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Full Length Research Paper

Methoxy-flavones identified from *Ageratum conyzoides* induce capase -3 and -7 activations in Jurkat cells

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New therapies for leukemia are urgently needed due to adverse side effects, tumor resistance and lack of selectivity of many chemotherapeutic agents in clinical use. *Ageratum conyzoides* has been used in folklore medicine for managing leukemia and other cancers. Thus, this study aimed to investigate the effects of fractions, sub-fractions and purified compounds from the ethanol leaf extracts of *A. conyzoides* against Jurkat cells-model for acute T cell leukemia. A two-dimensional purification process using normal phase flash, followed by reverse phase purification was necessary to isolate pure methoxy-flavones, which were further characterized by Nuclear Magnetic Resonance (NMR) and MS-MS. The effect of fractions or pure compounds on cell viability was determined using either the MTT reagent or CellTiter-Blue® assay, while the caspase-3 and -7 activation was tested with Caspase-Glo® 3/7 assay. Prediction of compounds' drug disposition profiles *in vivo* was measured with biomimetic affinity chromatography methodologies.

Key words: *Ageratum conyzoides*, methoxy-flavones, Jurkat, biomimetic affinity chromatography, cell viability.

INTRODUCTION

Medicinal plants remain an important source for the discovery of promising anticancer compounds. Notable examples include vincristine and vinblastine isolated from *Catharanthus roseus* as well as taxol isolated from *Taxus brevifolia* for the treatment of leukemia (Moudi et al., 2013). Although these therapies are beneficial, problems such as adverse side effects, tumor resistance and lack of cancer cell selectivity are often reported. To improve current treatment modalities, the search for novel anticancer agents with minimal side effects is imperative.

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Ageratum conyzoides L., commonly called Billygoat weed, is an annual herb that belongs to the family of Asteraceae, and is found in several tropical countries, including Ghana. This plant has been used in folklore medicine for the treatment of fever, pneumonia, cold, rheumatism, spasm, headache, and healing wounds (Shirwaikar et al., 2003).

Additionally, the crude ethanol leaf extracts and its fractions have been reported to show cytotoxic activity against different cancer cells lines (Acheampong et al., 2015; Adebayo et al., 2010). These suggest that *A. conyzoides* may possess anticancer activity which would be in line with its use by traditional Ghanaian herbalists to manage leukemia and other cancers. Given the limited scientific data, there is an urgent need to isolate the bioactive components responsible for the observed anticancer activity, in *A. conyzoides* and subsequently determine their mechanism of action.

A key mechanism by which chemotherapeutic agents may induce cancer cell death is through apoptosis-programmed cell death (Xue et al., 2014). The apoptosis mechanism is initiated by several factors involving a cascade of intracellular events, leading to the activation of downstream caspase enzymes (Gomes et al., 2010). Caspase-3 and -7 are effector mediators of apoptosis and represent a popular target for novel therapeutic strategies (Mukhopadhyay et al., 2014).

Moreover, in the drug discovery process, there are other important factors considered during the search of novel anticancer components as potential drug candidates. These include physiochemical properties of compounds like absorption, distribution, metabolism and excretion (ADME) and pharmacokinetics (PK). Often, animal models have been used to screen large libraries of compounds for their ADME/PK properties (Hollósy et al., 2006) and usually this processes are time consuming, labor-intensive, and ethically sensitive, which limits their usefulness in drug discovery.

Therefore, determination of these physiochemical properties in the early drug evaluation phase may allow for a more rapid selection of compounds that demonstrate suitable bioavailability and satisfactory ADME/PK properties *in vitro*, prior to further mechanisms of action studies *in vitro*.

In the present study, rarely studied methoxy-flavones were isolated and characterized from the ethanol leaf extracts of *A. conyzoides*, and their effect on cell viability as well as activation of caspase-3 and -7 in Jurkat cells were investigated. Finally, the physicochemical profiles of the isolated compounds were determined.

METHODOLOGY

Chemicals and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), culture media, RPMI-1640, penicillin, streptomycin and L- glutamine were purchased from Sigma-Aldrich (St. Louis, MO). The Caspase-Glo® 3/7 Assay system and CellTiter-Blue® cell viability assay kits were obtained from Promega (Madison, WI), while fetal bovine serum (FBS) was from ThermoFisher Scientific (Waltham, MA). All the chemicals were of analytical grade, including ethanol, heptane, ethylacetate, methanol, trifluoroacetic acid, acetonitrile (ACN), ammonium hydroxide, DMSO, formic acid, deuterated chloroform ($CDCl_4$), deuterated ACN (C_2D_3N) and tetramethyl silane (TMS) were also purchased from Sigma-Aldrich.

