

A hand holding several yellow capsules over a background of green leaves. The image is framed with rounded corners.

Journal of Medicinal Plant Research

Volume 11 Number 38, 10 October, 2017

ISSN 1996-0875



*Academic
Journals*

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Full Length Research Paper

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

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Received 27 July, 2017; Accepted 14 September, 2017

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 (CDCl₄), deuterated ACN (C₂D₃N) 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 40°C 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_3 or $\text{C}_2\text{D}_2\text{N}$). 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 37°C 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 $\mu\text{g}/\text{mL}$ of fraction A through F or with sub-fractions of D in a concentration range of 0 to 100 $\mu\text{g}/\text{mL}$. 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 $_{\text{Ex}}$ /590 $_{\text{Em}}$ 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 $_{\text{Ex}}$ /527 $_{\text{Em}}$ 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 \pm 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 $\mu\text{g}/\text{mL}$ 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, $\text{Log } V_d$, shows low values for all compounds. Finally, lipophilicity measurements at pH 7.4, $\text{Log } D_{7.4}$, 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

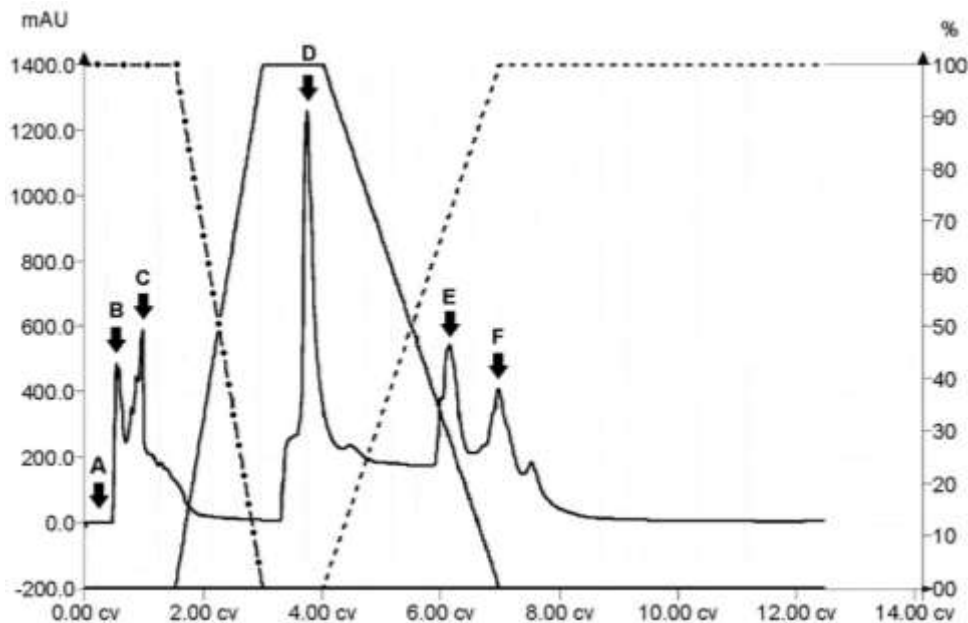


Figure 1. Normal phase flash chromatogram with heptane:ethylacetate:methanol as mobile phase. The column eluents were collected, pooled into 6 fractions (A-F) and evaporated at 40°C.

