Original Research

Isolation and characterization of the antibreast carcinoma cell growth components of *Vernonia amygdalina* extracts

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Abstract

Vernonia amygdalina (VA) is widely used for medicinal and food purposes in tropical Africa. Many health benefits (antioxidant, antimicrobial, anticancer activities and more) of VA extracts have been reported. The mechanisms of actions have also been described. We have previously reported that VA extracts elicited growth inhibitory activities in human estrogen receptor-positive (ER^+) cells (MCF-7 cells) and ductal carcinoma cells (BT-549) *in vitro*. The active components in the organic solvent (chloroform)-extracted VA have been previously determined. However, the active components in the ethanolic extracts of VA have not been previously studied. Hence, the objectives of this study are to isolate and characterize the active components of the ethanolic extracts of VA using liquid–liquid extraction, thin layer chromatography and column techniques. Fractionation of the ethanolic extracts of VA yielded three fractions named A1, A2 and A3, and A2 retained the DNA synthesis-inhibitory activity of the extracts. Subsequent fractionation of A2 yielded fraction A2B whose activity was 16 and three times more potent than the ethanolic fraction and fraction A2, respectively. The treatment of cells with 100 μg/mL of either the ethanolic VA extracts, fraction A2 or fraction A2B resulted in a 23% ($P < 0.01$), 86% ($P < 0.0001$) and 97% ($P < 0.0001$) inhibition of DNA synthesis compared with vehicle-treated controls, respectively. Further purification of A2B by high-speed countercurrent chromatography and confirmed by spectroscopic analysis revealed that the major active components of A2B (65% by weight) were steroid glucosides.

Keywords: anticancer, *Vernonia amygdalina*, steroid glucoside, active components, chromatography

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Introduction

Vernonia amygdalina (VA), a member of the Asteraceae (or Compositae) family and commonly called bitter leaf, is a small tree of two to five metres in height that grows throughout tropical Africa. Classified in the genus *Vernonia*, VA is widely used for medicinal and food purposes. Previous studies show that VA extracts possess antioxidant,[1–5] antimicrobial or antiparasitic,[6–11] anticancer,[10,12–14] hepatoprotective[15] and cathartic activities.[16] We have previously reported that VA aqueous extracts potently inhibit the growth of human estrogen receptor-positive (ER^+) cell line (MCF-7) *in vitro* at low concentrations (μg/mL) by modulating the extracellular signal-regulated kinase 1 and 2 activities,[14] cytochrome P450 enzymes[17] and by disrupting cell membrane.[18] Evidence shows that 32 compounds have been isolated and identified from VA. They are classified into three flavonoids,[1] one steroidal alcohol,[19] six sesquiterpene lactones,[2,6,12,20,21] 10 steroid glucosides[22–26] and 12 fatty acids.[3] The sesquiterpene lactones and flavonoids represent not only the most studied, but also the most chemically active classes of all. The sesquiterpene lactones and steroid glucosides possess anticancer[12] and antihelminthic[21] activities, respectively. There is paucity or non-existence of data on the effects of steroid glucosides on solid tumors such as adrenocarcinomas. Hence, the objective of this study is to isolate active fractions and components from VA against the proliferation of ER+ human breast adrenocarcinoma cell line (MCF-7) cells. We have previously utilized solvent

extraction, spectrophotometric and chromatographic techniques to fractionate and identify the anticancer activity components in VA extracts.[27]

Generally, the separation of natural compounds has relied on liquid–liquid extraction, thin layer chromatography (TLC), column chromatography and high-performance liquid chromatography. In this study, we have fractionated VA extracts to several small fractions and have determined their biological activities by bioactivity-guided assay (DNA synthesis). One of the fractions (high-speed countercurrent chromatography) contained steroid glucosides as confirmed by chromogenic reagent assay.

