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Antiproliferative Properties of Labdane Diterpenoids from *Croton sylvaticus* Hochst against Drug Sensitive and Resistant Leukemia cell lines

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Abstract

Background: Cancer treatment failure is majorly attributed to the emergency of multi-drug resistance of cancer cells towards most conventional drugs used in chemotherapy hence the need for more active principles with less side effective from natural sources.
Methods: Characterization of isolated compounds was achieved using NMR spectroscopy and comparison of acquired data with literature values. The antiproliferative properties were determined using resazurin reduction assay.
Results: Three labdane diterpenoids namely; 18-*nor*-labd-13(*E*)-ene-8α, 15-diol (**1**), labd-13(*E*)-ene-8α, 15-diol (**2**) and austroinulin (**3**) were isolated from the stem bark of *Croton sylvaticus.* The crude extract was active at 10 µg/mL with cell inhibition of 86.96±4.86 against drug sensitive CCRF-CEM and 77.57±2.84% against drug resistant CEM/ADR5000 leukemia cell lines. However, all the compounds displayed lower antiproliferative potencies as they exhibited cell inhibitions<70% of the cell population at 10 µM. The cell inhibition of doxorubicin, was 94.89±0.86% and 24.20±2.89% against CCRF-CEM and CEM/ADR5000 cells, respectively. From these observations, it is clear that CEM/ADR5000 are resistant to doxorubicin while the activities of the compounds were similar against the two cell lines. This could be attributed to the similarities in their skeletal structures. Furthermore, **1** and **3** were more active than doxorubicin against CEM/ADR5000 cells.
Conclusion: The labdane diterpenoids displayed low cytotoxicity against CCRF-CEM cells and CEM/ADR5000 as compared to the crude extract. Doxorubicin was inactive against CEM/ADR5000 cells. Furthermore, **1** and **3** were more active than doxorubicin against CEM/ADR5000 cells. These compounds could be modified to obtained analogues with improved activities against the drug resistant cells.

Keywords: *Croton sylvaticus* Hochst; antiproliferative properties; labdane ditepenoids

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Background

Cancer is rated second as the major cause of death after cardiovascular diseases. According to World Health Organization (WHO), 9.6 million deaths were globally recorded as being due to cancer and cancer-related ailments [1,2]. With this rate, if no interventions are made, the mortality rate is projected to increase to 11.5 million per annum by 2030 as predicted by the World Health Organization [1].

Croton sylvaticus Hochst. ex Krauss (Euphorbiaceae) is a semi-deciduous plant which normally grows in the woodland savannah, coastal forest and evergreen forest [3]. It is relatively common in many parts of tropical Africa [4]. It is one of the native plants to Ethiopia, Kenya, Lesotho, South Africa and Uganda [3]. Traditionally, it is one of the plants used in the treatment of some ailments such as malaria and cancer [5-7], tuberculosis, insomnia and abdominal disorders [7]. A wide range of biological activities have been reported for this plant including antibacterial [8,9], antifungal [8,10], anti-inflammatory [11,12], antioxidant [8,12], effects on the central nervous system (CNS) [13], larvicidal [14], mutagenic activities [13-16] and cytotoxicity against drug-sensitive and resistant cell lines [17]. Previous phytochemical studies have shown that *Croton sylvaticus* contain natural products belonging to the following classes; terpenoids [8], alkaloids [6] and flavonoids [18]. Some of the compounds that have been isolated from the genus *Croton* include julocrotine [5,6] with anti-leishmanial activity [19,20], penduliflaworonsin [8,21] possessing strong anti-angiogenic property and low cytotoxicity hence the potential of development of this compound for cancer treatment [22], hardwickiic acid [21] which have been shown to act at presynaptic opioid receptor [23] and hence is a potential candidate for the cure of central nervous system (CNS) diseases including mood disorders, drug abuse, anxiety, hypoxia and pain [24,25], lupeol [6] which has shown interesting cytotoxicity against a panel of drug sensitive and resistant phenotypes cancer cell lines [26], *ent*-3,13*E*-clerodadiene-15-formate [8,21], crotohalimaneic acid [8,21] which in previous investigations showed strong cytotoxicity against a number of human tumor cell lines [27] and labda-13*E*-ene-8α,15-diol [8,21] with a similar diterpene skeleton with *ent*-12R,16-dihydroxylabda-7,13-dien-15,16-olide known to inhibits proliferation of human lymphocytes present in peripheral blood mononuclear cells (PBMC) via induction of monocyte apoptosis [28-30].