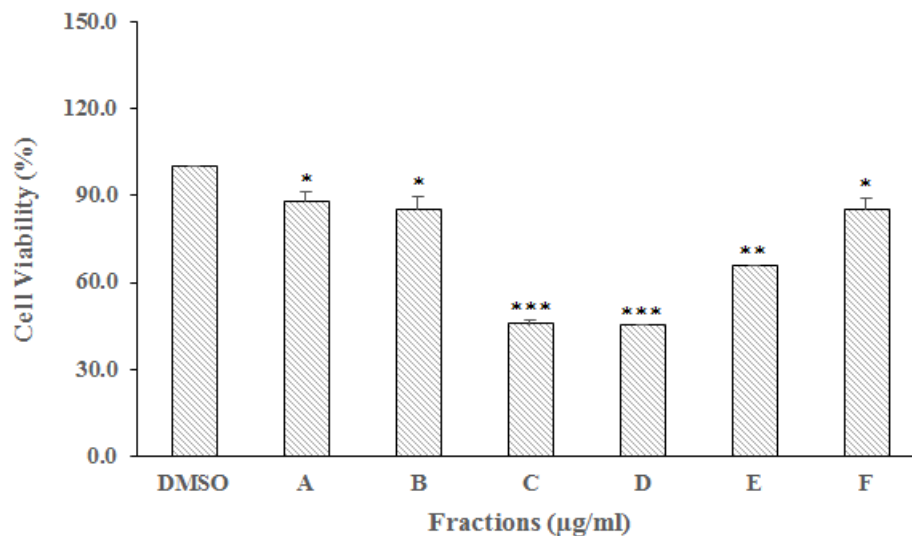


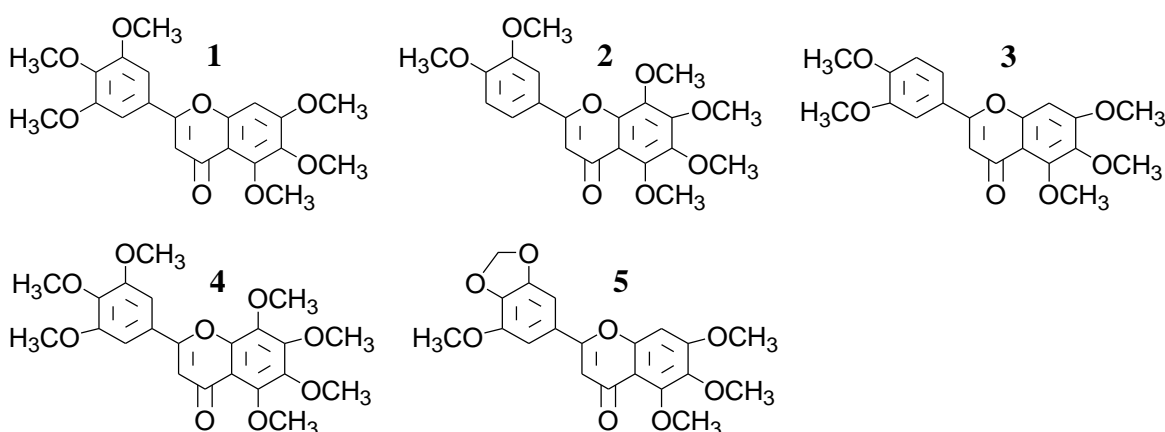
Figure 2. Effect of crude fraction A to F on the cell viability of Jurkat cells. The cells were treated for 72 h at concentrations of 200 µg/mL. The percentage of cell viability was determined by MTT assay and expressed as % compared to 0.1% DMSO-treated controls. Bar graphs represent means \pm SD of 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. IC₅₀ 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', 5'-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 reticulata* 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