Collection and preparation of plant extract

A. conyzoides leaves were handpicked from the main campus of KNUST, Kumasi in October, 2013. The leaf was authenticated at the Department of Pharmacognosy, KNUST, Kumasi, Ghana by Dr. George Sam (taxonomist) and a voucher specimen (KNUST/HMI/2014/ WP005) was deposited in the herbarium for reference purpose. The leaves were then washed three times under running water, air-dried for two weeks in the shade at room temperature, pulverized, and stored in air-tight containers. Pulverized leaf samples (100 g) were extracted twice with ethanol (50%, v/v), at room temperature on a shaker for 24 h. Supernatant was subsequently filtered to remove particulate matter, and evaporated with a rotary evaporator (Buchi R-205, Switzerland) at 40ºC to yield concentrated aqueous portion which was frozen and lyophilized in vacuum freeze-dryer (Labconco, England) to obtain 20 g of crude extracts.

Fractionation, isolation and characterization of compounds

The crude ethanol extract (5 g) was subjected to normal-phase flash chromatography (NPFC) (InterchimPuriflash 450, Montluçon, France) using a silica gel column (Biotage SNAP Ultra 25 g, 25 µm, Stockholm, Sweden). The menstruum consisted of heptane, ethylacetate: methanol with a gradient of 100% heptane to 100% ethylacetate to 100% methanol at a flow rate of 30.0 mL/min. The column eluents were sequentially collected and combined into six different fractions (A-F) based on UV absorbance (260 to 320 nm) (Ultraviolet-UV detector, Montluçon, France). The fractions were evaporated at 40°C under reduced pressure and stored at -20°C until it was needed for bioassays or further analysis.

Fraction D was purified based on its ability to reduce Jurkat cells viability. Initially, it was subjected to gradient scouting runs, and later to preparative reverse phase High-performance liquid chromatography-mass spectrometry (HPLC-MS) (Waters Prep LC-MS automated fraction collection system, MA). A volume of 1.5 mL at a concentration of 60 mg/mL were injected into column (XBridge, C18, 3.5 µm 3.0x30 mm, Waters Corp, MA) at a flow rate of 75 mL/min using a binary gradient with eluent A consisting of water and 5 mM ammonium hydroxide and eluent B consisting of ACN. The gradient was initiated at 5% and increased to 95% of eluent B over 5 min. Additionally, bioactive sub-fractions of D were purified by the same method using 1.5 mL injections at a concentration of 6 to 8.7 mg/mL with a gradient that began at 25% and increased to 50% of eluent B over 3.5 min. Structures were elucidated by mass and nuclear magnetic resonance spectroscopies.

For mass spectrometry characterization, the Sciex 6600 Q-TOF (AB Sciex LLC, MA, USA) was calibrated using the Sciex external calibration delivery device before running samples, and calibration was better than 5 ppm across the mass range of 150 to 950 Da in which 1 mg/mL of pure isolate was dissolved in absolute DMSO and further diluted to 10 µg/mL in absolute DMSO. Also, 10 µL of each sample was injected into the column at a flow rate of 0.6 mL/min over 6 min, (BEH C18, 2 × 50 mm, 1.7 µm, Waters, Milford, MA), and subjected to collection conditions of A: water with 0.1 % formic acid (v/v) and B: ACN with 0.1% formic acid (v/v).

For Nuclear Magnetic Resonance (NMR) spectroscopy characterization, compounds were dissolved in deuterated chloroform or acetonitrile ($CDCl_4$ or C_2D_3N). Tetramethyl silane (TMS) was used as an internal standard. These were subjected to 1-D NMR and 2-D NMR (COSY, NOESY, HSQC and HMBC) analyses at 400 MHz (^1H and ^{13}C) and spectra were recorded with a Bruker WM 400 (Bruker Optics Inc., Billerica, MA) (see supplementary Data information for spectra data of pure compounds).

