal formulation in alleviating the cancer symptoms.

Keywords: Benzene; chemoprevention; chemotherapy; polyherbal formulation; leukaemia.

1. INTRODUCTION

Cancer has emerged as a major global public health problem [1]. Its incidence and mortality rates continue to rise. A report by the World Health Organization (WHO) in 2008 shows that an estimated 12.7 million people were diagnosed with cancer and 7.6 million people died from cancer worldwide [2]. The WHO predicts that by 2030, an estimated 21.4 million new cases of cancer and 13.2 million cancer deaths will occur annually around the world [2].

Leukemia is a cancer that starts in early blood-forming cells found in the bone marrow, the soft inner part of certain bones. Most often, leukemia which may be acute or chronic is a cancer of the white blood cells, but some leukemia start in other blood cell types [3]. Any of the blood-forming cells from the bone marrow can turn into a leukemia cell [3]. It has been studied that exposure to some substances can cause cancer and these substances are termed carcinogen [4]. Example of a known human carcinogen is benzene [5]. Benzene is a colourless, flammable liquid with a sweet odour [6]. It is known to cause cancer especially leukemia based on evidence from studies in both humans and laboratory animals by causing chromosomal changes in bone marrow cells and such changes are commonly found in human leukemic cells [6].

Chemotherapy and radiotherapy are effective for cancer treatment, however, they also have serious side effects and complications which includes fatigue, pain, diarrhea, nausea, vomiting, and hair loss. Since some cancers are relatively chemo- or radio-resistant and highly refractory to cytotoxic chemotherapy or radiotherapy, systemic cytotoxic chemotherapy and radiotherapy are minimally effective at improving patient survival [7]. Therefore, more effective therapies or combination therapies with minimal side effects are needed to be developed to treat cancer swiftly.

In lieu of the adverse reactions and toxicity which may result from cancer chemotheraphy, researches regarding the use of herbs as cancer therapeutic agents are being developed although still at its infancy, hence no confident statement can be made. Nevertheless, complementary and alternative medicine use is common amongst cancer patients. In many surveys, herbal medicines are amongst the most commonly used group of treatments. Herbal remedies are believed by the general public to be safe, affordable, cause less side-effects and less likely to cause dependency [8].

Among the method used in cancer prevention, which is one of the problem of cancer researches is chemoprevention [9] and example of such drugs used in prevention of cancer by natural product experts is polyherbal.

Polyherbal formulation contains many phyto-constituents and naturally occurring substance,

but sometimes the individual phyto-constituent may not be enough to achieve the desired chemopreventive effect [10]. Polyherbal formulation (PHF) is therefore usually prepared to enhance the chemopreventive effectiveness and improved its bioavailability [10]. Besides this, synergism and reduction in undesirable side-effects is a key benefit for formulation compared with modern medicines [11].

Some research strongly supports high intake of fruits, vegetables and whole grains which are rich in polyphenols and also constituents of dietary supplement, as a link to lower risks of many chronic and degenerative diseases [12]. Some of these leukemia alternatives include herbal and natural foods that are rich in antioxidants and vitamins. Antioxidants reduce and control the total amount of reactive oxygen, reactive nitrogen, and free radicals in the body, which may have a positive effect on leukemia patients [13]. Consumption of antioxidants has been implicated in the prevention of several disorders, including cancer [14,15].

This study administered polyherbal formulations consisted of extracts of fruits such as; orange (*Citrus sinensis*), lime (*Citrus hystrix*), lemon (*Citrus limon*), pineapple (*Ananas comosus*), grape (*Vitsi*) and vegetables like pumpkin leaves, garden egg leaves all mixed in honey.

In order to serve as alternative to the conventional chemotherapeutic resolution this study aimed to evaluate the chemopreventive and chemotherapeutic effects of the poly herbal formulation in benzene induced leukemia bearing Wistar rats.