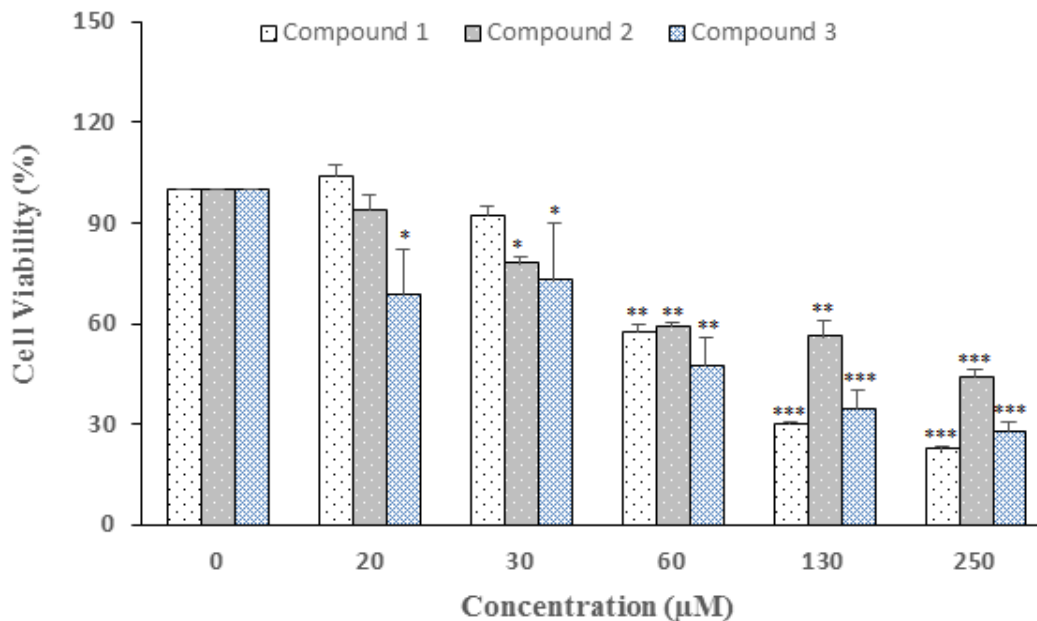


Figure 4. Effect of pure compounds 1, 2 and 3 on the cell viability of Jurkat cells. The cells were exposed to increasing concentrations for 24 h. The cell viability was determined by CellTiter-Blue® assay and expressed as % compared to 0.1% DMSO-treated controls. Bar graphs represent means \pm SD of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

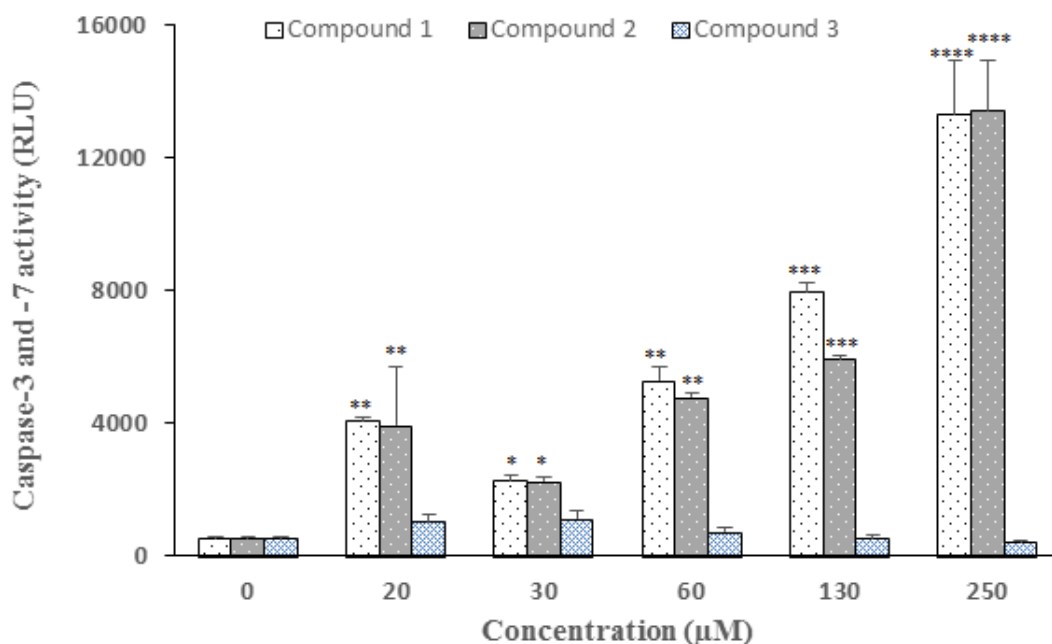


Figure 5. Effect of compounds 1, 2 and 3 on caspases -3 and -7 activity in Jurkat cells. The cells were exposed to increasing concentrations for 24 h. The caspases -3 and -7 activity was determined by Caspase-Glo® 3/7 assay and expressed as relative luminescence unit (RLU) compared to 0.1% DMSO-treated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

