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Ethno Botanical Survey, Anti-leukemia and Anticlastogenic Potential of Medicinal Plants used for Treatment of Leukemia in Oyo State Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author AA designed the study. Authors AA, OA, OAO and FAA conducted and managed the conduct of the study. Author OA conducted the statistical analysis and wrote the protocol. Authors OA and FAA performed the literature search. Authors AA and OAO read and approved the manuscripts.

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ABSTRACT

Ethnopharmacological Relevance: Leukemia, a cancer of the blood and bone marrow is responsible for about 222.000 deaths annually. The side effects of chemotherapy in leukemia treatment have necessitated the search for natural products especially medicinal plants as alternative therapy.
Aim: This study surveyed common plants used for treating leukemia in Oyo state, Nigeria and assessed the anti-leukemic and anti-clastogenic activities of fractions of *Nymphaea lotus.*
Methods: Semi-structured questionnaire (1000) was used to collect the ethnobotanical data among the traditional healers. Leukemia was induced in albino mice with 400 mg/kg body weight of

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benzene intraperitoneally. Aqueous extracts (120mg/kg/bw), fractions and 5-florouracil (25mg) was administered to mice of different groups. The anti-leukemic, anti-clastogenic, antioxidant, hepatoprotective activities and hematological parameters were determined. *N. lotus* was subjected to gas-chromatography mass spectroscopy analysis.

Results: The ethnobotanical survey recorded 89 plant species with their local names and parts used in the traditional therapeutic preparations. Seven plants (*Pistiastratiotes* (2.5%), *Nymphaea lotus* (1.4%), *Piper guineense* (1.7%), *Securinega virosa* (2.5%), *Calotropis procera* (3.4%), *Morinda lucida* (2.5%) and *Xylopia aethiopica* (4.5%) with the highest frequency of citation were selected for anti-leukemic and anti-clastogenic screening. The aqueous extract of *N. lotus* and *M. lucida* displayed anti-leukemic potential. *M. lucida* and *X. aethiopica* improved the hematological parameters. Ethyl acetate fraction of *N. lotus* significantly ($p<0.05$) reduced the number of micronucleated polychromatic erythrocyte in the bone marrow and showed anti-leukemic activity. Fractions of *N. lotus* restored the hematological parameters and exhibited significant ($p<0.05$) antioxidant activity. Histological observation revealed improvement in the liver general cyto-architecture of mice treated with ethyl acetate and butanol fractions of *N. lotus*. Some known compounds were identified in ethyl acetate fraction of *N. lotus.*

Conclusion: Most of the species tested had some anti-leukemic effect in mice, which to some extent supports their traditional inclusion in herbal preparations for treatment of leukemia. The study also identified potential anti-leukemic compounds in *N. lotus* extract. The study also identified potential anti-leukemic compounds in *N. lotus* extract.

Keywords: Ethnobotanical survey; anti-leukemia; medicinal plants; compounds; GC-MS; anti-clastogenic.

1. INTRODUCTION

Cancer is one of the causes of deaths worldwide, responsible for 7.6 million deaths in 2008. The World Health Organization (WHO) has predicted that deaths from cancer worldwide are expected to continue rising, with an estimated 21.7 million casualties in 2030 [1]. In Nigeria, over 100.000 people are diagnosed with cancer yearly, and about 80,000 die from this disorder, averaging 240 Nigerians every day or 10 Nigerians each hour [2].

Leukemia is a type of cancer that affects people in most parts of the world and was responsible for 281,500 deaths in 2010 worldwide [3,4]. Different types of leukemia have been linked to environmental factors and lifestyles [5]. Some of the causative factors include smoking, ionizing radiation, some chemicals (such as benzene), prior chemotherapy and Down syndrome [6]. Treatments of leukemia are mainly with chemotherapy, medical radiation therapy, hormone treatments and bone marrow transplantation [7]. Despite tremendous progress in cancer therapy research, there are long-term side effects of chemotherapy and other treatments in leukemia [8,9,10]. Medicinal plants with the ancient history of human use have been considered one of the important and reliable sources to discover promising therapeutic agents in disease conditions, including cancer chemo-preventive drugs [11]. Studies revealed that the plant extracts may be complementary to chemotherapies to treat cancer (Fennel *et al*., 2004).

Furthermore, the World Health Organization estimated that herbal medicine is still accepted by 75-80% of the world population, mainly in the developing countries for treatment of diseases including leukemia, with lesser side effects [12,13]. In Nigeria, medicinal plants have been used since time immemorial in the prevention and treatment of diseases including leukemia[14]. The traditional uses of medicinal plants in the treatment of cancer have been passed down through generations majorly by oral tradition [15]. Consequently, ethnopharmacological studies of medicinal plants used in the treatment of leukemia might be a good lead to discovery of anti-leukemic agents [14], [16]. Therefore, this study evaluated and ascertained the traditional use of some acclaimed anti-leukemic plants in Oyo State, Nigeria. In addition, this study identified the phytochemical constituents involved in the anti-leukemic activity of the most active plants.

2. MATERIALS AND METHODS

2.1 Ethnobotanical Survey

The ethnobotanical survey was carried out in Oyo state, Nigeria. Information on medicinal

plants useful in the treatment of leukemia was obtained from five hundred (500) respondents through interviews conducted within selected towns (Ogbomoso, Ibadan, Osogbo, Igboho, Oyo town and Igbeti areas) in Oyo state with herb sellers, herbalists, farmers, and the elderly ones. Semi structured questionnaire as described by Ashidi *et al*. [17] was used with slight modification. The data collected included the plant name, part used, route of administration and the mode of preparation. A qualified taxonomist did the identification of plants at the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso.

2.2 Plant Collection

Seven of the most cited plants were selected for bioactivity study. The leaves of *Calotropis procera* (Ait.) Ait*, Securinega virosa* Roxb. exWilld*, Morinda lucida* Benth*, Xylopia aethiopica* Dunal*, Nymphaea lotus* L.*, Piper guineese* Schum &Thonn and *Pistia stratiotes* Linn were collected within Ogbomosho. The sample of each plant was deposited in the herbarium of Biology Department of Ladoke Akintola University of Technology, Ogbomosho, Nigeria with voucher numbers LH0507, LHO509, LHO505, LH0409, LHO 508, LHO504 and LHO 512 for each plant respectively

2.3 Preparation of Extracts

The fresh leave of the plants were air-dried at room temperature for four weeks. The dried pieces were ground into powder and 50 g each of the plant material was macerated in 1000 mL distilled water for 72 hours with constant stirring. Mixture was kept in the refrigerator. After 72 hours, the mixture was filtered out using Whatman No 1 filter paper. The aqueous filtrate was thereafter freeze-dried. Each of the crude extracts was stored at 4^0C until use.