The assumption in the current investigation was that compounds isolated from *Croton sylvaticus* stem bark extract known to exhibit good antiproliferative properties should also exert similar antiproliferative potencies.

Therefore, in our continuous search for cytotoxic compounds from Kenyan plants that have exhibited cytotoxic potential, the antiproliferative properties of the stem bark extract (50% methanol in dichloromethane) of *C. sylvaticus* and isolated compounds were investigated against drug-sensitive CCRF-CEM and drug resistant CEM/ADR5000 human leukemia cells.

Methods

General

The compounds used in the present investigation included; 18-*nor*-labd-13(*E*)-ene-8α,15-diol (**1**) [31], labd-13(*E*)-ene-8α, 15-diol (**2**) [8,32] and austroinulin (**3**) [33]. The compounds were re-isolated from the stem bark of *C. sylvaticus* (50% MeOH in CH_2Cl_2) and characterized using spectroscopic techniques (such as NMR and MS) coupled with comparison of their TLC profile with authentic samples from the Natural Products laboratory of the Chemistry Department, University of Nairobi, Kenya (Figure 1). Doxorubicin 98.0%, which was used as the standard drug, was provided by the Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University (Mainz, Germany) and dissolved in Phosphate Buffer Saline (PBS; Invitrogen, genstein, Germany) at a concentration of 10 mM.

Plant Material

Croton sylvaticus Hochst was identified in Kakamega forest in Western Kenya with the help of Mr. Patrick Mutiso, a taxonomist in the School of Biological Sciences (SBS), University of Nairobi. The plant material was collected and the voucher specimen, KO-09-2016, deposited at SBS, University of Nairobi (UoN) herbarium for future reference.

Extraction and Isolation

Approximately 2000 g of air-dried and powdered stem bark of *Croton sylvaticus* was sequentially soaked in three liters of (1:1 v/v) methanol (MeOH) in dichloromethane (CH_2Cl_2) solvent and then filtered to obtain the filtrate. Similarly, the same plant material was soaked in 5% v/v water in MeOH for 24 hours at room temperature. The extract was filtered out to remove debris and concentrated *in vacuo* using a rotary evaporator yielding two brown extracts of 140g and 90g for 50%MeOH in CH_2Cl_2 and 5% H_2O in MeOH, respectively.

From thin layer chromatography (TLC) analysis, the two extracts obtained had similar profiles and therefore were combined. A column of 80 mm diameter was packed with 2 kg of silica gel using *n*-hexane (C_6H_{14}) and left standing overnight. 200g of the extract was adsorbed onto a similar amount of silica gel and loaded onto the packed column. Elution was done using ethyl acetate (EtOAc) in *n*-C_6H_{14} solvent system, initially with neat *n*-hexane and then gradually increasing polarity with EtOAc as follows: 0.02:0.98, 0.04:0.096, 0.06:0.94, 0.10:0.90, 0.12:0.88, 0.15:0.85, 0.20:0.80, 0.25:0.75, 0.30:0.70, 0.35:0.65, 0.40:0.60, 0.5:0.5, 0.6:0.4, 0.7:0.3, 0.8:0.2, 0.9:0.1 and finally using neat EtOAc. Each fraction consisting of approximately 500 ml were concentrated *in vacuo* using a rotatory evaporator and their profiles were compared using TLC.