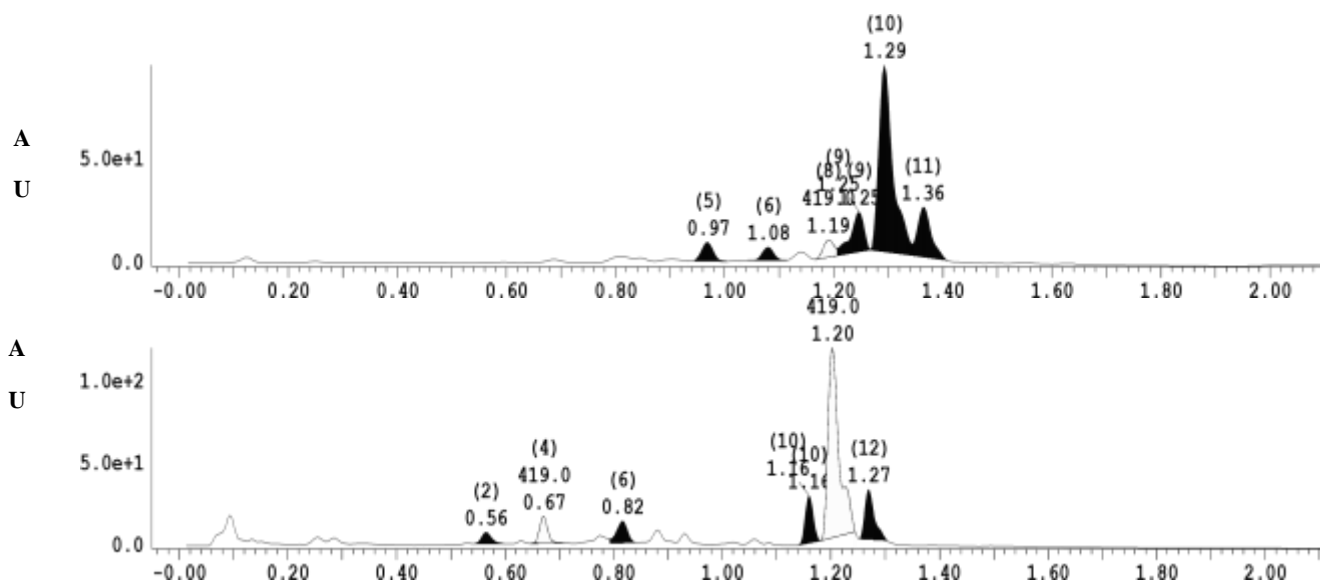
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Supplementary Data



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 1H 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 1H 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 1H 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 1H 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 1H NMR signals as well as COSY, HSQC, HMBC, and NOESY spectra agree with the literature (Lim, 2012).

Full Length Research Paper

The antibacterial and antifungal analysis of crude extracts from the leaves and bark of *Pimenta* species found in Jamaica

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Received 16 June, 2017; Accepted 27 September, 2017

Due to the rapid increase in the number of antibiotic-resistant bacteria, medicinal plants are being studied as new and promising alternatives to conventional antibiotic treatment. The crude hexane, ethyl acetate and methanol extracts from *Pimenta dioica*, *Pimenta jamaicensis* and *Pimenta racemosa* were quantitatively assessed to determine their antimicrobial susceptibility and potency using zones of inhibition methods, minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations minimum (MBC) or fungicidal concentrations concentration (MFC) against *Streptococcus A*, *Streptococcus B*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella* species, *Proteus mirabilis* and *Candida albicans*. The crude ethyl acetate extract of the *P. dioica* was most active against *Candida albicans* (MFC of 1.3 mg/mL and MIC of 0.63 mg/mL) while the crude hexane extract of *P. jamaicensis* was most active against *Streptococcus A* (MBC of 0.63 and MIC of 1.3 mg/mL). The crude ethyl acetate extract of *P. racemosa* was most active against *Streptococcus A* and *Salmonella* (MBC of 2.5 mg/mL and MIC of 1.3 mg/mL; MBC of 0.63 and MIC of 0.63 mg/mL, respectively). Extracts from selected species of *Pimenta* may potentially provide a source of new antimicrobial agents for the treatment of infectious diseases.

Key words: *Pimenta dioica*, *Pimenta jamaicensis*, *Pimenta racemosa*, antimicrobial.