2. MATERIALS AND METHODS

2.1 Experimental Animal

This is an experimental research in which procedures involving the use of animals and their care were conducted in compliance with the Guidelines for Care and Use of Laboratory Animals in Biomedical Research as promulgated by the Canadian Council on Animal Care [16]. Ethical approval was obtained from the ethical committee on health and research of Ladoke Akintola University of Technology (LAUTECH), Osogbo, Nigeria. A total of seventy-two Wistar rats weighing between 70 g and 80 g were purchased from the animal house of LAUTECH, Nigeria. The rats were randomly arranged in six compartment wooden cage according to their groups in 12 per group of 2 replicate of 6 each. They were allowed to acclimatize for seven days before the commencement of the experiment. The animals' room temperature was 28±2 degree Celsius with 12 hour light/dark cycle.

2.2 Administration of Benzene Solution to Induce Leukaemia

Benzene solution, a product of Sigma-Aldrich with Cat No 270709 and >99.9% was diluted in water and propanol for injection at a concentration of 1 ml of the benzene to 4ml of propanol and 5mL of water for injection. Exactly 0.2 ml was administered intravenously through the tail 48 hourly for 4 times (Table 1). The benzene exposed rats were observed for leukaemia development for 2-4 months on weekly basis during which period those that tested positive for leukaemia were separated for treatment. However, the chemopreventive groups were administered with the polyherbal formulation for 4 weeks prior to benzene exposure.

2.3 Polyherbal Formulation

This was developed and provided by a traditional healer in Osun state, Nigeria, West Africa. It contains different extract of fruits such as; orange (*Citrus sinensis*), lime (*Citrus hystrix*), lemon (*Citrus limon*), pineapple (*Ananas comosus*), grape (*Vitsi vinifera and seeds*) and vegetables such as; pumpkin leaves (*Telfairia occidentalis)*, garden egg leaves all mixed in honey. Exactly 1litre of the each of the herbal juice was extracted in raw and natural form which altogether was made up to 7litres and was thereafter suspended into 3 litres of pure honey. Phytoanalysis shows the formulation tested positive to the Flavanoids, Carotenoids, Coumarins, Limonoids and Phenollic acids.

2.4 Administration of Poly Herbal

Exactly 0.48 ml of the polyherbal formulation was administered orally once daily by gavage with the aid of cannular to the groups base on the experimental design (Table 1) for 3 weeks and 8weeks in the adverse reaction/toxicity group. The lethal dose of the polyherbal formulation was assessed and determined using fifty rats to be 0.48 mg/kg.

Table 1. Experimental protocol on the chemopreventive and chemotherapeutic activity of the polyherbal formulation

Treatment	Inference
Leukaemia induction using benzene	Positive control (PC)
Healthy rats on commercial feed and water	Negative control (NC)
Polyherbal formulation administration prior to benzene induction.	Chemopreventive activity (CP)
Leukaemic rats treated with polyherbal formulations for 4 weeks.	Chemotherapeutic activity (CT)
Polyherbal formulation for 8 weeks	Adverse Reaction/Toxicity (AR)

2.5 Blood Sample Collection

The animals were handled in which 2-3 mL of blood was collected into EDTA (Ethylene Diamine Tetra Acetic acid) anticoagulant bottle [16]. The hematocrit, total white blood cell (WBC) counts, red blood cell (RBC) counts, hemoglobin (Hb), platelets counts, MCH, MCV, and MCHC were estimated using the Outrao SH 800 plus analyzer [17]. The differential White Blood Cell Count was determined with Leishman staining technique, the counts were expressed as a percentage representative of each type [18]. Rat hematologic reference ranges: RBC, 6.76-9.75 x 10^{12}/L; PCV, 37.6-50.6%; WBC, 6.6-12.6 x 10^{9}/L; Hb, 11.5-16.1 g/dL; PLT, 150-460 x 10^{3}/mL; LYM, 4.78-9.12 10^{9}/L [19].

2.6 Animal Sacrifice

Animal suffering and distress were minimized as much as possible throughout the study. The animals that met specific clinical criteria for euthanasia were sacrificed ethically by cervical dislocation after administration of anesthetic (euthanasia) at 12 hours post- administration of the last treatment.

