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Research Article

Metabolomic Profiling of Kigelia africana Extracts with Anti-Cancer Activity by High Resolution Tandem Mass Spectroscopy

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ABSTRACT: Background: *Kigelia africana* is an African tree with a wide distribution across southern, central and western Africa. It has a history of therapeutic usage by multiple African ethnic groupings which inhabit the areas in which it grows. Amongst these groups there is a myriad of medicinal uses in the treatment of a wide variety of bacterial, fungal and protozoal infections, as well as in the treatment of inflammation and cancers. This study was undertaken to further examine *K. africana* fruit extracts for the ability to inhibit cancer cell growth, and to use an unbiased metabolomic profiling approach to detect and putatively identify as many individual components as possible, with the aim of developing a database of identified compounds. **Materials and Methods:** *K. africana* fruit powder was extracted and tested for inhibitory activity against the Jeg-3 choriocarcinoma cell line using a colorimetric cell proliferation assay. Toxicity was evaluated using an *Artemia franciscana* nauplii bioassay. Non-targeted HPLC separation of crude extracts coupled to high resolution time-of-flight (TOF) mass spectroscopy with screening against 3 compound databases was used for the identification and characterisation of individual components in crude plant extracts. **Results:** The methanol, water and ethyl acetate *K. africana* fruit extracts displayed significant anti-proliferative activity against Jeg-3 choriocarcinoma cells. The methanol and water extracts displayed the strongest anti-proliferative activity, inhibiting Jeg-3 growth to 42% and 46% of the untreated cell growth respectively. The ethyl acetate extract also significantly inhibited Jeg-3 cell proliferation, with decreases to 62% of the untreated control value. Neither the chloroform or hexane extracts had any effect on Jeg-3 cell proliferation. With the exception of the water extract (which displayed moderate toxicity), all extracts were non-toxic or of low toxicity. HPLC-MS/MS TOF analysis identified 356 unique mass signals in the extracts. The putative identities of 227 of these compounds are reported here. **Conclusion:** This report extends previous studies into the anti-cancer affects of *K. africana* fruit extracts. The generation of a database of the detected compounds will allow for rapid differentiation of compound profiles between active and less active extracts in future studies. This is expected to assist in the identification of the most important compounds for further separation and bioactivity studies.

KEYWORDS: Medicinal plants; Bigoniaceae; Sausage tree; Anti-proliferative activity; Metabolomic profile; LC-MS/MS; Toxicity, Artemia.

INTRODUCTION

Kigelia africana (family Bigoniaceae), commonly known as sausage tree due to the shape of its fruit, is an African plant with a wide geographical range, occurring from Southern Africa, through Central Africa, to Western Africa.[1] It is also known as worsboom (Afrikaans), modukguhlu (Northern Sotho), muvevha (Venda) and um vunguta or umfongothi (Zulu) in Southern Africa,[2] as pandoro in Western Nigeria, and as mvungunya, mwegea, mwicha and mranaa in Swahili.[3] Its use as both a therapeutic agent and as a food has been recorded over much of sub-Saharan Africa. *K. africana* is a large tree, growing to 20 m in height. It has smooth grey bark which regularly peels off older trees. The grey-brown fruit is a large woody berry, often more than 30 cm (and up to 1 m) in

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length and up to 18 cm in diameter, which hangs vertically on long peduncles. Some fruit have been reported to weigh as much as 5–10 kg. They are indehiscent, with a woody wall and multiple lenticles at the surface. Once mature, the fruit contain many oboid seeds embedded in the fibrous pulp.

A number of medicinal properties have been examined for various parts of *K. africana*, including antibacterial, antiviral, anticancer and antioxidant activities. Whilst the fruit is most often cited as having therapeutic properties, multiple parts of the *K. africana* tree have been used in traditional healing systems in the treatment of a variety of medical conditions and complaints. The powdered mature fruit is used to treat wounds, abscesses, and ulcers, whilst the green fruit is used to treat syphilis and rheumatism.[2,4,5] An infusion made from the ground bark and fruit is used to treat stomach problems in children.[2,5] Roots and bark are used to treat pneumonia.[2] In West Africa, leaves and twigs are used to treat wounds, dysentery, stomach and kidney disorders, snakebite, and rheumatism.[6] The fruit is used to treat constipation, gynaecological disorders, haemorrhoids, lumbago and dysentery.[6] Slices of mature baked fruits are used to ferment and flavour traditional African beer.[7] Due to its range of medicinal uses, *K. africana* may provide a source of useful phytochemicals with therapeutic properties which could be used as alternatives to currently used medicines.