INTRODUCTION

The spice of Jamaican cuisine is often times completed with a dash of *Pimenta*, also known as allspice, Jamaican

pepper, new spice or myrtle pepper. Its distinct flavor results from its unique blend of terpenes and other

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secondary metabolites. These compounds have also contributed to the medicinal properties of the plant and therefore used to treat various ailments in Jamaican folk medicine. The genus *Pimenta* contains about 14 species from the Myrtaceae family, all of which are indigenous to the Caribbean and Central America; Jamaica has 6 endemic species found within the genus (Adams, 1976). The species that may be found in Jamaica include *Pimenta dioica* (Allspice), *Pimenta racemosa* (bay rum), *Pimenta jamaicensis* (wild pimento), *Pimenta obscura*, *Pimenta richardii* and a species not yet identified (Institute of Jamaica Identification number: P22536B). These flowering plants may be monoecious or dioecious and contain many active ingredients. *Pimenta* is entrenched in many folklore remedies and used to treat cancer, diabetes, malaria and other chronic and infectious diseases.

The chemical profile of the *Pimenta* genus is distinct and contributes to the diversity in the use of the plant. The active components found in *P. dioica*, *P. obscura* and *P. richardii* are reported to be methyl eugenol, caryophyllene, and limonene identified via chemical analysis (Zhang et al., 2012). The medicinal properties for *P. dioica* and *P. racemosa* have been studied extensively. The essential oils and extracts from these plant species have been found to contain antimicrobial and antifungal properties (Martinelli et al., 2017; Hammer et al., 1999; Zabka et al., 2009), anti-nociceptive properties as well as anti-cancer activity against prostate cancer cell lines (Garcia et al., 2004). *P. dioica* also has significant cytotoxic, anti-oxidant, analgesic and anti-hypertensive effects (Suárez et al., 1997; Marzouk et al., 2007). The aqueous extract of the berries showed significant antitumor activity, while ethanolic extracts from the leaves and berries can also be used as a food preservative (Sessou et al., 2012). The macerated leaves of *P. dioica* have previously shown some antimicrobial activity with crude hexane and alcoholic extracts against *Pseudomonas fluorescens*, *Bacillus megaterium*, *Aspergillus niger* and *Penicillium* species (Boyd et al., 2014). Other studies have shown that essential oils obtained from *P. dioica* have the potential of being used as a natural pesticide and fungicide (Seo et al., 2009).

Essential oils of *P. racemosa* possess several types of bioactivity that have been validated experimentally. Alitonou et al. (2012) reported that the oil has low anti-inflammatory activity based on the lipoxigenase test results, as well as, antiradical, acaricidal and antimicrobial activity against both bacteria and fungi. It was postulated that the oils can be exploited industrially for its antioxidant activity to delay the degradation of fatty substances. Neither of the crude extracts (hexane, ethyl acetate and chloroform) of *P. racemosa* was found to have any significant effect as a cytotoxic agent. In a research conducted to assess the antimicrobial activity of 52 plants, *P. racemosa* was one of the three plants that showed anti-microbial activities on all the following

organisms: *Acinetobacter baumannii*, *Aeromonas veronii* biogroup *sobria*, *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* serotype *typhimurium*, *Serratia marcescens* and *Staphylococcus aureus*, using an agar dilution method (Hammer et al., 1999), which also formed a part of the basis for this study. Nesrin et al. (2017) have demonstrated that the most abundant component of essential oil obtained from *P. racemosa* is methyleugenol and suggested that the essential oil holds great promise as natural preservative and antimicrobial agents. *P. jamaicensis* contains eugenol and limonene which contributes to its characteristic smell and properties; other compounds include p-cymene, 1, 8-cineole, β -caryophyllene, and 4-terpineol as the main constituents (Tucker et al., 1992).

The potential for new antimicrobial agents is dependent on their experimental validation when extracts or compounds are exposed to different microorganisms. Plant-based secondary metabolites have immense potential for combating a broad spectrum antibiotic-resistant bacterium with little or no side effects (Chandra et al., 2017). The literature suggests that *Pimenta* species could hold significant promise as an antibiotic agent. In this study, the antifungal and antimicrobial activity of various crude extracts (hexane, methanol and ethyl acetate) was evaluated for *P. dioica*, *P. racemosa* and *P. jamaicensis*.