Materials and methods

Human breast cancerous cell line (MCF-7) was purchased from ATCC (Manassas, VA, USA). RPMI 1640 medium, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY, USA). [^{3}H]-thymidine (1 mCi/mL) was purchased from MP Biochemicals (Solon, OH, USA). Methanol (AR grade) and other chemicals were obtained from Sigma-Aldrich Chemicals (St Louis, MO, USA) or Fisher Scientific International Inc. (Hampton, NH, USA). Silica gel was obtained from Qingdao Haiyang Chemical Co., Ltd (Qiongdao, Shandong, China). The organic solvents (chloroform, methanol, ethanol and *n*-butanol) and other chemicals used in Guangxi University (Nanning, Guangxi, China) were purchased from Xilong Chemical (Shantou, Guangdong, China) and Nanning Lantian Experiment Equipment Co, Ltd (Nanning, Guangxi, China).

Sample collection and preparation

Pesticide-free fresh VA leaves were collected in Benin City, Nigeria, following the GAP (Good Agricultural Practices) protocol. The leaves were rinsed with distilled water and spread out evenly on galvanized-wire screens with the edges bent upward two inches on all sides. The galvanized-wire screens were placed in a specially constructed dryer and heated to 55–60°C for complete dryness within four hours. They were imported from Benin City, Nigeria (Phytosanitary Certificate No. IK2006/075, Plant Quarantine Service, Federal Ministry of Agriculture, Nigeria) and authenticated by Dr G Begonia, a botanist (Department of Biology, Jackson, State University, MS, USA), based on their vegetative characteristics (e.g. shape and color of leaves, stem).

Preparation of ethanolic extracts and liquid–liquid extraction

VA dried leaf powder (about 6 kg) was placed in a stainless-steel tank and soaked with 30 L 85% ethanol (EtOH) each time under 50–60°C. The sample was extracted five times, and all ethanol solutions were combined together. Ethanol was removed from the ethanol solutions by rotary evaporators to obtain a dark green condensate. The condensate was diluted with distilled water followed by hexane, chloroform and *n*-butanol extraction and named A1 for hexane, A2 for chloroform and A3 for *n*-butanol (Figure 1).

Column chromatography

The A2 fraction was separated further with chromatography of silica gel (160–200 or 300–400 mesh) into fractions A2A, A2B, A2C, A2D and A2E (Figure 2).

Preliminary bioactivity-guided assay data revealed two active fractions: A2A (modest activity) and A2B (most potent). Hence, there was further separation of fraction A2B into subfractions A2B5, A2B15, A2B25, A2B35 and A2B45 (Figure 2).

High-speed countercurrent chromatography

Subsequent chromatographic separation was made followed by high-speed countercurrent chromatography (HSC),

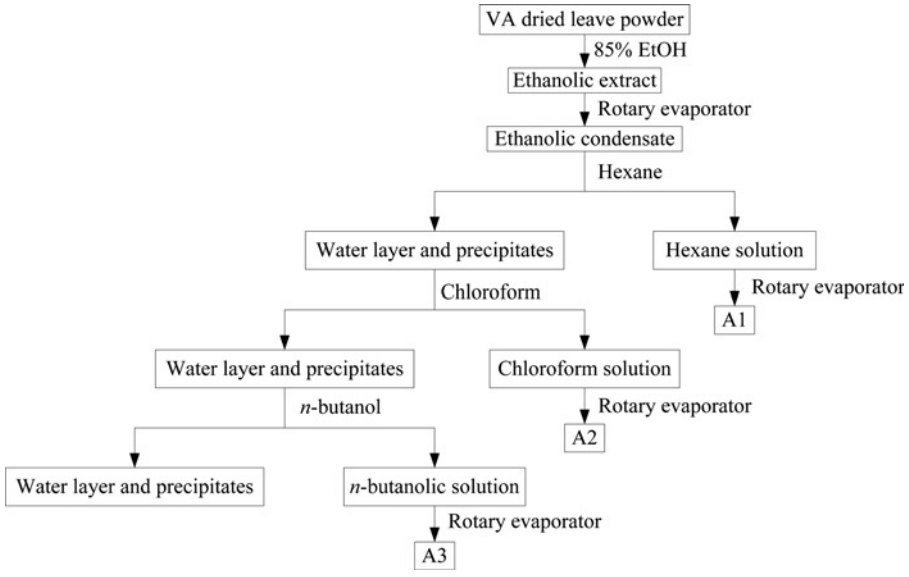

Figure 1 Flow diagram showing the liquid–liquid extraction and separation processes of fractions A1, A2 and A3 using different solvents