2.7 Statistical Analysis

The SPSS software package was used for statistical analysis. Values obtained from the study were expressed as mean ± standard deviation and compared using analysis of variance (ANOVA) and the significance was measured at $p < 0.05$. The variables have normal distribution.

3. RESULTS

The results of hematological parameters in the various experimental groups are shown in the Tables and Figures. The mean ± standard deviations of the hematological parameters in various experimental rat groups are presented in Table 2. The mean HCT, RBC, Hb and PLT in group B (Negative control) was observed to be normal, while WBC in group A (leukemia positive control) was observed to be significantly raised when compared to other groups which is indicative of leukemia development in the rat group.

Table 3 shows marked significant difference ($p<0.05$) in the estimated parameters when leukemia induced group A was compared with healthy group B, chemopreventive group C and toxicity group D. Also, significant difference was not observed between these groups B, C, and E when compared with each other.

In Table 4, there was significant difference ($p<0.05$) in the estimated parameters of the treated group, CT (Chemotherapeutic), compared with that of leukemia positive control (PC). These indicate that the leukemia induction in the treatment group was reversed after treatment with the Poly herbal formulation.

Fig. 1 demonstrates the distribution of some hematological parameters in the respective groups of experimental rats .The healthy rats treated with Poly herbal formulation only (group E) showed no significant alterations in estimated parameters when compared with the healthy rats in group B that were fed with commercial diet as illustrated in Fig. 2.

4. DISCUSSION

The earliest evidence of man's use of plant for healing dates back to the Neanderthal period [20]. Herbal medicine is now being used by an increasing number of patients who typically do not report to their clinicians [21] as natural plant products are perceived to be healthier than synthetic medicine [22]. Herbal medicine has benefited from the objective analysis of the medical science, while whimsical and emotional claims for herbal cures have been ignored, effective herbal treatments and plant medicine have been acknowledged.

Table 2. Haematological parameters of the various experimental rat groups

Groups	WBC (10^9/L)	RBC (10^{12}/L)	PLT (10^9/L)	HCT (%)	HGB (g/dl)	MCV (fl)	MCH (pg)	MCHC (g/dl)
PC	11.08±0.31	3.52±0.38	390.2±146.1	26.88±2.01	6.68±0.96	76.46±4.48	24.67±1.09	24.92±2.03
NC	6.84±0.30	6.30±0.39	555.1±24.2	43.74±1.72	13.62±0.69	69.44±2.40	20.62±1.56	31.22±1.64
CP	6.71±0.24	5.63±0.38	470.3±77.0	40.56±1.36	11.68±1.12	71.62±2.69	19.40±0.95	28.12±2.03
CT	7.45±0.65	5.10±1.02	425.1±60.8	37.48±8.05	11.80±2.96	73.99±3.12	23.36±0.93	31.43±2.11
AR	6.68±0.41	5.75±0.60	431.4±50.1	42.35±4.37	11.92±2.35	73.10±1.20	19.58±2.01	27.98±2.42

Values are Mean (X) ± S.D.

Legend: HCT, Haematocrit; WBC - Total White Blood Cell Count; RBC - Red Blood Cells; Hb – Haemoglobin; MCV - Mean Cell Volume; MCH - Mean Cell Haemoglobin; MCHC - Mean Cell Haemoglobin Concentration; PLT- Platelet; X – Mean, S.D. – Standard deviation

Table 3. Comparison between groups Negative controls, Chemotherapeutic and Adverse reaction groups with the Leukaemia positive group