2.4 Fractionation of Crude Extract

Methanol leave extract of *N. lotus* (400 g) was prepared by maceration for 72 hours, filtered and concentrated using rotary evaporator. The crude methanol extract was dissolved in 20 mL methanol and suspended in 150 mL distilled water. The mixture was thereafter successively partitioned with n-hexane, chloroform, ethyl acetate and n-butanol (200 mL, each) according to the method of Ahmed *et al*. [18]. *N .lotus* fractions were concentrated at 40^0C using rotary evaporator and stored separately at 4^OC until used.

2.5 Animals

One hundred (100) male Swiss albino mice obtained from Animal House of the Basic Medical Sciences, Ladoke Akintola University of Technology Ogbomoso were used. The animals weighed between 20- 25 g at the start of the experiment. The cages were cleaned and maintained in hygienic environment. Animals were maintained in normal 12-hr light/dark cycle and were allowed to acclimate to the experimental room conditions for a period of two weeks prior to induction and treatment. Animals were fed with standard commercial feed and water *ad libitium.*

2.6 Induction and Establishment of Leukemia

The mice were divided in groups of six animals each and treated respectively as described in Table 1. Animals were induced intraperitoneally (i.p) with 400 mg/kg/bodyweight [19] of benzene diluted in propanol and water at 2:1:1 respectively every 48 hours for four weeks. Tail blood smear of mice were prepared on slide, stained with Leishman and observed under microscope. The microscopic indication of leukemic blast in the peripheral blood established leukemia induction.

2.7 Sample Collection and Preparation of Sample

Forty-eight hours after the last treatment, animals were sacrificed by cervical dislocation and the blood was collected by cardiac puncture into Ethylene Diaminetetraacetic Acid (EDTA) vacuumed blood collection bottles for hematology examination. The liver sample for histological studies was fixed in 10% buffered formalin while the other liver tissue was washed in ice-cold saline. 10 % w/v homogenate was prepared in PBS homogenizing buffer (0.1 M phosphate buffers, pH 7.4 + 150 mM KCl) and the liver homogenate was centrifuged at 3000 rpm for 15 min. The supernatant was used for the antioxidant study.

2.8 Liver Antioxidant Assay

Superoxide dismutase (SOD) activity, catalase (CAT) activity and reduced glutathione (GSH)

concentration was evaluated by following the detailed instructions given in each Biodiagnostic kit (Glutathione Assay Kit: Batch No CS0260, SOD Assay Kit-WST: Batch No 19160, Catalase Assay kit: Batch No P6782).

2.8.1 Determination of reduced glutathione (GSH) concentration

One mL of supernatant liver homogenate was taken and 2ml of Tris-Hcl buffer was added. 0.05mL of DTNB 5, 5 dithiobis (2-nitrobenzoic acid) solution (Ellman's reagent) was added and mixed thoroughly. Optical density was read (within 2-3min after the addition of DTNB) at 412 nm against a reagent blank. Absorbance values were compared with a standard curve generated from known GSH [20].

2.8.2 Determination of total protein, Super Oxide Dismutase (SOD) and Catalase (CAT) and activities

The total protein content was measured by the method of Lowry *et al*. [21]. Determination of SOD activity followed the same method described by Kakkar *et al*. [22]. Catalase activity was assayed according to the method of Aebi [23].

2.9 Histopathological Study

The liver tissues were removed and smears in slides, which were processed and stained with Hematoxylin and eosin stains [24,25].

2.10 Bone Marrow Micronucleus Assay

The method described by Matter and Schmid [26] and modified by Heddle [27] was used for the micronucleus assay. Two hours before sacrifice, mice of both control and treated groups were injected with colchicine (4 mg/kg b. wt.) intraperitoneally. Both femurs were quickly excised and the epicondyle tips were cut open. The bone marrow content was smeared on the slide with a drop of 5% Fetal Calf Serum as the suspending medium [28]. Three slides were prepared from each animal. These were allowed to air dry, fixed in methanol and stained the following day with 0.4% May Grunwald stain, followed by 5 % Giemsa stain for 30 minutes. Excess stain was removed from the slides by washing with phosphate buffered saline and subsequent rinsing in distilled water. Glass cover slips were attached to the dried slide preparations by mounting in a mixture of DPX and xylene. The percentage of micronucleated polychromatic erythrocytes (MnPCE) in 1000 total erythrocytes was evaluated [29].

2.11 GC-MS Analysis

The GC-MS analysis of the ethyl acetate and aqueous fraction of *N. lotus* was performed on Agilent Technologies 789OA coupled with MSD VL5975C. HP5MS column was used with 30 m length, diameter of 0.0320 mm and thickness of 0.25 µm. The sample injection volume was 1 ml. Helium was used as the carrier gas with a constant flow rate of 2 ml/min. The oven temperature was programmed at 80ºC for 2 mins with an increase rate of 10ºC/min to 240ºC with holding time of 6 min. Mass spectral scan range was set at 35-550 (m/z). The spectrum of the separated components was compared with the spectrum of MS library 2014 National Institute of Standards and Technology (NIST).

2.12 Statistical Analysis

The results of this study were expressed as mean ± SEM. Statistical analysis was performed using One-way Analysis of Variance in Graph Pad Prism 5. Probability values of less than 0.05 (P < 0.05) were considered statistically significant.

Table 1. Animal grouping and treatments

Group	Treatment
A	Leukemia induction+120mg/kg body weight of *P. stratiotes*
B	Leukemia induction+120 mg/kg body weight of *N. lotus*
C	Leukemia induction+120mg/kg body weight of *P. guineense*
D	Leukemia induction+120mg/kg body weight of *S. virosa*
E	Leukemia induction+120mg/kg body weight of *C. procera*
F	Leukemia induction+120mg/kg body weight of *M. lucida*
G	Leukemia induction+120mg/kg body weight of *X. aethiopica*
H	Leukemia induction without treatment
I	Control (distilled water only)
J	Leukemia induction + 25mg/kg body weight of 5-fluorouracil
Fractions	Leukemia induction + 120 mg/kg body weight of each fraction

3. RESULTS

3.1 Ethnobotanical Survey

Table 2 also showed the frequency of citation of the plants collected during the survey. A total of 84 plant species belonging to 53 different families were obtained from the survey. The use of leaves was more prominent in the result. The family Euphorbiaceae had the highest occurrence of plant species followed by Annonaceae and Asclepiadaceae. The traditional mode of preparation included infusion, decoction and concoction. Plants with citation more than 10 were listed in Table 2.

3.2 Anti-leukemic Activity of the Selected Plants

Table 3 showed the percentage blast in the bone marrow and peripheral blood of leukemic and treated mice. Bone marrow blast > 20% and incidence in the peripheral blood indicates leukemia. Only *N. lotus* and *M. lucida* treated groups showed potential anti-leukemic effects with blast less than 20% in bone marrow.