Cell culture

Human leukemia-immortalized T lymphocyte (Jurkat), Clone E6-1 (ATCC TIB-152) was purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture was carried out as previously described with slight modifications (Ham et al., 2012). The Jurkat cells were cultured in RPMI-1640 medium, supplemented with 1% penicillin streptomycin L-glutamine (PSG) and 10% fetal bovine serum (FBS). The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and sub-cultured on reaching 90% confluency.

Determination of cell viability

The effects of fractions A to F and subfractions of D on cell viability were tested with MTT reagent, as described previously (Ayisi et al., 2011). Similarly, pure compounds were tested against Jurkat cells using the CellTiter-Blue® assay in accordance with the manufacturer's protocol. Briefly, 100 µL of cells were seeded in 96-well plates at a density of 10,000 cells per well. Afterwards, cells were treated for 72 h with 200 µg/mL of fraction A through F or with sub-fractions of D in a concentration range of 0 to 100 µg/Ml. Pure compounds were incubated with Jurkat cells in a concentration range of 0 to 250 µM for 24 h. Uv absorbance were measured at 570 nm in a microplate reader (Tecan-PC infinite M200 Pro, Switzerland), while fluorescence at $560_{Ex}/590_{Em}$was determined in a microplate reader (PerkinElmer, Waltham, MA). Cell viability was expressed as percentage of DMSO-treated controls.

Caspase -3 and -7 activation by compounds

Caspases -3 and -7 activation was examined with the Caspase-Glo® 3/7 assay kit, according to the manufacturer's protocol. 10,000 cells per well were seeded in 96-well plates and incubated with the pure compounds in a concentration range of 0 to 250 µM for 24 h. Afterwards, 100 µL of Caspase-Glo® 3/7 reagent was added and incubated for 1 h at room temperature. Luminescence at $485_{Ex}/527_{Em}$ was measured in a microplate reader (PerkinElmer, Waltham, MA). The measured Caspase -3/7 activation was compared to DMSO-treated controls.

Prediction of *in vivo* drug distribution by biomimetic affinity chromatography

Drug like profiles of compounds including albumin binding, affinity for phosphatidylcholine, volume of distribution and lipophilicity were determined as described (Valko et al., 2000, 2003; Jiang and Reilly, 2012; Kerns and Di, 2003; Hsiao et al., 2014). In summary interaction of compounds with immobilized artificial membrane (IAM) and human serum albumin (HSA) were calculated from calibrated gradient HPLC retention times using affinity chromatography. Volume of distribution predictions were calculated from IAM and HSA values. Additionally, lipophilicity of compounds was determined from gradient HPLC retention times on a standard C18 reverse phase method.

Statistical analysis

Statistical analyses were carried out with Graph Pad Prism 5 (La Jolla, CA, USA); results are expressed as the mean ± SD of triplicates of two independent experiments. One-way analysis of variance (ANOVA) was used for statistical analyses and levels of significance were considered at $p < 0.05$.

RESULTS

Fractionation of the crude extract by normal-phase flash chromatography resulted in six fractions; A to F, of which A, B and C eluted within heptane, D within ethylacetate and E and F within methanol (Figure 1). Each fraction was further evaporated and resulting portions were screened for cytotoxic activity against Jurkat cells using 200 µg/mL for 72 h.

Figure 2 demonstrates that fractions C and D significantly reduced the cell viability of Jurkat cells ($p<0.001$) as compared to the other fractions. Fraction D was chosen for further purification using reverse phase HPLC-MS to obtain sub-fractions 1 through 11. These were subsequently investigated for their effect on cell viability. The cell viability of Jurkat cells was unaffected by sub-fractions 1 and 2, as well as 4 through 6. However, sub-fractions 8 and 10 significantly reduced the cell viability of Jurkat cells (Table 1), whereas IC_{50} values of sub-fractions 3 and 7 were slightly higher. The active sub-fractions (3, 7, 8 and 10) were subjected to further purification which resulted in the isolation of five known but rarely studied methoxy-flavones (Figure 3). Compounds 1, 2 and 3 were found to significantly affect the cell viability of Jurkat cells in a concentration dependent pattern (Figure 4), while no such effect was observed for compounds 4 and 5 (data not shown). Moreover, only compounds 1 and 2 significantly induced caspase -3 and -7 activations in Jurkat cells in a dose-dependent manner compared to solvent control (Figure 5). Additionally, the physicochemical properties of the pure compounds were determined to estimate their disposition *in vivo*. Table 2 shows that all the tested compounds had acceptable albumin binding (% HSA), staying under 95%, and a low to medium affinity for phosphatidylcholine, CHI $IAM_{7.4}$ scores less than 60. Moreover, the predicted volume of distribution, Log V_d, shows low values for all compounds. Finally, lipophilicity measurements at pH 7.4, Log $D_{7.4}$, are under three, indicating acceptable values for these "drug like" small molecules, particularly since they have molecular weight under 500 Da.