Several pharmacological examinations have reported on the antibacterial, antiviral and antioxidant activities of various parts of *K. africana* although many of these reports have focussed on the bark. The antimicrobial activity has been particularly well studied. Bark and root solvent extracts have been reported to inhibit the growth of *Escherichia coli*, *Enterobacter aerogens, Klebsiella pneumoniae*, *Salmonella typhi, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus cereus*.[8] In a similar study, solvent extracts prepared from stem and root bark have also been shown to inhibit growth of *E. coli*, *P. aeruginosa*, *S. aureus* and *Candida albicans*.[9,10] Other studies have also reported antibacterial activity for *K. africana* leaf extracts.[3,11] Of particular interest, polar *K. africana* leaf extracts have been shown to inhibit the growth of the bacterial trigger of rheumatoid arthritis.[11] Grace et al.[3] reported that extracts prepared from the *K. africana* fruit inhibited the growth of a panel of Gram positive and Gram negative bacteria. Recent studies in our own laboratory have also confirmed the broad spectrum antibacterial and antifungal activities of fruit extracts.[12] Furthermore, butanolic *K. africana* bark extracts[13] and methanolic and aqueous extracts[12] have been reported to inhibit the growth of *Entamoeba histolytica* and *Giardia duodenalis* respectively, indicating the therapeutic potential of this species against gastrointestinal protozoal parasites. Indeed, verminoside which was present in the butonolic extract had twice the efficacy of the reference drug metranidazole.[13] Similarly, a crude *K. africana* fruit aqueous extract had similar efficacy to pure metranidazole with IC50 values of 13.7 and 12.7 μg/ml respectively. *K. africana* wood extracts have also been reported to have better antimalarial activity than chloroquine and quine against resistant *Plasmodium falciparum* strains.[14]

Strong analgesic activities have been reported for ethanolic *K. africana* bark and fruit extracts and their components.[4] Recent studies have also reported anti-inflammatory activity for ethanolic fruit extracts[15] and for isolated verminoside by inhibiting NO release from induced J774. A1 marcophages.[16] Further studies have demonstrated the extract and its components can inhibit the synthesis/release of inflammatory mediators including the prostaglandins.[9] It is likely that these anti-inflammatory activities may contribute to the analgesic properties of *K. africana* extracts.

Cytotoxic and anti-proliferative effects have also been reported for *K. africana* extracts against several carcinoma cell lines. *K. africana* bark extracts have cytotoxic activities and have shown promising results in treating melanoma and renal carcinoma.[17] Aqueous root bark extracts significantly inhibit the growth of melanoma cells and renal carcinoma cells.[13,17] Recent studies have also demonstrated cytotoxic activity for *K. africana* fruit extracts against a melanoma and 2 breast cancer cell lines[18] That study also used a bioactivity driven separation approach to identify demethylkigelin, kigelin, ferulic acid and 2-(1-hydroxyethyl)-naphtho[2,3-b] furan-4,9-dione as possessing cytotoxic activity.[18] Of these, 2-(1-hydroxyethyl)-naphtho[2,3-b] furan-4,9-dione was a particularly potent cytotoxic agent. Potent anti-proliferative activity against CaCo2 and HeLa carcinoma cell lines was noted for *K. africana* fruit extracts.[12] of further interest, the chloroform and hexane *K. africana* fruit extracts also tested in that study demonstrated a stimulatory effect on cell proliferation. Therefore, these extracts may prove useful in accelerating wound healing and further compound isolation and identification studies are needed.

Whilst further studies are required to fully characterise the phytochemistry of *K. africana*, a number of interesting compounds have already been identified. The bark and roots contain significant quantities of the naphthaquinone lapachol and the coumarin kigelin.[2] Several other compounds including isopinnatal, kigelinone, pinnatal,

β-sitosterol, stigmasterol and vernolic acid have also been isolated from *K. africana* bark.[2,13] Several compounds have also been isolated and identified from fruit extracts, including vermonoside, 6-p-coumaroyl-sucrose, sitosterol-7-O-glucoside, 19a-dihydroxyurs-12-ene-28oic acid, caffeic acid and chlorgenic acid.[17,19-21] Despite the identification of these constituents, much work remains to fully characterize the bioactive *K. africana* extracts. Crude extracts are complex mixtures and whilst it was originally thought that norviburtinal was the main *K. africana* anticancer compound, it is now believed that multiple other compounds may also contribute and that the anti-cancer activity is due to complex interactions between a myriad of extract components. Thus a full understanding of all compounds present in *K. africana* extracts is required.