MATERIALS AND METHODS

Plant collection and extraction

The plant materials were obtained and verified by the taxonomist at The University of the West Indies, Mona Herbarium and samples were given reference numbers 35922, 35923 and 35924 for *P. dioica*, *P. racemosa*, and *P. jamaicensis*, respectively. The samples (leaves and bark) were dried at room temperature and milled into a powder prior to solvent extraction with hexane, ethyl acetate and methanol to obtain crude extracts of each species. The first solvent, hexane, was percolated through the plant material for 24 h, filtered, concentrated in vacuum under reduced pressure using rotary evaporator and dried in a desiccator to obtain the crude hexane extract. The same plant material was then treated with ethyl acetate and methanol sequentially for the same time period to obtain the crude ethyl acetate and methanol extracts. A sample of 0.1 g of the plant extracts was dissolved in 10 ml of dimethyl sulphoxide (DMSO) and a serial dilution was carried out to obtain 6 extract concentrations (10,000, 5000, 2500, 1250, 625, and 312.5 $\mu\text{g/mL}$) which was then used in the microbial test.

Bacterial and fungal isolates

Bacterial isolates (Gram-positive: *Streptococcus* group A (ATCC 12386), *Streptococcus* group B (ATCC 19613), *E. faecalis* (ATCC 29212), *S. aureus* (ATCC 25923) and Gram-negative: *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC BAA 1705), *Salmonella* species (5567 Nov 2011), *Proteus mirabilis* (7002) and fungal isolate (*C. albicans*; ATCC 14053)) used

for this study were obtained from The University of the West Indies, Mona (Microbiology Department).

Anti-microbial assays

All the extracts were screened to assess their antibacterial and antifungal properties using agar-well diffusion method (Perez et al., 1990). The samples were further tested at 1 mg/mL (the maximum concentration at which an extract may be considered active controls) (DeMarsh et al., 2001). The minimal inhibitory concentration (MIC) of the active samples was determined using extracts obtained from leaves and bark of the respective *Pimenta* spp.

Well diffusion assay

Suspensions made from 24 h bacterial or fungal cultures were standardized using 0.5 McFarland Standard (ca. 10^8 CFU/mL) and inoculated on the surface of Mueller-Hinton agar (bacteria) and Sabouraud dextrose agar (fungi) plates using sterile cotton swab (Baker et al., 1983). The diluted crude extract and the control (100 μ l of each) were then introduced into the wells of 6 mm diameter that were created in the agar. The plates were kept at 4°C for 1 h to permit diffusion of the extracts, then incubated at 37°C for 24 (bacteria) and 48 h (fungi) and the diameter of the zone of growth inhibition was then measured using a caliper. The experiment was repeated for reliability.

Tube dilution assay

The extracts that showed antimicrobial activity were further tested to determine the MIC for each bacterial or fungicidal sample. 1 mL of each extract was added to 1 mL of sterile brain heart infusion broth and used to prepare two-fold broth micro-dilutions of the various extracts (Ferraro, 2000). The tubes were inoculated with a drop of microbial suspension and incubated for at 37°C for 24 h, after which the MICs were measured macroscopically to assess the efficacy and effectiveness of the extracts in comparison with the sterility and growth controls which were included for each assay (DeMarsh et al., 2001). All the tubes were evaluated for growth indicated by turbidity. The samples at each concentration, sterile and growth controls were then seeded onto blood agar plates and incubated at 37°C for 24 h after which the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) was recorded. The MBC or MFC was defined as the lowest concentration of the extract associated with no bacterial or fungal growth on a freshly inoculated agar plate.

RESULTS

The antibacterial and antifungal activity of *P. jamaicensis*, *P. dioica* and *P. racemosa* are outlined (Tables 1 and 2). All the leaf extracts of the investigated *Pimenta* spp. showed significant antimicrobial activity against one or more microbes, except for the crude hexane extract of *P. dioica* and crude methanol extract of *P. racemosa* (Table 1). *P. racemosa* was the most active of the three *Pimenta* spp., with the ethyl acetate extract being most active against *Salmonella* and *Streptococcus B* and the hexane extract being most active against *C. albicans*. The ethyl acetate extract of *P. dioica* and hexane extract of *P.*

jamaicensis were most active against *C. albicans* and *Streptococcus A*, respectively. There was no significant antimicrobial activity observed for *E. faecalis*, *E. coli*, *P. mirabilis* and *K. pneumonia* using any of the extracts.