DISCUSSION

It has been reported that the crude ethanol leaf extracts

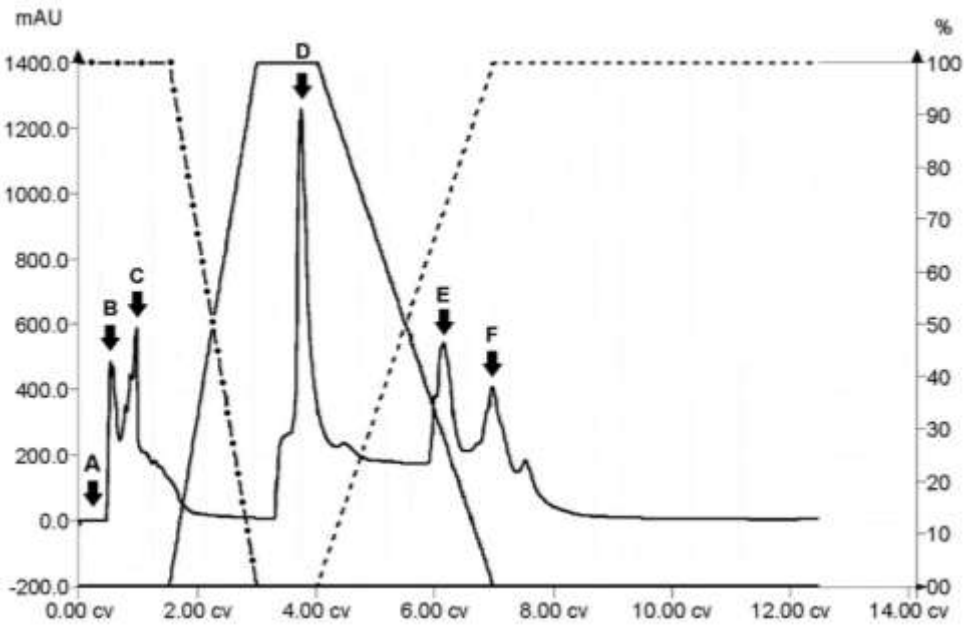

Figure 1. Normal phase flash chromatogram with heptane:ethylacetate:methanol as mobile phase. The column eluents were collected, pooled into 6 fractions (A-F) and evaporated at 40ºC.

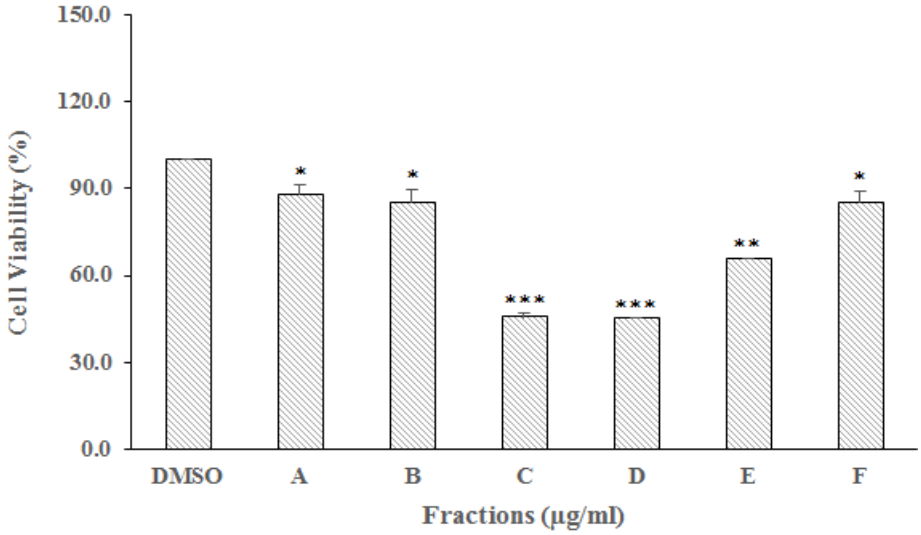

Figure 2. Effect of crude fraction A to F on the cell viability of Jurkat cells. The cells were treated for 72 h at concentrations of 200 µg/mL. The percentage of cell viability was determined by MTT assay and expressed as % compared to 0.1% DMSO-treated controls. Bar graphs represent means ± SD of triplicates from two independent experiments.