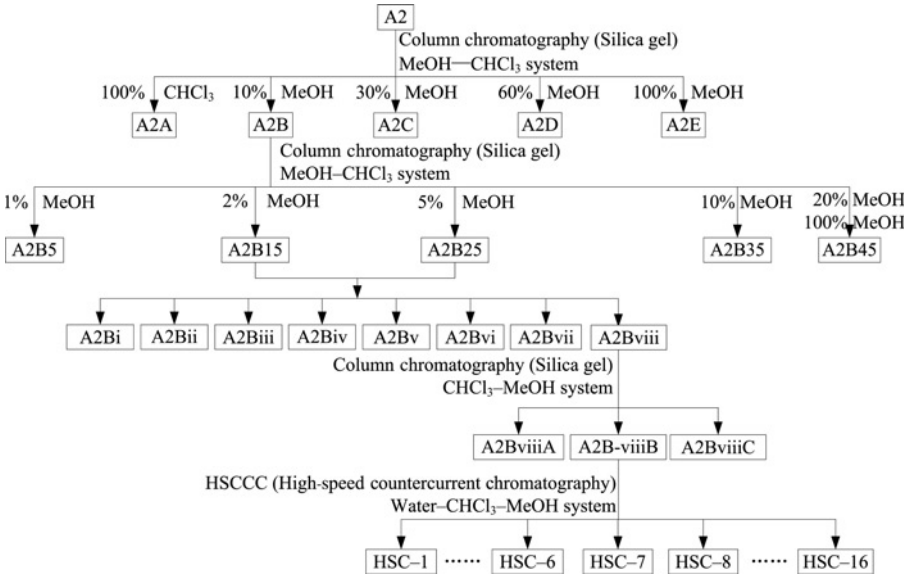

Figure 2 Flow diagram showing the column chromatographic and further separation processes of fractions A2 and A2B using different solvents. Bioactivity-guided assays indicate that the fraction A2B is the most active in A2

a technique used to separate components of a mixture based on the partitioning of individual solutes in immiscible solvent phases at 25°C. The solvent system was $CHCl_3$–MeOH–water (2:2:1). The stationary phase was the bottom layer. The mobile phase was the top layer. The sample (100 μg) was dissolved in 2 mL stationary phase, filtrated by a 15 mm PTFE syringe filter (pore size: 0.45 μm), and the filtrate was injected into the instrument. Sample analyses were carried out on an HSC-TBE-300A System (Shanghai Tauto Biotech Co, Ltd, Shanghai, China). The flow of the mobile phase was 2.0 mL/min; the wavelength and speed were 254 nm and 700 r.p.m., respectively.

Preparation of phosphomolybdate reagent (steroid reagent), color development and component analysis

Five to fifteen grams of phosphomolybdic acid hydrate were dissolved in 100 g ethanol to prepare 5–15% phosphomolybdic acid hydrate ethanol solution. The TLC plates were sprayed with 5–15% phosphomolybdic acid hydrate ethanol solution. After that, the TLC plates were heated for five minutes under 100°C. Steroids or steroid glucosides revealed blue spots.[28] The sample used in the component analysis experiment was dissolved in methanol and then applied to the TLC plates by capillaries. The TLC plates were developed with methanol–chloroform solution (1:9).