The MBCs and MICs exhibited by the extract against test bacteria ranged between 0.63 and 2.5 mg/mL and 0.63 and 1.3 mg/mL, respectively (Table 2). The growth of *C. albicans* was inhibited by *P. dioica* leaf ethyl acetate extracts giving a MFC of 1.3 mg/mL and MIC of 0.63 mg/mL. Of the three plant species, the *P. racemosa* leaf ethyl acetate extracts were the most potent antibacterial agents against the Gram-positive bacteria, *Streptococcus B* (MBC of 0.63 mg/mL and MIC of 0.63 mg/mL). This extract also showed an antibacterial effect against the Gram-negative bacteria, *Salmonella* spp., but was less potent (MBC of 2.5 mg/mL and MIC of 1.3 mg/mL).

DISCUSSION

Pimenta spp. have shown significant promise based on the preliminary tubal broth dilution assays and subsequent inhibitory and bactericidal tests conducted. The MIC indicates the lowest concentration that can inhibit growth, while the MBC determines the concentration at which a compound or extract will kill the bacteria being tested. The MIC and MBC are complementary in that the extract or compound would be most effective as the MIC determines if it is anti-proliferative and then the MBC determines if it is also bactericidal.

The results reveal that both Gram-positive and Gram-negative bacteria and the fungi *C. albicans* were susceptible to the effects of the extracts obtained from *Pimenta* plant species. As outlined in Table 1, all three crude leaves extracts of the endemic *P. jamaicensis* showed antibacterial activities against microbes such as Streptococci which are known to cause numerous ailments. *Streptococcus A* (Group A Streptococcus, GAS) was susceptible to the effect of the *P. jamaicensis* crude extracts, however, the hexane extract was the most active, while the crude ethyl acetate and methanol extracts were partially active against *Streptococcus B*. *Streptococcus A* is usually spread via mucus from the nose, throat or via skin, and is often treated with antibiotics. Over 500,000 deaths have been reported per year for GAS related diseases (Cohen-Poradosu and Kasper, 2007). The *P. jamaicensis* leaf hexane extract could be an effective natural astringent or disinfectant which could aid with growth control of *Streptococcus A*. *Streptococcus B* or *Streptococcus agalactiae* (Group B Streptococcus, GBS) are bacteria also found commonly in the human body that may cause infections, especially with pregnant women and newborns. The highest cases of GBS infections were observed in non-pregnant women above 65 years old, with the incidence decreasing exponentially with age. The *P. racemosa* ethyl acetate

Table 1. Antibacterial and antifungal activity of *Pimenta dioica*, *Pimenta jamaicensis* and *Pimenta racemosa* leaf extracts at 1 mg/mL through well diffusion method.

Plant specimen	Diameter of the zone of inhibition (mm) in 1 mg/mL		
	Methanol	Ethyl acetate	Hexane
<i>Pimenta jamaicensis</i>	<i>S. aureus</i> (9)	<i>Streptococcus</i> A (10),	<i>Streptococcus</i> A (15)
	<i>Streptococcus</i> A (11)	<i>Streptococcus</i> B (10),	-
	<i>Streptococcus</i> B (12)	<i>P. aeruginosa</i> (10)	-
<i>Pimenta dioica</i>	<i>Salmonella</i> (10)	<i>Candida albicans</i> (14)	NA
	<i>Streptococcus</i> A (11)	<i>P. aeruginosa</i> (9)	-
	<i>Streptococcus</i> B (9)	-	-
<i>Pimenta racemosa</i>	NA	<i>Salmonella</i> (16),	<i>Candida albicans</i> (15)
	-	<i>Streptococcus</i> B (13)	<i>Streptococcus</i> B (9)
	-	-	<i>P. aeruginosa</i> (10)

6-8, Not active (NA); 9-12, partially active; 13-17, active; >17, very active.
Source: DeMarsh et al. (2001).

Table 2. The MBCs/MFCs and MICs of the most effective crude extract of *P. jamaicensis*, *P. dioica* and *P. racemosa* using tube dilution assay.