Current metabolomic approaches allow plant extracts to be rapidly screened for metabolites of the major metabolic pathways including amino acids, organic acids, sugars, sugar alcohols and glycosides.[22] Individual metabolomic studies are limited in the types of compounds which may be separated and detected. GC-MS is generally used for the detection of low polarity compounds. Thus, a large amount of compounds of medium polarity or high polarity are often not detected by GC-MS metabolomic analysis. Liquid chromatography – mass spectroscopy (LC-MS) is better suited for the analysis of compounds of medium and high polarity. Coupling LC with high mass accuracy spectroscopy techniques using both mild ionisation and electrospray ionisation (ESI) can generate large amounts of useful information for compound identification and metabolomic analysis. Using these methods, molecular ions can be detected and their empirical formulas accurately determined and compared to databases. Furthermore, coupling this with ESI analysis also allows for the detection and characterisation of characteristic fragments, allowing for rapid identification of unknown compounds in a crude extract.

LC coupled to hybrid mass spectrometry has the ability to detect and analyse large amounts of individual compounds in a crude extract. When metabolomic profiles are subsequently compared between extracts with different bioactivity profiles, it is possible to accurately detect differences between the extracts and thus highlight compounds which may be responsible for these bioactivity differences. This technique has been successfully used before in our laboratory to narrow the focus of compounds present in an extract,[23] thus simplifying the purification and identification process. Extracts can be screened for bioactivity and their metabolomic profiles subsequently compared. The differences in these profiles may be used to simplify the compliment of possible compounds that may be responsible for the bioactivity. The current study examines the anti-proliferative properties of several *K. africana* extracts of varying polarity towards Jeg-3 choriocarcinoma cells. This study was undertaken with the aim of establishing a HPLC-MS/MS method for the metabolic profiling of *K. africana* compounds and to use this method to qualitatively differentiate and identify a significant number of these compounds.

MATERIALS AND METHODS

K. africana fruit was collected from a mature tree at St Lucia, Brisbane and stored at –30°C until processing. The fruit was thawed at room temperature, cut into small pieces and dried in a Sunbeam food dehydrator. The dried pieces were subsequently ground into a coarse powder. The powdered plant material was extracted by standardised methods.[24–26] Briefly, an amount of 1 g of powdered plant material was weighed into each of five tubes and five different extracts were prepared by adding 50 ml of methanol, water, ethyl acetate, chloroform, or hexane respectively. All solvents were obtained from Ajax and were AR grade. The ground dried nut material was extracted in each solvent for 24 hours at 4 °C with gentle shaking. The extracts were filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 ml deionised water.

Qualitative Phytochemical Studies

Phytochemical analysis of *K. africana* extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[27-31]

Screen for anti-Anticancer Bioactivity Jeg-3 choriocarcinoma cell line

The Jeg-3 human choriocarcinoma cell line used in this study were originally obtained from American Type Culture Collection (Rockville, USA) by Dr Giovanna Di Trapani, Griffith University, Australia, and kindly donated for use in these studies. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies), supplemented with 20 mM HEPES, 10 mM sodium bicarbonate, 50 μg/ml streptomycin, 50 IU/ml penicillin, 2 mM glutamine and 10% foetal calf serum (Life Technologies). The cells were maintained as monolayers in 75 ml flasks at 37 °C, 5% CO_2 in a humidified atmosphere until approximately 80% confluent.

Evaluation of anti-Jeg-3 proliferative activity

For anti-proliferation studies, 1 ml of trypsin (Sigma) was added to the culture flasks and incubated at 37°C, 5% CO_2 for 15 min to dislodge Jeg-3 cells. The cell suspension was then transferred to a 10 ml centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9 ml of fresh media. Aliquots of the resuspended cells (70 µl, containing approximately 5000 cells) were added to the wells of a 96 well plate. A volume of 30 µl of the test extracts or cell media (for the negative control) was added to individual wells and the plates were incubated at 37°C, 5% CO_2 for 12 hours in a humidified atmosphere. A volume of 20 µl of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 hours. Absorbances were recorded at 490 nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed in at least triplicate and triplicate controls were included on each plate. The anti-proliferative activity of each test was calculated as a percentage of the negative control using the following formula:

$$\text{Proliferation (\% untreated control)} = (A_{ct}/A_{cc}) \times 100$$

where A_{ct} is the corrected absorbance for the test extract (calculated by subtracting the absorbance of the test extract in media without cells from the extract cell test combination) and A_{cc} is the corrected untreated control (calculated by subtracting the absorbance of the untreated control in media without cells from the untreated cell media combination).

Toxicity Screening

Reference toxins for biological screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 2 mg/ml solution in distilled water and was serially diluted in synthetic seawater for use in the *A. franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[32-35] Briefly, *A. franciscana* cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/l distilled water were prepared prior to use. An amount of 1 g of *A. franciscana* cysts were incubated in 500 ml synthetic seawater under artificial light at 25°C, 2000 Lux with continuous aeration. Hatching commenced within 16–18 h of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 h of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. The extracts and positive control were also serially diluted in artificial seawater for LC50 determination. A volume of 400 µl of seawater containing approximately 38 (mean 37.8, n = 156, SD 14.6) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 4 mg/ml in seawater for toxicity testing, resulting in a 2 mg/ml concentration in the bioassay. 400 µl of diluted plant extract and the reference toxins were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 µl seawater) was run in at least triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered moribund if no movement of the appendages was observed within 10 sec. After 48 h all nauplii were sacrificed and counted to determine the total number per well. The LC50 with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical Analysis

Data are expressed as the mean ± SEM of at least three independent experiments. One way ANOVA was used to calculate statistical significance between control and treated groups with a P value < 0.01 considered to be statistically significant.