DNA synthesis assays

DNA synthesis was determined by the incorporation of [^{3}H]-thymidine into cellular nucleic acids. The cells were grown to about 60% confluence in 35 mm diameter tissue culture plates with RPMI 1640 medium containing 1% antibiotic–antimycotic solution containing 10,000 U/mL penicillin, 10,000 μg/mL streptomycin and 25 μg/mL amphotericin B (fungizone), and 10% FBS in a humidified CO_2 incubator. Stock solutions (10 mg/mL) were prepared by dissolving 20 mg of fractions (either ethanol extract, A2, A2A or A2B) of VA extracts in 2 mL ethanol, and the cells were treated with either 50 or 100 μg/mL final concentrations of ethanol extract, A2, A2A or A2B ethanolic solution. In other experiments, stock solutions (5 mg/mL) were prepared by dissolving 10 mg of fractions (A2B and A2B15–45) of VA extracts in 2 mL ethanol, and the cells were treated with either 25 or 50 μg/mL final concentrations of either fraction A2B or A2B15–45 ethanolic solution. Untreated cells served as negative controls while ethanol only-treated cells served as positive controls. The cells were then incubated for 18 h in a 37°C humidified 5% CO_2 incubator before the addition of 2 μCi/mL of [^{3}H]-thymidine per 35 mm well, followed by an additional six-hour incubation time. The incubation was stopped by media aspiration, and then followed by three sequential washes with cold PBS, pH 7.4. Two (2 mL/35 mm) of ice-cold 10% trichloroacetic acid (TCA) was added and the cells were incubated for 10 min at 4°C. TCA solution was aspirated and the cells were washed three times with ice-cold distilled water, and then solubilized with 1 mL of 0.5 mol/L NaOH solution at 37°C for 30 min. Upon solubilization, 1 mL per well aliquot samples was transferred to scintillation vials into which 5 mL of scintillation cocktail was added. DNA synthesis in treated and controlled cells was quantified by a liquid scintillation analyzer (Try-Carb 2700TR).

Experimental design

Three experiments were conducted using MCF-7 cells for each experiment. Each treatment group was further subdivided into three replicates. The cells were grown under similar conditions. The experimental units (cells) possess similar genetic and other traits with only a difference of

experimental treatments to determine the true effects of treatments.

Statistical analysis

Data collected were analyzed using one-way analysis of variance with post-test (GraphPad Prism, version 4). Means were further classified using Tukey–Kramer honestly significant difference test. P values of <0.05 were considered significant.

Results

Ethanol VA extract, fractions A2, A2A and A2B inhibited DNA synthesis

The treatment of cells with either ethanolic VA extracts, subsequent fraction A2 or subfraction A2B inhibited DNA synthesis in a concentration-dependent fashion. The starting ethanolic VA extracts (100 μg/mL) inhibited DNA synthesis by 23% ($P < 0.01$) compared with ethanol only-treated control. Fraction A2 (derivative of the ethonolic VA extracts) inhibited DNA synthesis by 86% ($P < 0.0001$) compared to ethanol only-treated control. This corroborates our previous report that fraction A2 represents the most active fraction of the three fractions (A1, A2 and A3) derived from ethanolic VA extract fractionation.[27] Subfraction A2B (derivative of A2) inhibited DNA synthesis by 97% ($P < 0.0001$) compared with ethanol only-treated control (Figure 3). All treatments (ethanolic VA extracts, fraction A2 and sub-fraction A2B) were compared at the same concentration of 100 μg/mL (Figure 3).

Further fractionation of fraction A2B into A2B5, A2B15, A2B25, A2B35 and A2B45 fractions did not improve the activity of A2B. For example, the most active subfractions of A2B were A2B15 and A2B25. At equal concentrations of 50 μg/mL, neither significantly affected DNA synthesis ($P > 0.05$) compared with fraction A2B-treated cells (Figure 4). In contrast, fractions A2B35 and A2B45 decreased ($P < 0.001$) the activity of A2B at equal concentration.

The ultraviolet spectrum of HSC-7

The Unico (Shanghai, China) UV-Visible spectrophotometer (UV-2102PCS) with a 1 mL quartz cuvette was used and an absorption peak of about 200–300 nm was recorded. Anhydrous methanol was used as a reference solvent for HSC-7. The ultraviolet (UV) result (Figure 5) shows four absorbance peaks around 227, 235, 242 and 251 nm. These UV data were very similar to 10 steroid glucosides previously isolated from VA.[22–26] The comparison of HSC from this study with the previously isolated steroid glucosides is presented in Table 1.