The fractions of the major column eluted with 0.20:0.80v/v EtOAc in *n*-C_6H_{14} yielded white amorphous solids of labd-13(*E*)-en-8α, 15-diol (**2,** 43mg).The fraction of the main column eluted with 0.25:0.75 EtOAc in *n*-C_6H_{14}yielded white crystals of 18-*nor*-labd-13(*E*)-en-8α,15-diol (**1,** 50mg) which were filtered by sanction and washed using 1% MeOH in CH_2Cl_2.

The rest of the fractions were combined based on the similarity of their TLC profiles into the following fractions; KNO-1A (18.2g), KNO-2A (20.4g), KNO-3A (15.5g) and KNO-4A (25.6g. Fraction 2A with an interesting TLC profile of only two spots was subjected to further purification using column chromatography with silica gel as the stationary phase and eluting with EtOAc/*n*-C_6H_{14} solvent system in increasing polarity. The earlier fractions (1-20) of the minor column afforded austroinulin (**3,** 34mg), isolated as white crystals.

Cell cultures

The cell lines used in the current work, their origins and their treatments were previously reported [26,34]. They included drug-sensitive CCRF-CEM leukemia and its multidrug-resistant *p*-glycoprotein over-expressing subline CEM/ADR5000 [35,36]

Resazurin reduction assay

Resazurin reduction assay [37] was performed to assess the cytotoxicity of the studied samples toward various sensitive and resistant cancer cell lines. The assay is based on reduction of the indicator dye, resazurin, to the highly fluorescent resorufin by viable cells. Non-viable cells rapidly loose the metabolic capacity to reduce resazurin and, thus, produce no fluorescent signal. Adherent cells were detached by treatment with 0.25% trypsin/EDTA (Invitrogen) and an aliquot of 1 x 10^4 cells was placed in each well of a 96-well cell culture plate (Thermo Scientific, Germany) in a total volume of 200 mL. Cells were allowed to attach overnight and then were treated with different concentrations of the studied sample. For suspension cells, aliquots of 2 × 10^4 cells per well were seeded in 96-well-plates in a total volume of 100 mL. The studied sample was immediately added in varying concentrations in an additional 100 mL of culture medium to obtain a total volume of 200 mL/well. After 24 h or 48 h, 20 mL resazurin (Sigma-Aldrich, Taufkirchen, Germany) 0.01% w/v in ddH_2O was added to each well and the plates were incubated at 37 °C for 4 h. The fluorescence was measured on an Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was done at least two times, with six replicate each. The viability was evaluated based on a comparison with untreated cells. IC_{50} values represent samples' concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve by linear regression using Microsoft Excel. The protocol has been published by us [38,39].

Results

Isolated compounds: The stem bark (50% MeOH in CH_2Cl_2) yielded three labdane type diterpenoids characterized as; 18-*nor*-labd-13(*E*)-ene-8α,15-diol (**1**) [24], labd-13(*E*)-ene-8α, 15-diol (**2**) [8,32] and austroinulin (**3**) [33] (Figure 1)

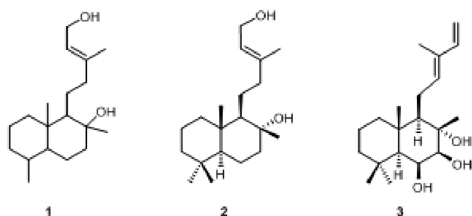

Figure 1. Structures of labdane diterpenoids, 18-*nor*-labd-13(*E*)-ene-8α,15-diol (**1**), labd-13(*E*)-ene-8α, 15-diol (**2**) and austroinulin (**3**) from the stem bark of *Croton sylvaticus* Hochst