of *A. conyzoides* affects the viability of several cancer cell lines (Acheampong et al., 2015; Adebayo et al., 2010). In the present study, bioassay guided fractionation was used to isolate the active components through NPFC and preparative HPLC-MS. From these techniques, the fraction and sub-fractions with the strongest effect on the cell viability were further purified. Interestingly, fraction D and its sub-fractions especially 3, 7, 8 and 10, contained higher concentrations of methoxy-flavones with known structures, which were further, tested against Jurkat cells. The most promising compounds were identified as 5, 6, 7, 3', 4', 5'- and 5, 6, 7, 8, 3', 4'-hexamethoxyflavones

Table 1. IC_{50} sub-fractions values of fraction D against Jurkat cells.

Sub-fractions	IC_{50} values (µg/ml)
1	> 100
2	> 100
3	12.6±0.4
4	> 100
5	> 100
6	> 100
7	10.7±3.2
8	4.7±0.4
9	65.0±3.3
10	4.2±1.8
11	64.3±0.8

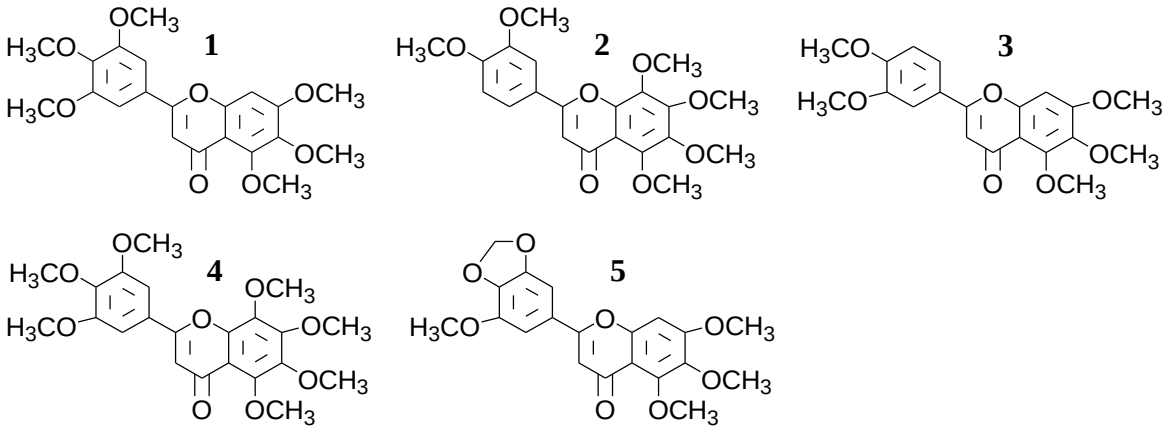

Figure 3. Structures of isolated compounds: 5, 6, 7, 3', 4', 5'-hexamethoxyflavone (**1**); 5, 6, 7, 8, 3', 4'-hexamethoxyflavone (**2**); 5, 6, 7, 8, 3', 4', 5'-heptamethoxyflavone (**3**); 5, 6, 7, 3', 4'-pentamethoxyflavone (**4**); 5, 6, 7, 3'-tetramethoxy-4', 5'-methylenedioxyflavone (**5**).

and were found to induce caspase -3 and -7 activities, which correlated to their reduced cell viability. While 5, 6, 7, 8, 3', 4'-hexamethoxyflavone, commonly known as nobiletin has been recognized for some time to affect cell proliferation of several cell lines (Hsiao et al., 2014; Chen et al., 2014) by inducing cell-cycle arrest, inhibition of extracellular signal-regulated kinase (ERK) activity and activation of caspases. Herein, it reports its effect on Jurkat cells, a model for T-cell leukemia.