Fig. 1. presented the representative photomicrograph showing the presence of leukemic blast and well differentiating cell in the bone marrow and peripheral blood respectively. The black arrow indicated blast while the blue arrow showed differentiating cell (Fig. 1). The blue arrow indicated blast while the black arrow showed differentiating cells.

3.2.1 Effect of plant extracts on hematological parameters of benzene induced leukemic mice

Fig. 2 Present the hematological parameters of leukemic mice that were treated with or without plant extracts. A significant ($p<0.05$) decrease in white blood cell (WBC) count was observed across the treated groups when compared with the untreated control. When compared with the positive control, no significant difference was observed except in the *Piper guineense* and *M. lucida* treated groups. No significant difference was also observed in red blood cell concentration (RBC) of all the extract treated groups except *M. lucida* that showed significant increase. There was significant ($p<0.05$) increase in hemoglobin concentration (HGB) of all the extract treated groups except *S. virosa* treated group that showed significant decrease. Significant increase in packed cell volume was observed in *M. lucida*, *P. guineense* and *P. stratiotes* treated groups while a decrease was observed in the *X. aethiopica* treated group when compared with the untreated control.

3.3 Anti-leukemic Activity of Fractions of *N. lotus*

Table 4showed the percentage of blast in the bone marrow and peripheral blood of leukemic and treated mice. Bone marrow blast > 20% and incidence in the peripheral blood indicates leukemia. Only the ethyl acetate treated group showed anti-leukemic effects with blast less than 20% in bone marrow and absence in the peripheral blood.

3.4 The Effect of Extracts Fractions of *N. lotus* on Hematological Parameters of Benzene Induced Leukemic Mice

The effects of fractions of *N. Lotus* on hematological parameters are showed in Table 5. The result shows no significant changes in the WBC count across the treated groups when compared with the untreated and control groups. The RBC, HGB, PCV levels were insignificantly ($p<0.05$) increased in all the extract treated groups when compared with the untreated control group. A significant decrease was also observed in the HGB, RBC and PCV of the 5-florouracil treated group when compared with the normal control group.

3.5 Anti-clastogenic Activity of Fractions of *N. lotus* in Leukemic Mice

Fig. 3 showed the percentage of micro-nucleated polychromatic erythrocyte (% MNPCE) in mice treated with the fractions of *N. lotus*. There was a significant decrease in % MNPCE across the treated groups when compared with the untreated group. No significant difference was observed when the fractions were compared with 5-florouracil and control group.

3.6 Liver Antioxidant Status of Leukemic Mice Treated with Fractions of *N. lotus*

The antioxidant effect of fractions of *N. lotus* on benzene induced leukemic mice is showed in Table 6. There was significant ($p< 0.05$) increase in reduced glutathione concentration and SOD activity of the n-hexane, chloroform and ethyl acetate fractions when compared with the

Table 2. List of plants used by traditional healers in Oyo State, Nigeria for the treatment of leukemia

S/N	Family	Botanical name	Vernacular names	Frequency of citation	Plant parts used	Usage
1.	Zingiberaceae	*Aframomum. Melegueta* (Loskoe) K. Schum	Atare	14	Seed	Infusion (Topical)
2.	Apocynaceae	*Alafia barteri* Baker	Agbari-etu	14	Leaves	Infusion(oral)
3.	Liliaceae	*Allium ascalonicum* Linn	Alubosaelewe	50	Bulb	Raw (Oral) and concoction (Oral)
4.	Liliaceae	*Allium cepa* Linn	Alubosaonisu	12	Leaves	Infusion(oral), infusion(Topical)
5.	Liliaceae	*Allium sativum* Linn.	Ayu	14	Bulb	Infusion(oral), Concoction(oral)
6.	Liliaceae	*Aloe barteri* (Baker)	Etierin	12	Leaves	Infusion (oral)
7.	Apocynaceae	*Alstonia congensis* De wild	Awun	11	Bark	Decoction(oral)
8.	Bromeliadceae	*Anana scomosus* (L.) merr	Ope-oyinbo	25	Juice	Raw(oral)
9.	Araceae	*Anchomanas difformis* (Blume) Eng	Eego	13	Leaves	Decoction(oral)
10.	Longaniaceae	*Anthocleista djalonensis* A.cheu	Sapo	13	Leaves	Decoction(oral)
11.	Moraceae	*Antiaris africana* Engl	Oro	25	Bark	Decoction(oral)
12.	Poaceae	*Bambusa vulgaris* Linn.	Oparun	11	Bark	Decoction(oral)
13.	Leguminosae-Caesalpinioideae	*Berlinia grandi flora* (Vahl) Hutch. & Dalziel	Apado	13	Bark	Decoction(oral)
14.	Sapindaceae	*Blighia sapida*	Ewe isin	11	Leaves	Infusion(oral)
15.	Euphorbiaceae	*Bridelia ferruginea* (Benth)	Ira	16	Bark	Decoction(oral)
16.	Crassulaceae	*Bryophyllum pinnatum* (Lam) Oken	Abamoda	13	Root	Infusion(oral)
17.	Leguminosae-Mimosoideae	*Calliandra haematocephala* (Jacq) Benth	Tude	15	Leave, bark and seed	Concoction(oral, topical)
18.	Asclepiadaceae	*Calotropis procera* R.B	Bomubomu	12	Leaves	Decoction(oral), add with honey leak(oral)
19.	Solanaceae	*Capsicum frutescens* (Benth)	Ata ijosi	12	Fruit	Infusion (Topical, Oral)
20.	Caricaceae	*Carica papaya*	Ibepedudu	11	Leaves	Decoction(oral, topical)
21.	Caesalpinaceae	*Cassia occidentalis*	Ewe rere	11	Leaves	Infusion(oral)