Groups	WBC (10^9/L)	RBC (10^{12}/L)	PLT (10^9/L	HCT (%)	HGB (g/dl)	MCV (fl)	MCH (pg)	MCHC (g/dl)
PC	11.08±0.31	3.52±0.38	390.2±146.1	26.88±2.01	6.68±0.96	76.46±4.48	24.67±1.09	24.92±2.03
NC	6.84±0.30	6.30±0.39	555±24.2	43.74±1.72	13.62±0.69	69.44±2.40	20.62±1.56	31.22±1.64
CP	6.71±0.24	5.63±0.38	470±77.0	40.56±1.36	11.68±1.12	71.62±2.69	19.40±0.95	28.12±2.03
AR	6.68±0.41	5.75±0.60	431.4±50.1	42.35±4.37	11.92±2.3	73.10±1.20	19.58±2.01	27.98±2.42
p-value	0.002	0.001	0.001	0.001	0.001	0.05	0.002	0.002

Significant difference at $p<0.05$; Values are Mean (X) ± S.D.

Legend: HCT - Haematocrit, WBC - White Blood Cell, RBC - Red Blood Cell, HGB -Haemoglobin, PLT - Platelet, X - Mean, S.D. - Standard deviation

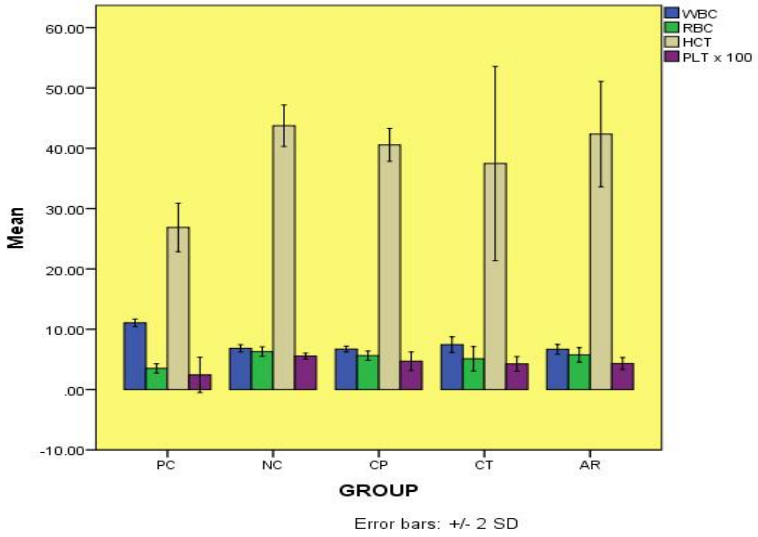

Fig. 1. Distribution of some haematological parameters in the different groups of the experimental animals

Legend: HCT- Haematocrit (%); WBC- white blood cell (10^9/L); PLT- platelet (10^9/L); RBC-red blood cell count (10^{12}/L); SD - standard deviation.

PC– leukaemia positive control; NC – Leukaemia negative control; CP –administration of polyherbal formulation suspension prior to leukemia induction (chemopreventive activity); CT– Leukaemia rats treated with polyherbal formulation (Chemotherapeutic activity);AR- Adverse reaction group

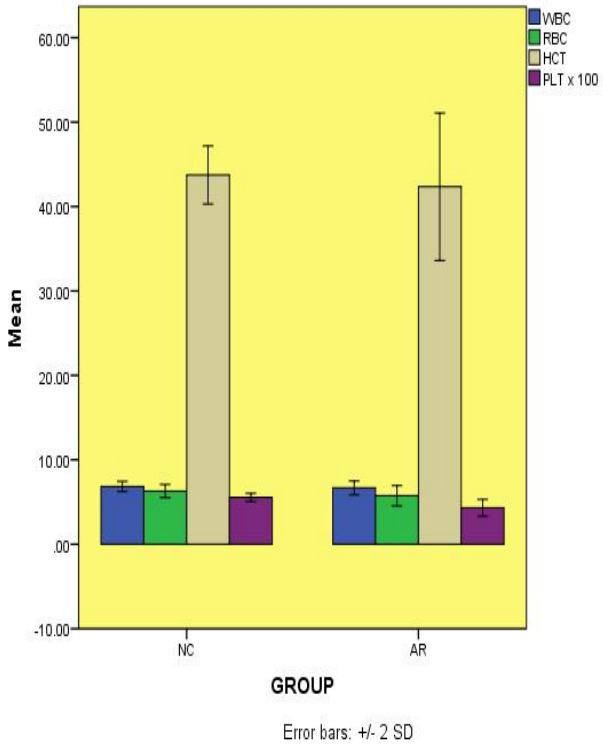

Fig. 2. Effect of the polyherbal formulations on experimental animals

NC – Negative Control; AR- Adverse reaction/Toxicity group on poly herbal formulations only.