Notably is the higher potency of the related 5, 6, 7, 3', 4', 5'-hexamethoxyflavone on the cell viability and caspase -3 and -7 activation, at lower concentrations. Limited data are available on this flavone. The few studies report its isolation from *Lantana ukambensis*, *Citrus reticulate* and *A. conyzoides*, and its cytotoxicity against P-388 mouse lymphocytic leukemia and A549 lung carcinoma cells, and inhibition of histamine release in RBL-2HR cells (Adebayo et al., 2010; Sawadogo et al., 2015; Itoh et al., 2008).

The favorable activities of these pure components against T-cell leukemia support its use by herbalists to manage leukemia. This warrants further investigation for rational drug development and it is encouraging to observe good biomimetic physicochemical properties of these flavones determined by affinity chromatography techniques.

All tested components showed low predicted volume of distribution values, resulting from albumin binding under 95% (% HSA) and the low to medium affinity for phosphatidylcholine (CHI $IAM_{7.4}$). These results predict lower non-specific tissue targeting and therefore hopefully higher specific binding to target tissues, due to their ability to permeate the phospholipid bilayer and lower propensity to distribute into all organ tissues (Jiang and Reilly, 2012; Reilly et al., 2011).

Additionally, the lipophilicity represented by the distribution coefficient log $D_{7.4}$ below 3 is deemed to be appropriated for "drug like" small molecules with a

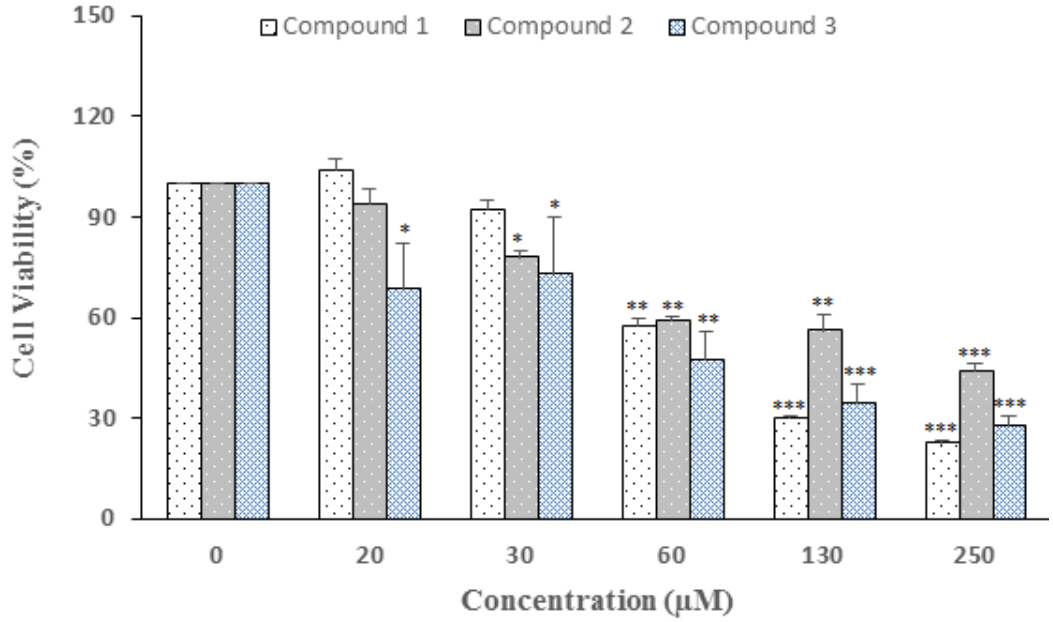

Figure 4. Effect of pure compounds 1, 2 and 3 on the cell viability of Jurkat cells. The cells were exposed to increasing concentrations for 24 h. The cell viability was determined by CellTiter-Blue® assay and expressed as % compared to 0.1% DMSO-treated controls. Bar graphs represent means ± SD of triplicates from two independent experiments.