22.	Celastraceae	*Celastrus indica* Linn.	Ponju owiwi	13	Root	Infusion (topical, oral), decoction (oral, topical)
23.	Chenopodiaceae	*Chenopodium ambrosioides* Linn.	Arunpale	12	Bark	Decoction(oral)
24.	Sapotaceae	*Chrysophyllum albidum* Linn.	Agbalumo	11	Seed	Decoction(oral)
25.	Rutaceae	*Citrus aurantifolia* (Christm.) Swingle.	Orombo	11	Juice	Raw Juice (oral)
26.	Rutaceae	*Citrus aurantium* Linn.	Osanijaganyin	14	Root	Infusion(oral)
27.	Palmae (Araceae)	*Cocos nucifera* Linn.	Agbon	12	Leaves & juice	Decoction(oral), Raw Juice(oral)
28.	Rubiaceae	*Coffea bracteolate* Linn.	Poropiwo	12	Leaves	Infusion(oral)
29.	Malvaceae	*Cola nitida*	Apopo obi	15	Stem, bark, seed, kernel	Grind into powder(topical)
30.	Tiliacaeae	*Corchorous Olitorius*	Ooyo	11	leaves	Concoction(oral)
31.	Amaryllidaceae	*Crinum jagus (*Thomson*)* Dandy	Isumerin	12	Tuber	Concoction(oral)
32.	Araceae	*Culcasia scandens* P. Beauv	Agumona	14	Leaves	Infusion(oral)
33.	Zingiberaceae	*Curcuma domestica* Bull. Jard	Atalefunfun	13	Seed/Pod	Poultice(topical)
34.	Meliaceae	*Ekebergia senegalensis*	Epojebo	11	Bark	Decoction(oral)
35.	Palmae	*Elaeis guineensis* (Jacq)	Ope	14	Bark	Concoction (oral)
36.	Myrtaceae	*Eugenia aromatic* Linn	Kanafuru	14	Flower	Infusion(topical)
37.	Euphorbiaceae	*Euphorbia unispina* (L) Pax	Oro adete	12	Root	Concoction(oral)
38.	Guttiferae	*Garcinia kola* Heckel	Orogbo	15	Root, bark	Infusion(oral), decoction (oral)
39.	Meripilacaeae	*Grifola-frondosa*	Olu	16	leaves	Take with soup: concoction (oral)
40.	Hyperiaceae	*Harungana madagascarensis* Lan. ex poir	Aroje	16	Bark	Decoction(oral)
41.	Menispermaceae	*Jateorhiza palmate* (Lam.) Miers.	Wowo	14	Bark	Decoction(oral)
42.	Meliaceae	*Khaya grandifoliola* C.D.C	Oganwo	14	Bark	Decoction(oral)
43.	Bignoniaceae	*Kigelia africana* Benth	Pandoro	18	Leave, bark	Decoction(oral)
44.	Lythraceae	*Lawsonia inermis* Linn.	Lali	13	Bark	Decoction(oral)
45.	Anacardiaceae	*Magnifera indica* Linn.	Mongoro	16	Bark, Leaves	Decoction(oral)
46.	Anacardiaceae	*Lanneae gregia (Hiern)* Engl	Ekudan	11	Leaves	Decoction(oral)
47.	Cucurbitaceae	*Momordica charantia* Schum & Thonn.	Ejirinwewe	12	Leaves	Infusion (topical, oral)
48.	Rubiaceae	*Morinda lucida* Benth	Oruwo	19	Bark	Infusion(**O**); decoction(**O**)

49.	Moringaceae	*Moringa oleifera*	Ewe moringa	11	Root, seeds and leaves	Decoction (oral)
50.	Musaceae	*Musa paradisiaca* Linn	OgedeAgbagba	11	Leaves	Decoction (oral)
51.	Musaceae	*Musa paradisiac*	Ogede wewe	12	Root	Concoction (oral)
52.	Rubiaceae	*Nauclea latifolia* Smith	Egbesi	15	Root, Bark	Infusion(oral), decoction (oral), concoction (oral)
53.	Solanaceae	*Nicotiana tobacum*	Ewe taba	11	Leaves	Infusion(oral)
54.	Nympheaceae	*Nymphaea lotus*	Ewe osibata	15	Leaves	Decoction (oral, topical)
55.	Labiatae	*Ocimum basilicum* Linn.	Efinrin	19	Leaves	Squeeze and press water(oral)
56.	Olacaceae	*Olax subscorpioidea* Oliv.	Ifon	15	Leaves, Root	Infusion (oral), Decoction(**O**)
57.	Cactaceeae	*Opuntia dillenii*	Oro agogo	12	Root and Leaves	Decoction(**T**)
58.	Cactaceeae	*Opuntia dillenii* Haw	Orokoro	11		
59.	Periplocaceae	*Parquetina nigrescens* (Afzel) Bullock	Oogbo	13	Leaves	Infusion(oral)
60.	Sapindaceae	*Paullina pinnata* Linn	Kakasela	12	Leaves	Infusion(oral)
61.	Phytolacaceae	*Petiveria alliacea* Linn	Awogba	14	Root, Bark	Infusion(O), decoction (oral)
62.	Piperaceae	*Piper guineense* Schum &Thonn	Iyere	16	Seed	Poultice(**O**); decoction(**O**)
63.	Araceae	*Pistia stratiotes*	Ojuoro	19	Leaves	Decoction (oral)
64.	Plumbaginaceae	*Plumbago zeylanicca* Linn	Inabiri	15	Root	Concoction (topical)
65.	Meliaceae	*Pseudocedrela kotschyi* Engl	Emigbegiri	11	Bark	Decoction(oral, topical)
66.	Guttiferae	*Psorospermum febrifugum* Spach	Legun-oko	13	Bark	Decoction(topical, oral)
67.	Leguminosae	*Pterocarpus osun*	Ewe osun	11	Leaves	Infusion (topical)
68.	Poaceae	*Saccharum offinarum* Linn.	Ireke	14	Juice	Raw(oral)
69.	Asclepiadaceae	*Secamonea fzelii* K Shulfes	Arilu	12	Leaves	Infusion(oral)
70.	Polygalaceae	*Securidaca longepedunculata* Frer	Ipeta	17	Root, Bark	Concoction (oral), infusion(oral)
71.	Euphorbiace	*Securinega virosa* (Roxb) Bail.	Iranje	19	Bark, Leaves	Concoction(oral), decoction (oral)

72.	Leguminosae-Caesalpinioideae	*Senna alata* Linn.	Asunwon	14	Leaves and Root	Concoction(oral)
73.	Leguminosae-Caesalpinioideae	*Senna fistula* Linn.	Aidan tooro	14	Leaves, Bark	Infusion (oral), decoction (topical)
74.	Bignoniaceae	*Spathodea companulata* P.beauv	Orudu	16	Leave bark	Decoction(oral)
75.	Leguminosae - Mimosoideae	*Tetrapleura tetraptera* Schum&Thonn	Aidan onigun	13	Seed	Decoction (topical, oral)
76.	Meliaceae	*Tricalysia macrophylla* K. Schum	Olojaebano	11	Bark	Decoction(oral)
77.	Asclepiadaceae	*Tylophora spp*	Olubara	17	Leaves	Infusion(oral)
78.	Malaceae	*Urena lobata*	Ewe Akeeri	11	Leaves	Infusion(oral)
79.	Annonaceae	*Uvaria afzelii* Elliot	Gbogbonse	12	Root	Concoction(oral)
80.	Annonaceae	*Uvaria chamae* P. Beauv	Eruju	13	Bark	Concoction(oral)
81.	Compositae	*Vernonia amygdalina* Linn	Ewuro	12	Leaves	Infusion(oral)
82.	Annonaceae	*Xylopia aethiopica* (Dunal) A. Rich	Eeru-lamo	11	Fruit	Raw(oral)
83.	Zingiberaceae	*Zingiber officinale* Roscoe	Ataile	11	Rhizome	Concoction(**O**), Decoction(**O**)