The infrared spectrum of HSC-7

The fraction, HSC-7, was analyzed by a Nicolet (Madison, WI, USA) Fourier transform infrared (FT-IR) spectrometer (NEXUS470 FT-IR). It showed absorption peaks at 3429 cm^{-1} for hydroxyl groups, 1075 and 1039 cm^{-1} for C–O bond stretching, 1635 and 3031 cm^{-1} for C=C double bound and 1777 and 1731 cm^{-1} for the carbonyls (Figure 6). The absorption of C=O groups in a non-cyclic ester occurred at about 1735 cm^{-1} and that of γ-lactones was around 1770 cm^{-1}.[29] The C–O stretching vibrations in non-cyclic esters or γ-lactones occurred at 1258 and 1160 cm^{-1}, respectively. The peaks at 2962, 2934, 2876, 1445 and 1377 cm^{-1} are the absorption bands due to aliphatic CH, CH_2 and CH_3 groups. These data are very similar to the 10 steroid glucosides previously isolated from VA[22–26] (summarized in Table 1).

Component analysis of HSC-7

HSC-7 was dissolved in methanol and then applied to the TLC plates by capillaries. The TLC plates were developed with methanol–chloroform solution (1:9), and then phosphomolybdate reagent was sprayed on the TLC plates and

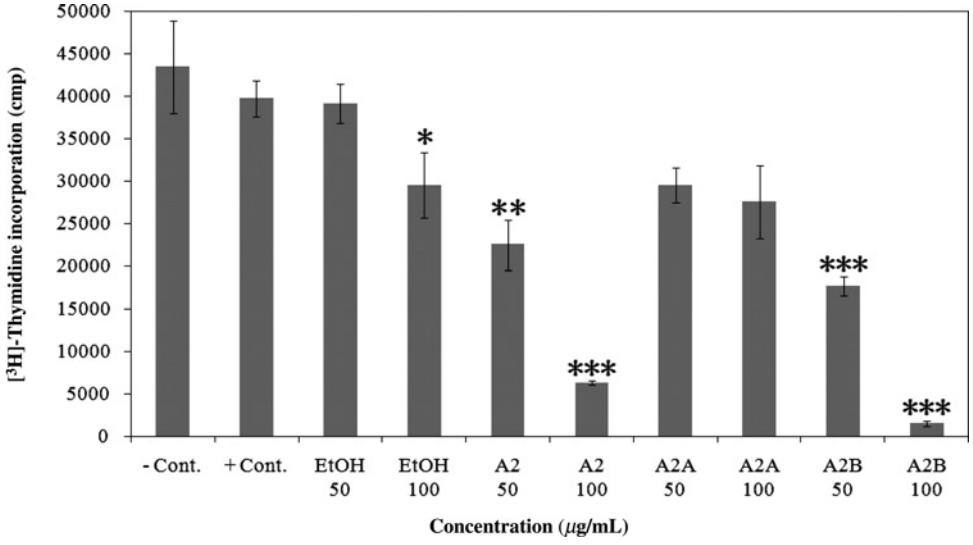

Figure 3 Ethanol extract, A2, A2A and A2B inhibited DNA synthesis. Cells at the logarithmic growth phase were treated with either 50 or 100 μg/mL for 18 h before the addition of 1 μCi/mL [^{3}H]-thymidine for six hours. Each data point represents the mean of three independent experiments done in duplicates ($n = 9$). $^*P < 0.03$; $^{**}P < 0.001$; $^{***}P < 0.0001$