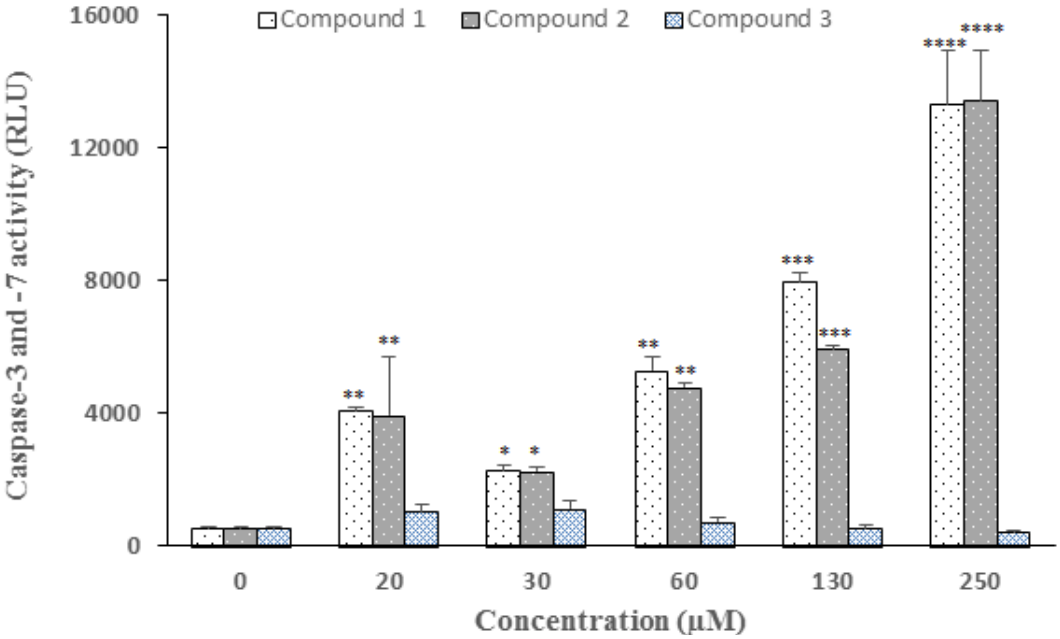

Figure 5. Effect of compounds 1, 2 and 3 on caspases -3 and -7 activity in Jurkat cells. The cells were exposed to increasing concentrations for 24 h. The caspases -3 and -7 activity was determined by Caspase-Glo® 3/7 assay and expressed as relative luminescence unit (RLU) compared to 0.1% DMSO-treated controls.

molecular weight below 500 (Merz et al., 2010). In all, the physicochemical measurements predict that the isolated components will have desirable ADME properties and are likely to retain their pharmacological properties when

Table 2. Physicochemical profiles of compounds isolated from *A. conyzoides*

Compound	HSA (%)	CHI $IAM_{7.4}$	Log V_d	Log $D_{7.4}$
1	86.3	31.9	0.02	2.60
2	88.7	32.3	-0.01	2.72
3	91.5	32.9	-0.06	2.98
4	88.5	31.4	-0.04	2.34
5	93.4	35.6	-0.01	2.83

tested *in vivo.*

Conclusion

The goal of this research was to isolate the anticancer components of *A. conyzoides* test against Jurkat cells and elucidate their caspases -3 and -7 activation as well as their physicochemical profiles. 5, 6, 7, 3', 4', 5'- and 5, 6, 7, 8, 3', 4'-hexamethoxyflavones significantly reduced the cell viability of Jurkat cells and this correlated to their respective caspases -3 and -7 activation. Furthermore, their physicochemical profiles predict on-target actions with fewer propensities for off-target promiscuity due to a low CHI $IAM_{7.4}$ affinity to phospholipid.