Table 3. Percentage blast in the bone marrow and peripheral blood of benzene induced leukemic and leukemic treated mice

Group	(%) Blasts in bone marrow	(%) Blasts in peripheral blood
Control group	17	0
Untreated group	40	10
5-florouracil	17	17
P. stratiotes	22	2
N. lotus	18	3
P. guineense	35	3
S. virosa	28	5
C. procera	22	3
M. lucida	17	3
X. aethiopica	23	7

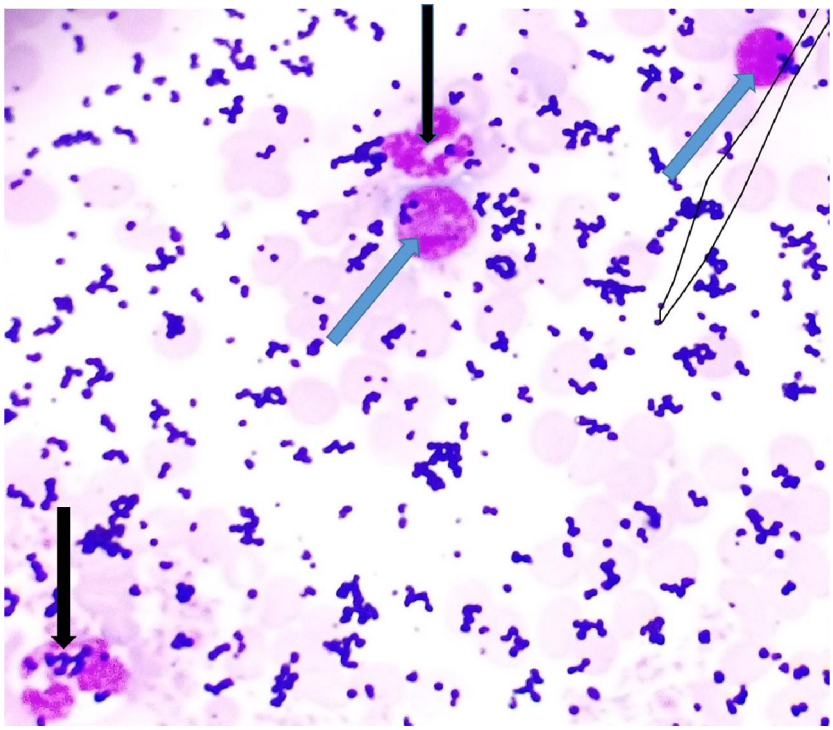

Fig. 1. Photomicrograph of representative of peripheral blood smear showing the presence of blast (blue arrow) and differentiating cells (black arrow)

Table 4. Percentage blast in the bone marrow and peripheral blood of leukemic and treated mice

GROUP	Blasts (%) bone marrow	Blasts (%) peripheral blood
n-Hexane	20	28
Chloroform	22	29
Ethyl-acetate	11	0
n-Butanol	18	49
Aqueous	21	33
Untreated	31	41
Control	3	0
5-florouracil	16	0

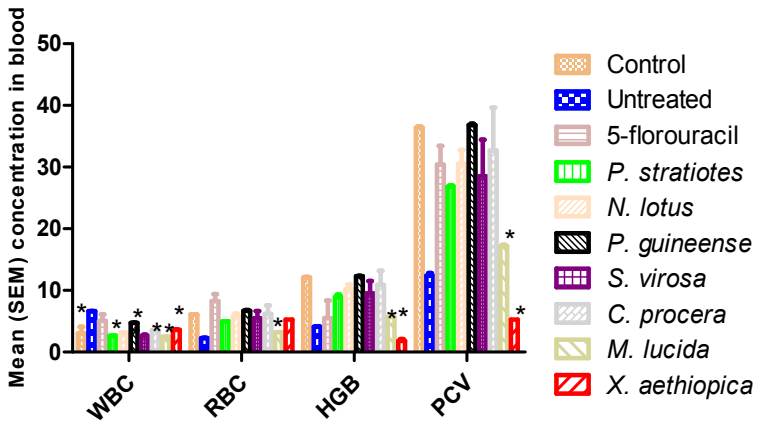

Fig. 2. Effect of plant extracts on hematological parameters of benzene induced leukemic mice

**means significant difference when compared with the untreated group.*
WBC= White Blood Cell; RBC= Red Blood Cell
HGB= Hemoglobin
PCV= Packed Cell Volume

Table 5. The effect of extracts fractions of *N. lotus* on hematological parameters of benzene induced leukemic mice

Fractions	WBC×10^9	HGB g/dL	RBC×10^{12}/L	PCV %	PLT× 10^9/L
n-Hexane	8.47±3.87#	8.93±2.16#	5.56±1.08#	29.3±6.32#	601±93.83#
Chloroform	5.56±0.60#	9.73±1.07#	7.12±1.14#	31.2±2.48#	499.33±133.12#
Ethyl-acetate	5.7±0.2#	11.3±0.26#	8.8±0#µ	38.6±0.1#	666±300#
n-Butanol	4.97±0.90#	10.87±0.84#	7.36±0.74#	37.70±1.88#	1141±509#
Aqueous	3.31#	8.65±0.65#	5.36±0.05#	26.42±0.53#	474±162.5#
Untreated	5.26±1.86	7.15±0.25#	4.91±0.42	23.4±1.2	388
Control	4.23±1.19#	13.24±0.89#µ	8.42±0.34#µ	41.07±3.14µ	510±116#
5-florouracil	5.1±0.61#	7.3±2.42#	4.84±1.53#	27.3±8.17	865#

*Values were expressed as mean ± SEM. ***significant difference when compared with the untreated group, #no significant difference when compared with the untreated group, ˙significant difference when compared with the normal control group and µsignificant difference when compared with 5-florouracil treated group*

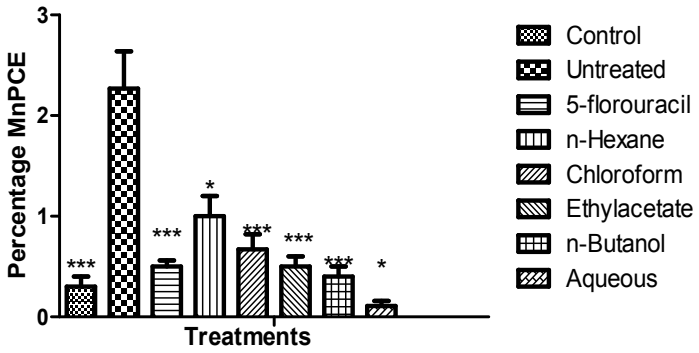

Fig. 3. Percentage of micro-nucleated polychromatic erythrocyte (% MNPCE) in fractions of *N. lotus*. MnPCE means micronucleated polychromatic erythrocyte.