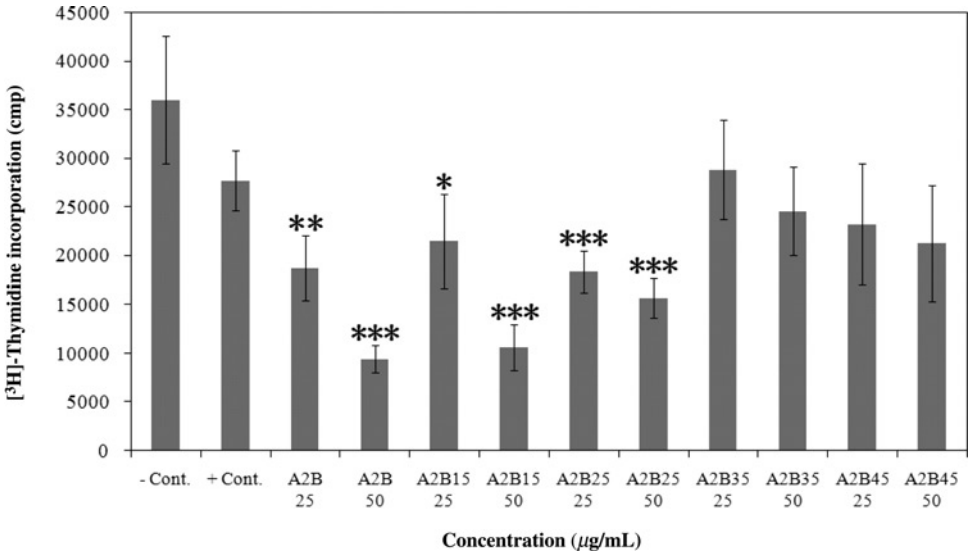

Figure 4 A2B, A2B15, A2B25, A2B35 and A2B45 inhibited DNA synthesis. Cells at the logarithmic growth phase were treated with either 25 or 50 μg/mL for 18 h before the addition of 1 μCi/mL [^{3}H]-thymidine for six hours. Each data point represents the mean of three independent experiments done in duplicates ($n = 9$). $^*P < 0.03$; $^{**}P < 0.001$; $^{***}P < 0.0001$

heated for five minutes under 100°C to give a round blue spot which was at $R_f = 0.7$ position.

Discussion

Medicinal benefits of VA leaf extracts (antioxidant, antimicrobial, anticancer activities and more) have been reported.[1–16] Evidence suggests that these health benefits are mediated by a variety of compounds representing five classes of compounds: flavanoid, three compounds; steroidal alcohol, one compound; sesquiterpene lactone, six compounds; sterioid glucoside, 10 compounds and fatty acid, 12 compounds. The flavonoids and sesquiterpene lactones represent the most studied compounds of all; studies show that sesquiterpene lactones possess the anticancer activity of the VA extracts. Another interesting class of compounds is the steroid glucoside. These compounds (steroid glucosides) are believed to be responsible for the antihelminthic activity of the VA extracts.[21] Jisaka *et al.*[21] using adults pairs of schistosomes (*S. japonicum*) obtained from mice infected with cercariae, demonstrated that steroid glucosides (vernodalin and vernonioside B_1) profoundly inhibited the movement and egg-laying capabilities of schistosomes at 200 ppm concentration *in vitro*. Thus, this provided evidence for antihelminthic activity of steroid glucosides. These findings also uncovered the potential use of steroid glucosides as treatment against schistosomiasis. Schistosomiasis is one of the most common parasitic infection diseases throughout Africa; its symptoms include loss of appetite, lethargy, constipation, dark-colored urine and abdominal discomfort.[21,30]

In this study, ethanolic extracts of VA were separated by liquid–liquid extraction and column chromatography