Antiproliferative Potencies of studied samples

The crude extract was active at the tested concentration of 10 µg/mL with cell inhibition of 86.96±4.86 against the drug sensitive CCRF-CEM and 77.57±2.84% against the drug resistant CEM/ADR5000 leukemia cell lines. However, all the compounds displayed lower antiproliferative potencies as they exhibited cell inhibition <70% of the cell population at 10 µM. The cell inhibitions were as follows; 48.60±4.08% for **1**, 8.12±4.27% for **2** and 55.11.89±2.31% for **3** against CCRF-CEM and 33.83±4.79% for **1**, 20.26±1.77% for **2** and 46.03±07% for **3** against CEM-ADR5000. The cell inhibition of the standard drug, doxorubicin, was 94.89±0.86% and 24.20±2.89% against CCRF-CEM and CEM-ADR5000 cells, respectively. From these observations, it is clear that CEM/ADR5000 are resistant to doxorubicin while the activities of the isolated compounds were similar against the two cell lines. The similarities in their activities may be attributed to the similarities in their skeletal structures. Furthermore, labdane diterpenoids, **1** and **3** were more active than the standard drug against CEM/ADR5000 cells. These compounds could be modified to obtained analogues with improved activities against the drug sensitive and resistant cells.

Table 1. Cell viability of the stem bark extract (50 % MeOH in CH_2Cl_2) and compounds from *C. sylvaticus* Hochst and doxorubicin against CCRF-CEM, CEM/ADR5000

Samples	Cell Viability (%)	
	CCRF-CEM	CEM/ADR5000
Crude extract	13.04±4.86	22.43±2.84
1	51.40±4.08	66.17±4.79
2	91.88±4.27	79.74±1.77
3	44.89±2.31	53.97±0.70
Doxorubicin	2.64±0.86	78.97±2.89

Discussion

*Croton sylvaticus*is a potential herb in the treatment of cancer [40]. Previous studies have shown that the stem bark extract (50% MeOH in CH_2Cl_2) inhibited the proliferation of drug sensitive CCRF-CEM leukemia cancer cell lines by more than 50% at 10 µg/mL, following incubation for 72 h [17].

In the preliminary screening, the three labdane diterpenoids namely 18-*nor*-labd-13(*E*)-en-18α,15-diol (**1**), labd-13(*E*)-en-18α,15-diol (**2**), and austroinulin (**3**) isolated from stem bark of *Croton sylvaticus* displayed lower activity against both drug sensitive and resistant human leukemia cells as compared to the crude extract. However, the stem bark extract was more active with cell inhibitions of 86.96±4.86 against CCRF-CEM and 77.57±2.84% against CEM/ADR5000 leukemia cell lines. It was also noted that two of these compounds (**1** and **3**) comparatively were more active against the drug resistant phenotype cells than the standard drug, doxorubicin. However, **1** and **3** were substantially less active against the drug sensitive phenotype. Future studies can aim at modifications of these compounds towards improving their activities against the leukemia CCRF-CEM and CEM/ADR5000 cells. Previous studies have shown the potential of compounds with labdane type skeletons as anticancer compounds. For example, ent-12R,16-dihydroxylabda-7,13-dien-15,16-olide is known to inhibits proliferation of human lymphocytes present in peripheral blood mononuclear cells (PBMC) via induction of monocyte apoptosis [28-30]. Therefore, structural modifications of the three labdane diterpenoids are likely to yield analogues with cytotoxic effects.

The fact that the extract exhibited higher cytotoxicity as compared to the isolated compounds would be due to their synergistic effect which might have been lost during the purification process. Alternatively, the cytotoxic compounds would have been minor and therefore were not successfully isolated in the current study.

Conclusions

The labdane diterpenoids displayed low cytotoxicity against CCRF-CEM cells as compared to the stem bark extract (50% methanol in dichloromethane). Doxorubicin showed minimal cell inhibition against CEM/ADR5000 cells. Furthermore, **1** and **3** were more active than doxorubicin against CEM/ADR5000 cells. The lower activities of the compounds as compared to the crude extract would either imply that the compounds were experiencing synergistic interactions or that the active compounds would have been minor compounds that was not successfully isolated in the current study.

Authors' Contribution

KNO carried out the isolation experiment; KNO, EOK and LKO contributed to the identification of isolated compounds; KNO and LKO wrote and edited the manuscript; LKO and EOK designed the experiments.

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Conflict of interest

The authors have no competing interests

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