Crude extract	Organism	MBC/MFC (%)	MIC (%)
<i>Pimenta dioica</i> leaf ethyl acetate	<i>Candida albicans</i>	1.3 mg/mL (100)	0.63 mg/mL (99)
<i>Pimenta racemosa</i> leaf ethyl acetate	<i>Salmonella</i> spp.	2.5 mg/mL (100)	1.3 mg/mL (50)
	<i>Streptococcus</i> B	0.63 mg/mL (100)	0.63 mg/mL (100)
<i>Pimenta jamaicensis</i> leaf hexane	<i>Streptococcus</i> A	1.3 mg/mL (100)	0.63 mg/mL (90)

extract was active against *Streptococcus* B with MBC of 0.63 mg/mL and MIC of 0.63 mg/mL which is comparable to that of fluconazole (Barchiesi et al., 1994), while the hexane extract showed partial activity. The *P. jamaicensis* ethyl acetate leaf and methanol extracts and *P. dioica* methanol leaf extract also showed partial activity for GBS.

All three *Pimenta* species crude leaves extracts were partially active against *P. aeruginosa*, which has multiple-mechanisms of resistance to antibiotics and can only be treated by a selected number of anti-pseudomonal agents. This gram-negative bacterium has about 13 different strains, which is often implicated in ailments such as pneumonia, urinary tract infections, gastrointestinal infections, hemorrhage, and nosocomial infections. It thrives on moisture and as such is often found in persons hospitalized for over a week via cross infection due to catheters and other medical equipment. Its presence inside the body can be fatal when associated with some of the major organs such as the kidney and lungs. *P. aeruginosa* growth may be reduced or retarded by the *P. racemosa* hexane leaf extract, *P.*

dioica ethyl acetate leaf extract, and the *P. jamaicensis* ethyl acetate leaf extract.

Interestingly, the gram-negative *Salmonella* spp. which is often associated with gastrointestinal diseases, are susceptible to the effect of *P. dioica* and *P. racemosa* which are often added to food to enhance the flavor. The crude methanol extract of *P. dioica* was partially active while the crude ethyl acetate *P. racemosa* was active against *Salmonella* spp. This bacteria has been implicated in over one million cases of food poisoning annually (Centre for Disease Control and Prevention, 2016). According to the Centre for Disease Control and Prevention, *Salmonella* infection can be extreme and cause death, as reported in 2013 where over 19,000 patients were hospitalized for salmonellosis, from which 378 died. These infections are preventable with simple hands hygiene techniques, the separating of uncooked and cooked foods, cooking food properly and keeping foods refrigerated. Nonetheless, these crude extracts may have applications as an antimicrobial agent, to reduce *Salmonella* growth and as such reduce the prevalence of food borne illnesses.

C. albicans is a yeast-like fungus that forms a part of the normal intestinal microbiota of 70% of the human population. Its overgrowth may cause infections when there is favorable change in the internal conditions of the body, which results in candidiasis making this fungus the fourth leading cause of nosocomial infection (Pfaller and Diekema, 2007). Infections involving skin or mucosal surfaces are usually combated with oral or topical agents. *C. albicans* may, however, become invasive, traveling throughout the bloodstream causing damage to major organs requiring parenteral antifungal therapy. The *P. racemosa* and *P. dioica* were the most effective against *C. albicans* with the crude hexane leaf extract and the crude ethyl acetate leaf extract, respectively showing significant activity in the initial screening of the microbes. In the second stage of antimicrobial confirmation, the MICs of the *P. jamaicensis* ethyl acetate crude leaf extract was 99% (0.63 mg/mL) against *C. albicans* which was comparable to fluconazole, an antifungal, which usually has MIC of 92% against this organism (Barchiesi et al., 1994).

Conclusion

The use of *Pimenta* spp. has scientific credential as an antimicrobial agent. This is particularly important as globally there has been an increase in antimicrobial resistance, while fewer antimicrobial agents are being made, creating a possible scenario where there are no options for treatment. Most significant activity for *P. jamaicensis* was against the *Streptococcus A* bacteria, *P. dioica* was active against *C. albicans* and *P. racemosa* was active against *Salmonella*, *Streptococcus B* and *C. albicans*. These *Pimenta* species showed significant inhibitory and antimicrobial properties against the respective microbes and may be further studied in order to see whether they can be effectively incorporated into disinfecting, antimicrobial or antiseptic formulations to aid in combating diseases caused by these microbes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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A hand holding several yellow, oval-shaped pills over a background of large green leaves. The image is framed with rounded corners.

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