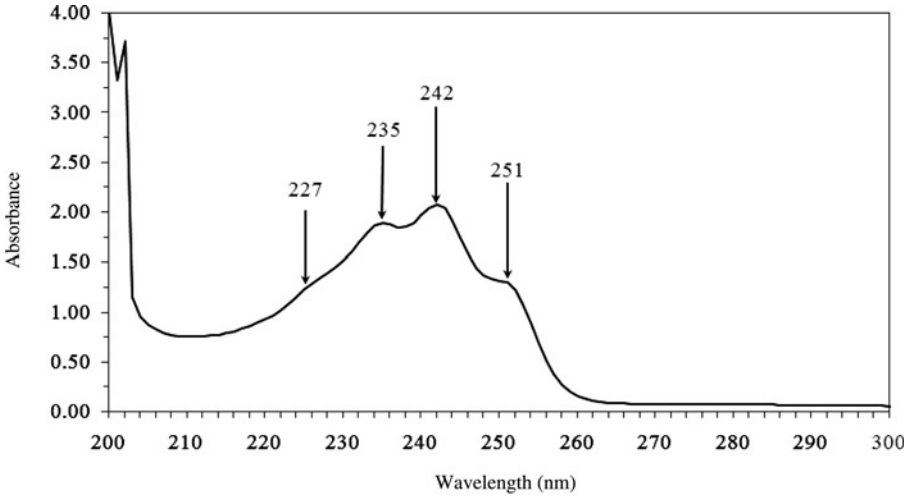

Figure 5 Ultraviolet (UV) spectrum of HSC-7. The sample UV spectrum shows four peaks at 227, 235, 242 and 251 nm. The data were measured on a Unico UV-Visible spectrophotometer (UV-2102PCS) from 200–300 nm. Reference solvent used was anhydrous methanol

Table 1 UV and IR data comparison of HSC-7 and 10 known steroid glucosides in VA[20–24]

	UV data (nm)	IR data (cm^{-1})
HSC-7	242, 235, 227, 251	1771, 1731
Vernonioside A_1	243, 235 (236), 250 (251)	1760
Vernonioside A_2	243, 235, 250	1735
Vernonioside A_3	242, 235, 250	1740
Vernonioside A_4	241, 234, 228, 251	1735
Vernonioside B_1	243, 235, 250	1775
Vernonioside B_2	242, 235, 251	N/A
Vernonioside B_3	242, 235, 227, 250	1760, 1725
Vernonioside C	244, 252, 336	1770
Vernonioside D	241, 234, 251	N/A
Vernonioside E	241, 234, 251	N/A

N/A: not application; IR, infrared; UV, ultraviolet; HSC, high-speed countercurrent chromatography; VA, *Vernonia amygdalina*

followed by bioactivity-guided assays to determine the active fractions and compositions. Serial purification of the active ethanolic fraction significantly increased its potency (DNA synthesis-inhibitory activity by several folds). The whole extract (VA leaves extracted with 85% ethanol), also referred to as ethanolic extracts of VA, inhibited ($P < 0.01$) DNA synthesis by 23% at 100 μg/mL. Purification of the ethanolic fraction to produce fraction A2 improved activity to greater than 85% (inhibition of DNA synthesis) ($P < 0.0001$) at the same concentration of 100 μg/mL (Figure 3). Further purification of fraction A2 to A2B extended the DNA synthesis-inhibitory activity to 97% ($P < 0.0001$) at the same concentration. Further purification and separation of fraction A2B not only prevented increase in activity, but also compromised the activity of A2B ($P < 0.05$) (Figure 4) compared to intact fraction A2B. This phenomenon suggests that these steroid glucosides work in concert with other components in A2B for synergism. Thus, fraction A2B may contain critical components required for maximum activity.

Using HSC, UV, infrared (IR) and component analyses, we have determined that the major components of A2B are steroid glucosides which represent 65% of A2B by weight, followed by the purification of these glucosides. Work is underway in our laboratories to uncover the identity and structure of the remaining 35% of the A2B. Further purification of A2B by HSC and confirmed by spectroscopic and chromogenic analysis revealed that the major active components of A2B (65% by weight) were steroid glucosides. Further separation and purification activities revealed that A2Bviii was the main component of A2B in quantity and its content was up to 65% by weight. Some pigments of A2Bviii were also removed before HSC separation. The middle fraction A2BviiiB was used for HSC separation. Sixteen samples (HSC-1 to HSC-16) were collected. Each fraction was lyophilized; HSC-6, HSC-7 and HSC-8 were ivory powder. HSC-7 was chosen for the UV, IR and component analyses.