Legend: HCT- Haematocrit (%); WBC- white blood cell (10^9/L); PLT- platelet (10^9/L); RBC-red blood cell count (10^{12}/L); SD - standard deviation

Table 4. Comparison between the Leukaemia induced group, and chemopreventive group

Parameters	Group PC Mean±SD	Group CT Mean±SD
WBC (10^9/L)	11.08±0.31	7.45±0.65*
RBC (10^{12}/L)	3.52±0.38	5.10±1.02*
PLT (10^9/L)	390.2±146.10	425.1±60.8*
HCT (%)	26.88±2.01	37.48±8.05*
HGB (g/dl)	6.68±0.96	11.80±2.96*

**Significant difference at p<0.05*
Legend: HTC, Hematocrit; WBC, White blood cell; RBC, Red blood cell count; HGB, Hemoglobin; PLT, Platelet; SD, Standard Deviation

Some nutritional supplements used as herbal medicines protect the body from cancer by enhancing detoxification functions of the body. Certain biological response modifiers derived from herbs are known to inhibit growth of cancer by modulating the activity of specific hormones and enzymes. Some herbs reduce toxic side effects of chemotherapy and radiotherapy [23].

The chemotherapeutic and chemopreventive activities of a poly herbal formulation on benzene induced leukemia bearing Wistar rats was evaluated in this study by the analysis of its effect on some hematological parameters. Based on results obtained from this study (Tables 2 and 3), anemia of chronic disease was observed in rats in the leukemia positive control group (PC), where leukemia was induced due to exposure to benzene solution, which is a potent carcinogen and this is shown by a significant reduction ($p < 0.05$) in hematocrit, hemoglobin and red cell counts. Leukocytosis, which is associated with leukemia were observed in rats in PC group when compared with NC (negative control) group. This established the fact that leukemia was successfully induced. This finding is supported by the report of a previous work conducted by Akanni et al. [24] on anti-leukemic and immunomodulatory effects of fungal metabolites of *Pleurotus pulmonarius* and *Pleurotus ostreatus* on benzene induced leukemia in wistar rats, in which anemia of chronic disease and leukocytosis was also observed in rats induced with leukemia caused by exposure to benzene solution.

Oranges contain diverse phytochemicals, including carotenoids (beta-carotene, lutein and beta-cryptoxanthin), flavonoids e.g. naringenin [25] and numerous volatile organic compounds producing orange aroma, including aldehydes, esters, terpenes, alcohols, and ketones [26] and these substances have been studied to possess antioxidant properties against diseases and bacterial infections [27,28,29].

Lemon is cultivated mainly for its alkaloids, which are having anticancer activities [30] and its citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic and antiviral activities [31,32]. In addition, Grape antioxidants have been shown to induce cell cycle arrest and apoptosis in cancer cells [33] as well as prevent carcinogenesis and cancer progression in rodent models [34,35].

Furthermore, studies have shown that the bromelin content of pine apple has the capacity to modify key pathways that support malignancy as well as on the modulation of immune, inflammatory, and hemostatic systems [36] while the fiber, ascorbic acid, phenols, anthocyanin, glycoalkaloids and α-chaconine content of garden egg [37] has been reported to possess significant analgesic, anti-inflammatory, anti-asthmatic, anti-glaucoma, hypoglycemic, hypolipidemic, and weight reduction effects animals and humans [38,39,40].