** means slightly significant difference when compared with the untreated group and *** means highly significant difference when compared with the untreated group*

Table 6. Total protein level and effect of fractions of *N. lotus* on antioxidant parameters in benzene induced leukemic mice

Fractions	Total Protein g/dl	Activity of SOD (U/g of proteins)	Reduced Gluthatione (µmol/mg of proteins)	Catalase (U/mmol H2O2)
n-Hexane	7.83±0.03#••	23.05±0.95***•µ	35.95±0.55***• µ	3.50±0.17•
Chloroform	7.6±0.10#••	19.8±0.30***• µ	22.45±0.15***• µ	3.48±0.05•
Ethyl acetate	8.02±0.02#•	64.1±0.90*** µ	49.65±0.85***	5.50±0.50***
n-Butanol	8.15±0.26#	32.65±1.15***• µ	11.25±0.96• µ	3.74±0.67•
Aqueous	7.93±0.28#•	13.03±0.48#• µ	12.83±0.48• µ	3.06±0.02• µ
Untreated	7.52±0.02	11.52±0.48	11.83±0.27	3.09±0.11
Control	9.0±0.10**	63.7±0.20***	48.97±0.23***	6.10±0.20***• µ
5-florouracil	8.05±0.02#•	56.35± 0.75***•	45.95±0.35***	4.32±0.09•

*Values were expressed as mean ± SEM. ***significant difference when compared with the untreated group, #no significant difference when compared with the untreated group, •significant difference when compared with the normal control group and µ significant difference when compared with 5-florouracil treated group.*

Table 7. Phytocomponents identified by GC-MS in the ethyl acetate fraction of *N. lotus*

Name of compounds	Retention time (min)	Relative abundance (%)
Caryophyllene	12.603	0.313
Humulene	13.099	4.509
1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-methylene-, (4aS-cis)-	13.708	0.194
(Z)-1-Methyl-4-(6-methylhept-5-en- 2-ylidene)cyclohex-1-ene	14.459	0.339
1(2H)-Naphthalenone,3,4,4a,5,8,8a-hexahydro-8a-methyl-, trans-	14.896	0.505
cis-Z-.alpha.-Bisabolene epoxide	15.258	1.065
Tetradecanoic acid	17.457	0.093
Hexadecanoic acid, methyl ester	19.596	1.341
n-Hexadecanoic acid	20.633	1.838
cis-13-Octadecenoic acid, methyl ester	22.497	2.780
Methyl stearate	22.891	0.230
9-Octadecenoic acid, (E)-	23.996	4.222
Oleic Acid	24.021	1.535
3-Decanone, 1-(4-hydroxy-3-methoxyphenyl)-	25.149	13.807
2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-	26.028	2.421
1-(4-Hydroxy-3-methoxyphenyl)dec-4-en-3-one	26.253	20.259
1-(4-Hydroxy-3-methoxyphenyl) decan e-3,5-dione	26.845	18.997
Squalene	31.121	0.386

untreated group. Groups treated with the aqueous and butanol fractions showed insignificant ($p < 0.05$) changes in glutathione concentration and SOD activity. Only the ethyl acetate fraction group demonstrated a significant increase in catalase activity, other fraction groups showed an insignificant ($p < 0.05$) change when compared with the untreated group.

3.7 Liver Histological Studies on Leukemic Mice Treated with Fractions of *N. lotus*

Fig. 4 A-H are the representative photomicrograph of leukemic, treated and control mice characterized by severe hemorrhage and fibrosis (Group F), presence of some necrotic tissue, degenerating blood vessel walls and some pyknotic hepatocytes as well as infiltration of some dark cells localized around the degenerating region (Black arrow). This observation in Fig. A-E and H is mild compared to the untreated group.

3.8 Gas Chromatography-Mass Spectroscopy Identification of Compounds in the Fractions of *N. lotus*

The identification of compounds in the ethyl acetate fraction was based on the elution order in a HP-5MS column. Table 7 showed the name,

retention time, and relative abundance of the identified compounds. The peaks of the chromatogram in Fig. 5 correspond to each compound.

4. DISCUSSION

Despite increasing understanding on cancer therapy and discovery of a number of chemotherapeutic agents, there is still an impetus to identify, develop and test more potent and safest anticancer therapeutics [30,31]. This study carried out ethnobotanical survey of plants used in the treatment of leukemia in Oyo State and conducted bioactivities of the extracts in mice *in vivo* to study potential phyto-pharmaceutical in the plants to combat leukemia. Therefore, seven of the plant species with the most frequent citation in the survey were evaluated for their anti-leukemic potentials. Benzene was selected for leukemia induction because it is known to cause pancytopenia, aplastic anemia, preleukemia myelodysplastic syndrome and finally leukemias [32], [33].