In conclusion, we have shown that serial fractionation/purification of ethanolic extracts of VA improved its DNA synthesis inhibitory (anticancer) activity by 22.4-fold. The increasing order of activity compared to ethanol-only-treated controls is as follows: ethanolic VA extracts $<$(5.3-fold) fraction A2 $<$(4.2) A2B. Fraction A2B of VA extracts, composed mainly of steroid glucosides as confirmed by spectroscopic chromogenic reagent assay techniques, possesses anticancer activity. This observation is supported by earlier reports of cytotoxic actions of steroid glucosides in L-1210 and P-388 leukemia cells, although

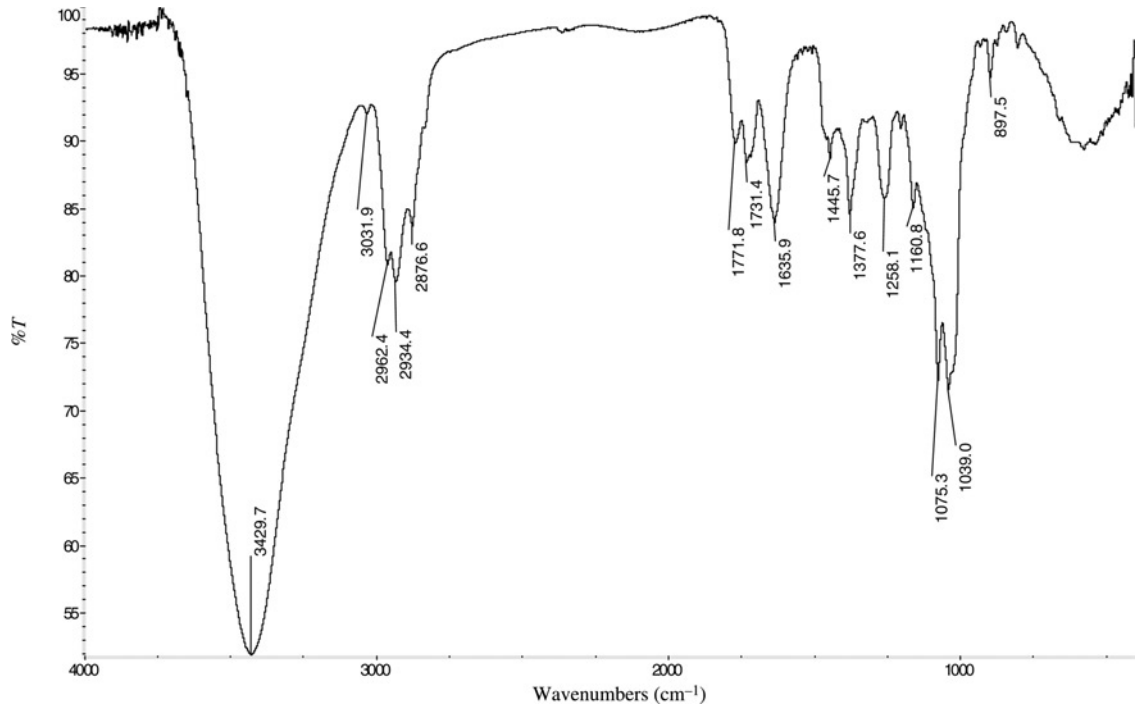

Figure 6 IR spectrum of HSC-7. The data were measured on a Nicolet (Madison, WI, USA) FT-IR spectrometer (NEXUS470 FT-IR) from 4000–400 wave numbers. It was made on plates having 7 mm diameter with KBr (potassium bromide, IR grade) before being detected by the FT-IR spectrometer. FT-IR, Fourier transform infrared

leukemia represents non-solid tumors (reviewed by Jisaka *et al.*, 1992). To the best of our knowledge, the present study presents evidence for the first time that, like the sesquiterpene lactones, steroid glucosides possess anticancer activity against breast adrenocarcinoma (solid tumor). Future studies will focus on the areas of further separation/purification of fraction A2B to identify additional component(s); determination of whether or not steriodglucosides exclusively constitute the active components of fraction A2B; and the investigation of synergism if other active compounds exist.

Author contributions: All authors participated in the experimental design, analyses and interpretation of the data, and review of the manuscript.

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