Several studies have also demonstrated the chemotherapeutic effect of honey through its apoptotic, antimetastatic effects as well as via inhibition of angiogenesis [41,42,43].

Additionally, results obtained in Table 3 and Fig. 1 depict that anemia of chronic disease, marked increase in lymphocyte count and leucocytosis which are usually associated with leukemia was observed to have been prevented in rats treated with the polyherbal formulation suspension before exposure to leukemia inducing agent as shown by the significant differences at $p>0.05$ in the hematocrit, hemoglobin, red cell count, lymphocyte and total white blood cell count of rats in chemopreventive (CP) group when compared with that of positive control. This shows that the polyherbal formulation possess chemopreventive activity against leukemia.

Similarly, leukocytosis, anemia of chronic disease and reduced platelets was observed being reversed in leukemic rats treated with the polyherbal formulation as observed in the total white blood cell counts, red cell count, haemoglobin, haematocrit and platelet count of rats in chemotherapeutic (CT) group when compared with that of PC group ($p<0.05$) as specified in Table 4 and Fig. 1. This chemotherapeutic effect of the polyherbal

formulations shows that the polyherbal formulations used in this study is highly effective in the treatment of leukemia and this is corroborated by reports that the chemopreventive properties of fruits and vegetables arise from their high content of phytochemicals such as phenolic compounds [44,45] that target several key events involved in the development of cancer [46,47]. Potential mechanisms for cancer prevention of phytochemicals include prevention of DNA adduct formation [48], enhanced carcinogen elimination [49], inhibition of inflammatory processes [50], interference with tumor angiogenesis [51,52], as well as through a direct cytotoxic effect on tumor cells [53].

A similar study by Akanni et al. [54] also demonstrated the effect of *Moringa oleifera* extract on benzene induced leukaemia as chemopreventive and antileukaemic by ameliorating the induced leukaemic condition in rat models owing to the bioactive constituents of the extract.

Some studies on the effect of some herbs and spice on cancer have also substantiated this study whereby Gingerols from ginger (*Zingiber officinale*) inhibits proliferation and induce apoptotic cell death of the malignant cells in cancers of the ovary, cervix, colon, rectum, liver, urinary bladder, oral cavity, neuroblastoma and leukaemia. It is reported to be effective even in chemotherapy resistant ovarian cancer [55,56]. Ginger was also observed to possess antioxidant, antimutagenic and anti- inflammatory properties and reduces side effects of chemotherapy & radiotherapy [57] while Ginseng acts as potent chemo-preventive agent and effectively regress stomach, liver, pancreas, and colon cancer by inhibiting the inflammation-to-cancer sequence [58].

Furthermore, when rats that were administered the poly herbal formulation only in group E were compared with rats fed with commercial feed and water only in group B , there was no significant difference ($p>0.05$) in hematocrit, hemoglobin, total white cell counts and platelet counts (Table 3 and Fig. 2). This finding indicates that the poly herbal formulation has no adverse or toxic effects on healthy hemopoietic cells thereby sustaining the fact that they are needed for maintaining a healthy blood component and haemopoietic environment. This can be supported by a work done by Ajayi et al. [59], where aqueous extract of the pumpkin leaves (*T. occidentalis)* increased the hematocrit value, the reticulocyte count of rabbits [59] as well as increasing the hemoglobin (Hb) levels in $FeSO_4$ hemoglobin depleted anemic mice [60].

5. CONCLUSION

It can be concluded based on the results obtained from this study that the poly herbal formulation investigated possesses chemopreventive and chemotherapeutic properties thereby alleviating leukemia in benzene induced leukemia bearing Wistar rats and that it is well tolerated by the rats as it did not show any adverse or toxic effect on the rats.

6. RECOMMENDATION

Since it has been established that the poly herbal formulation used in this study is made from naturally occurring edible fruit and vegetables, that possesses anti-leukemic potential, the full phyto - constituents and pharmacologic active components of the formulation needs to be evaluated and then subsequent clinical trials to evaluate its effects on humans.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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