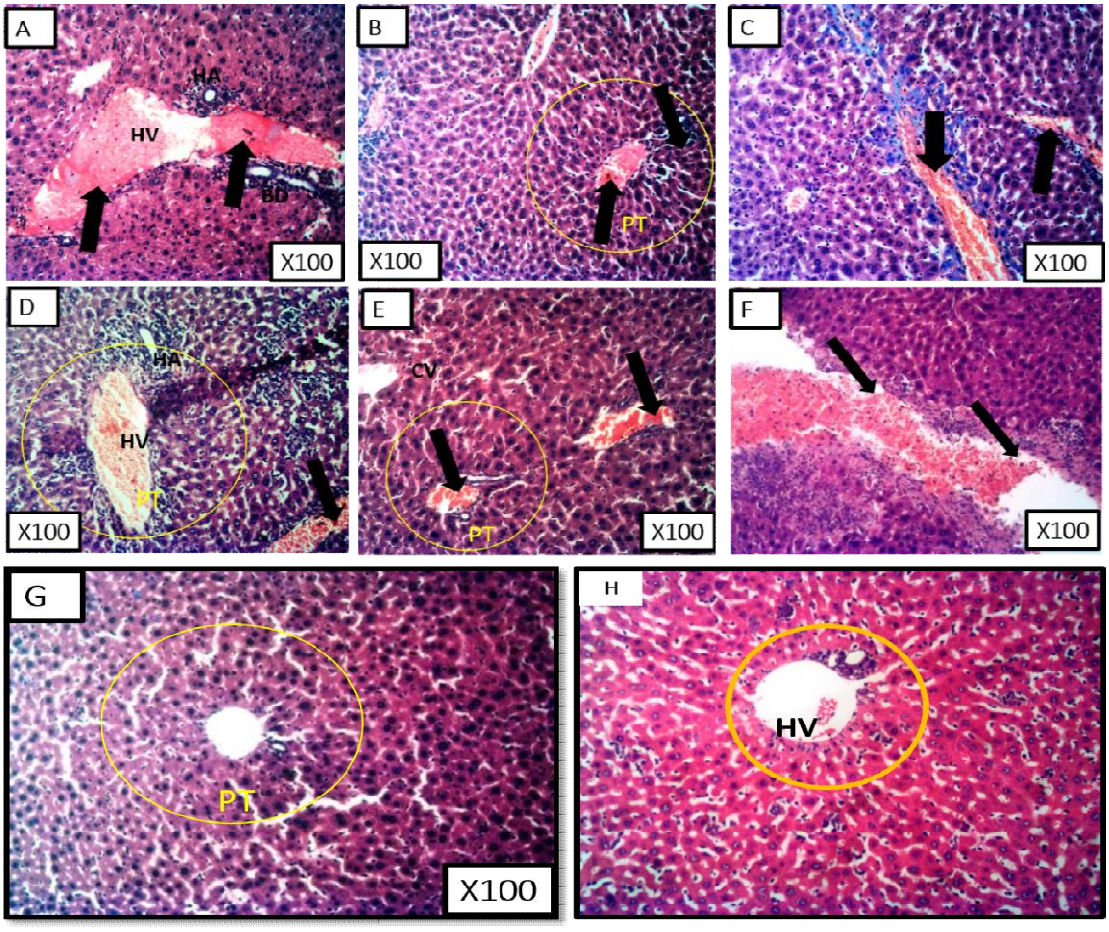

Fig. 4. Photomicroghraph of representative groups of leukemic mice treated, without treatment and group fed with water and commercial feed showing the hepatocytes (H), portal triad (PT) comprised of hepatic vein (HV), hepatic artery (HA) and the bile duct (BD). Also observed across the micrographs is the central vein (CV) and branches of blood vessels

Fig 4 A = mice induced with benzene and treated with n-hexane fraction of N. lotus
Fig 4 B = mice induced with benzene and treated with chloroform fraction of N. lotus
Fig 4C = mice induced with benzene and treated with ethyl acetate fraction of N. lotus
Fig 4D = mice induced with benzene and treated with butanol fraction of N. lotus
Fig 4E = mice induced with benzene and treated with aqueous fraction of N. lotus
Fig 4F= mice induced with benzene and not treated
Fig 4 G = mice fed with commercial feed and water
Fig 4 H = mice induced with benzene and treated with 5-fluorouracil

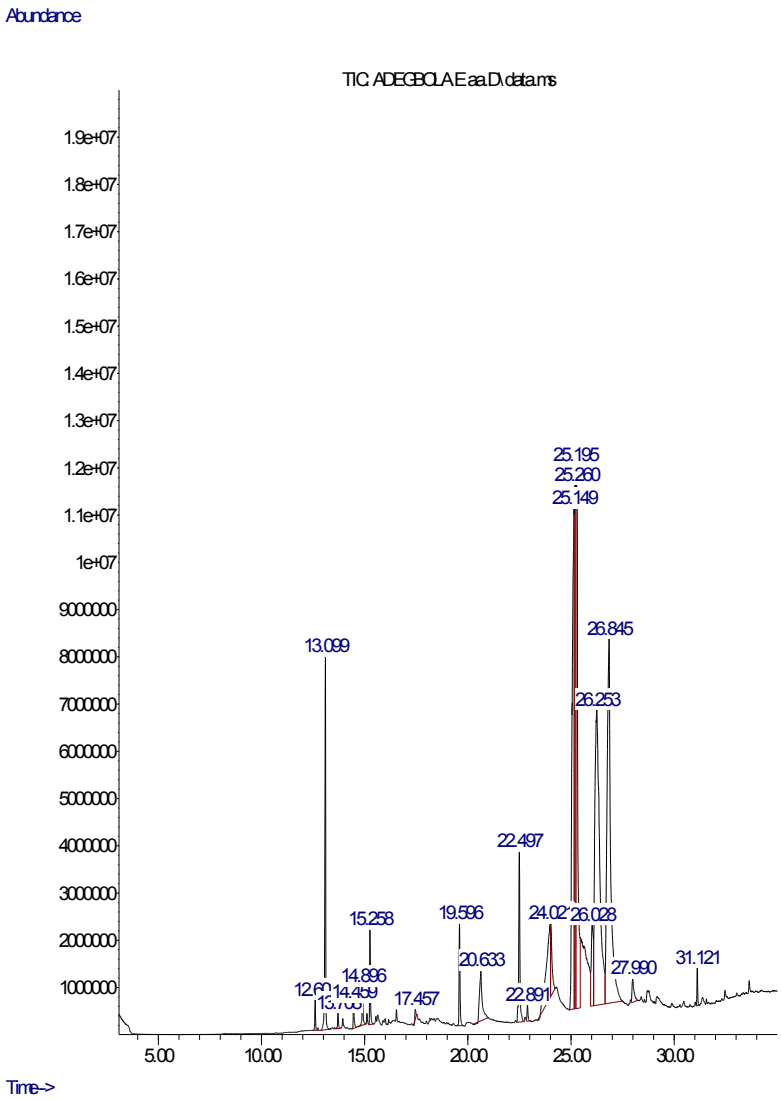

Fig. 5. Chromatogram of the compounds present in ethyl acetate fraction of methanolic leave extract of *N. lotus*

In addition, benzene is a known spindle fiber disruptor, and an inhibitor of topoisomerase II, and both effects may cause the stem cell/progenitor cell population to give rise to micronucleated cells. The indices of leukemic myeloblast by the World Health Organization (WHO) are the presence of more than 20% in the peripheral blood and or the bone marrow (Harris *et al*., 1999). In this study, *X. aethiopica* and *P. guineense* extracts did not reverse the leukemic blast cells in benzene-induced leukemia in mice. Conversely, *N. lotus* and *M. lucida* extracts exhibited anti-leukemic property by reversing the incidence of leukemic blast in the blood and by reducing the blast in the bone marrow to the percentage lesser than twenty in mice. Previous *in vitro* study has confirmed the anti-leukemic potential of *M. lucida* in HL-60 cell line through reduction of proliferation and induction of apoptosis [34]. In this study, treatment of the leukemic mice with extracts of *P. stratiotes, N. lotus, C. procera, S. virosa* and *X. aethiopica* led to increase in concentration of the hemoglobin, RBC count and the PCV. Peng *et al*. [35] explained that an early indication of benzene poisoning is the formation of aplastic anemia leading to pancytopenia that is decrease in RBC. Therefore, the extract may have protected the mice from forming leukemia by reversal of

aplastic anemia and myelodysplasia (pre-leukemic symptoms).