Thus, the results obtained in this study give an initial scientific insight on the usefulness of traditionally used *A. conyzoides* and provides a basis for further investigation of the active components which could be useful in the development of new leukemic therapies. This study also highlights the usefulness of the 2D chromatographic purification approach with NPLC followed by RP HPLC to isolate pure natural products from complex mixtures.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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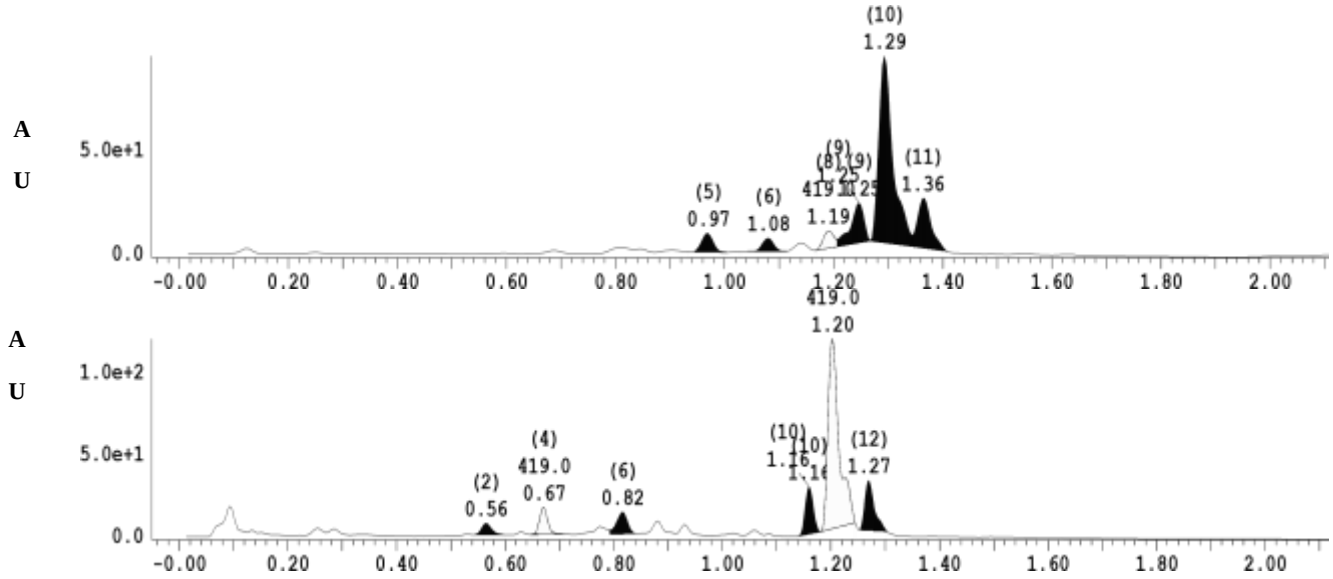
Supplementary Data



Example of chromatograms by reverse phase HPLC-MS. Total ion chromatogram of basic and acidic pH gradient scouting runs of fraction D (**Top**) basic run with eluent A: water and 5 mM ammonium hydroxide and (**Bottom**) acidic run with eluent A: water and 0.05% trifluoroacetic acid. Eluent B consisted of ACN. The gradient initiated at 5% and increased to 95% of eluent B over 2.0 min.

Spectral data of isolated compounds

Compound **1** (5, 6, 7, 3', 4', 5'-Hexamethoxyflavone), white powder, gave a molecular ion peak at *m/z* 402.13147 with corresponding molecular formula of $C_{21}H_{22}O_8$. The assignments of ^{13}C NMR and ^{1}H NMR signals agree with the literature (Gonzalez et al., 1991).

Compound **2** (5, 6, 7, 8, 3', 4'-Hexamethoxyflavone), white powder, $C_{21}H_{22}O_8$, structural isomer of compound 1, gave a molecular ion peak at *m/z* 402.13147. The assignments of ^{13}C NMR and ^{1}H NMR signals as well as COSY, HSQC, HMBC, and NOESY spectra agree with the literature and is commonly called nobiletin (Gonzalez et al., 1991).

Compound **3** (5, 6, 7, 3', 4'-Pentamethoxyflavone), white powder, also gave a molecular ion peak at *m/z* 372.1209 with molecular formula of $C_{20}H_{20}O_7$ based on HRESI-MS data. The assignments of ^{13}C NMR and ^{1}H NMR signals as well as COSY, HSQC, HMBC, and NOESY spectra are in agreement with literature and is commonly called sinensetin (Gonzalez et al., 1991).

Compound **4** (5, 6, 7, 8, 3', 4', 5'-heptamethoxyflavone), white powder, had a molecular formula of $C_{22}H_{24}O_9$ and gave molecular ion peak at *m/z* 432.14203, based on HRESI-MS data. The assignments of ^{13}C NMR and ^{1}H NMR signals as well as COSY, HSQC, HMBC, and NOESY spectra agree with the literature (Moreira et al., 2007).

Compound **5** (5, 6, 7, 3'-tetramethoxy-4', 5'-methylenedioxyflavone), yellow solid, with molecular formula $C_{20}H_{18}O_8$, gave a molecular ion peak at *m/z* 386.10017 according to HRESI-MS data. The assignments of ^{13}C NMR and ^{1}H NMR signals as well as COSY, HSQC, HMBC, and NOESY spectra agree with the literature (Lim, 2012).