N. lotus extract displayed the most potent activities in all the plant extracts selected for screening in this study, therefore, fractions of *N. lotus* was selected for further study on anti-leukemic bioassays and analysis of the chemical composition. To these effects, *N. lotus* fractions (n-hexane, chloroform, ethyl acetate and butanol) were studied for their anti-leukemic, antioxidant, anti-clastogenic and hepatoprotective activities. In the anti-leukemic study of the fractions, only the ethyl acetate fraction reversed the leukemia formed in mice with percentage blast less than 20 percent in bone marrow and absent in the peripheral blood. DNA damage in bone marrow cells of mice as initiated by acute exposure of the mice to benzene was reduced by the fractions of *N. lotus* and reflected in varying degrees of reduction in the number of micronucleated polychromatic erythrocyte in benzene induced leukemic mice. This might be due to the ability of these extracts to prevent the covalent binding of the reactive metabolites of benzene to the DNA of leukemic mice [36,37,38]. Similarly, Chromosomal damage in mice bone marrow was most prevented by the ethyl acetate fraction of *N. lotus.* Micronucleated polychromatic erythrocyte are seen as a result of clastogenic activity of benzene [39] that act during cell division and cause fragment of chromosome to lag behind, the lagging chromosome are not integrated into daughter nucleus forming micronuclei that can be viewed due to lack of main nucleus [40]**.** Studies on the effects of fractions on the hematological parameters revealed that the ethyl acetate and butanol fractions protected the RBC, HGB and the PCV status of the mice. This support the possible restoration of bone marrow function in benzene induced damage.

The ethyl acetate fraction revealed antioxidant activity, which may contribute to the anti-leukemic activity demonstrated by this fraction. The significant antioxidant effect of the ethyl acetate fraction showed that it could inhibit the pathway of leukomogenesis. Although the possible anti-leukemic mechanism is yet to be fully understood, the DNA protective effects of the fraction, might contribute to the observed results. Treatment with butanol and ethyl acetate fractions of *N. lotus* reversed the severe infiltration of inflammatory cells, hemorrhage, necrosis as well as fibrosis including some degenerative changes observed in the untreated control group. The chloroform and aqueous fractions caused a slight improvement in the degenerative changes that accompany benzene toxicity. The result revealed the possible hepatoprotective effects of the fractions of *N. lotus*.

GC–MS analysis of ethyl acetate fraction showed the presence certain compounds among which humulene, 3-decanone, 9-octadecenoic acid, squalene, oleic acid, caryophyllene and hexadecanoicacids are vital to anti-leukemic activity. Some of the identified compounds had been previously proven to possess pharmacologic activities, which may contribute to the anti-leukemic potential of *N. lotus*. Squalene identified in *N. lotus* has been shown to have antioxidant property and can effectively inhibit chemically induced skin, colon and lung tumor in rodents [41,42]. Lingadurai *et al. [43]* explained the antileukemic activities of caryophyllene through DNA fragmentation and apoptosis. Humulene possessed cytotoxic activities against PC-3, A-549, DLD-1 and M4BEU tumor cells [44]. Chowdhury *et al. [45], [46]* reported anti-leukemic activities of humulene through increase ROS and decrease cellular reduced gluthathione. Cytotoxic activity of hexadecanoic acid was reported by Harada *et al*. [47] against human leukemic cells, murine leukemic cell and leukemia cancer cells MOLT-4. Hexadecanoic acids interact with DNA topoisomerase I and thus prevent proliferation of cancer cells and induce apoptosis [48].

In addition, octadecadienoic acid was identified in ethyl acetate fractions of *N. lotus*. This compound was known to exhibit antioxidant [49,50], antitumor and anti-proliferative activities in previous cancer research [51,52,8]. It could be suggested that octadecadienoic in the fractions of *N. lotus* reversed leukemia in mice by anti-proliferation and inhibitory effects.

Benzene effect on leukemogenesis is not a singular process and can occur throughout the leukemia generation stages [53]. For instance, the anti-leukemic potential demonstrated by the ethyl acetate fraction of *N. lotus* can be correlated directly with the unique anti-clastogenic and antioxidant activity demonstrated in this study. Chromosomal damage has been reported to be involved in the mechanism of benzene carcinogenesis through the production of reactive metabolites [45], Li *et al*., [39]. Therefore, prevention of chromosomal damage through the scavenging of radical metabolites by

antioxidant paves way for inhibition of the leukemia pathway and thus might explain one of the possible mechanisms of anti-leukemic potential of ethyl acetate fraction of *N. lotus.*

Furthermore, it is clear from this study that the active phytochemicals of *N. lotus* might reside in the ethyl acetate fraction of *N. lotus* and therefore might contribute the pharmacological effects observed. Phytochemicals identified in *N. lotus* have been shown to induce cell cycle arrest, cause apoptosis and affect the differentiation and proliferation of cells mediated by the effect of intracellular reactive oxygen species on the signal transduction pathway [54].

5. CONCLUSION

This study had shown few of the selected plant extracts have some anti-leukemic activity. Comparative analysis of the seven extracts showed that *N. lotus* extract possessed the most potent and significant anti-leukemic activity at the concentration tested. Phytochemical analysis of fractions of *N. lotus* showed that ethyl acetate fractions had significant anti-leukemic activity and certain compounds with potential anticancer activities. This lends support to the ethno-medicinal uses of the plants for which they are known and used for and therefore, potentially *N. lotus* could be sources for pharmacologically active products suitable for development as chemotherapeutic or chemopreventive agents.

ETHICAL APPROVAL

Ethical approval was obtained prior to the use of animals for the experiment

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX A. SUPPORTING INFORMATION

Appendix A: Semi-structured questionnaire used for assessing anti-leukemic medicinal plants in ogbomoso

GENERAL DATA

Name..... Family name.....
Age.....Sex: ( ) Male ( ) Female
Date of Birth.....

Position in your village:

1.Priest( )
2.Family Head( )
3.Opinion Leader( )
4.Chieftancy Title Holder( )
5.Others(please specify)( )

VILLAGE NAME.....
LOCAL GOVERNMENT AREA.....
STATE.....

HIGHEST EDUCATIONAL LEVEL(please tick one)
1.Primary School ( )
2.Secondary School ( )
3. Technical/Teacher's college ( )
4. Diploma ( )
5. National Certificate of Education ( )
6. First Degree ( )
7. Higher Degree ( )
8.No Formal Education ( )

OCCUPATION (please tick one)
1.Farming ( )
2.Traditional Healer ( )
3. Artisan ( )
4. Private sector employee ( )
5. Governor official ( )

How did you obtain the knowledge? (please tick)
1.formal training ( )
2.apprehenticeship ( )
3. from your elders ( )
4.family herbal practice records ( )
5.others(please specify) ( )

Are you a registered Tradomedical practitioner?
( ) Yes ( ) No

With which of the following council are you registered?
Community council of traditional healers
Traditional healers association of Nigeria
Herb sellers Association
Others(please specify)

KNOWLEDGE ABOUT MEDICINAL PLANTS USED FOR LEUKEMIA TREATMENT
Do you have any knowledge about the use of medicinal plants for the following ailment?
Cancer /leukemia ( ) Yes ( ) No

S/N	General Name	Local Name	Extraction/Usage	Dosage	Side effects
S/N	General Name	Local Name	Usage	Dosage	

Peer-review history:
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