HPLC-MS/MS Analysis

Chromatographic separations were performed using 10 µL injections of sample onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C18 column (2.1 x 100 mm, 1.8 µm particle size). The mobile phases consisted of (A) ultrapure water and (B) 95:5 acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% (v/v) glacial acetic acid for mass spectrometry analysis in positive mode and with 5 mM ammonium acetate for analysis in negative mode. The chromatographic conditions utilised for the study consisted of the first 5 min run isocratically at 5% B, a gradient of (B) from 5% to 100% was applied from 5 min to 30 min, followed by 3 min isocratically at 100%. Mass spectrometry analysis was performed on an Agilent 6530 quadrapole time-of-flight spectrometer fitted with a Jetstream electrospray ionisation source in both positive and negative mode.

Data was analysed using the Masshunter Qualitative analysis software package (Agilent Technologies). Blanks

using each of the solvent extraction systems were analysed using the Find by Molecular Feature algorithm in the software package to generate a compound list of molecules with abundances greater than 10,000 counts. This was then used as an exclusion list to eliminate background contaminant compounds from the analysis of the extracts. Each extract was then analysed using the same parameters using the Find by Molecular Feature function to generate a putative list of compounds in the extracts. Compound lists were then screened against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (650 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds). Empirical formula for unidentified compounds was determined using the Find Formula function in the software package.

RESULTS

Liquid Extraction Yields and Qualitative Phytochemical Screening

Extraction of 1 g of dried plant material with various solvents yielded dried plant extracts ranging from approximately 56 mg to 213 mg (Table 1). Water and methanol gave the highest yields of dried extracted material (213 and 199 mg respectively). Ethyl acetate, chloroform and hexane extracted lower masses (approximately 56, 129, and 82 mg respectively). The dried extracts were resuspended in 10 ml of deionised water resulting in the extract concentrations shown in Table 1.

Table 1: The mass of dried extracted material, the concentration of extracts after resuspension in deionised water and qualitative phytochemical screenings of solvent extractions.

	Methanol	Water	Ethyl Acetate	Chloroform	Hexane
Mass of dried extract (mg)	198.9 ± 0.4	213.3 ± 37.6	56 ± 25.2	128.7 ± 42.4	81.7 ± 42.5
Resuspended extract concentration (mg/ml)	19.9	21.3 ± 3.8	5.6 ± 2.5	12.8 ± 4.2	8.2 ± 4.3
Qualitative phytochemical screens					
Total phenolics	+++	+++	+	++	++
Water soluble phenolics	++	++	-	+	+
Water insoluble phenolics	+++	+	+++	+++	+++
Cardiac glycosides	-	-	-	-	-
Saponins	-	+	-	+	-
Triterpenoids	-	-	-	-	-
Polysterols	-	-	-	-	-
Alkaloids (Mayer test)	++	++	-	+	-
Alkaloids (Wanger test)	++	++	-	+	-
Flavanoids	+++	++	++	+	-
Tannins	+++	++	+	+	-
Anthraquinones	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

Phytochemical studies (Table 1) show that methanol and water extracted the widest range and largest amount of phytochemicals in this study. The methanol extract showed high levels of total phenolics (water soluble and insoluble phenolics), flavanoids and tannins. Whilst still containing relatively high phytochemical levels, the aqueous extract displayed slightly lower levels of phenolics, flavanoids and tannins. Moderate levels of alkaloids were also detected in the methanol and water extracts. In contrast to the methanol extract, saponins were detected in the aqueous extract, albeit at lower levels. Similar classes of phytochemicals were detected in the ethyl acetate and chloroform extracts, albeit generally at lower levels. Most of the phytochemical classes were not evident in the hexane extract. Indeed, this extract only contained detectable levels of phenolics.

Inhibition of Jeg Choriocarcinoma Cell Proliferation

K. africana fruit extracts was tested for their ability to inhibit Jeg-3 choriocarcinoma cell growth (Figure 1). The methanol, water and ethyl acetate extracts displayed significant ($p<0.01$) inhibitory activity, with proliferation inhibited to as low as 42% of the untreated control cell growth (for the methanol extract). The aqueous extract also was very effective at inhibiting Jeg-3 proliferation (to approximately 46% of untreated cell proliferation). Whilst still displaying significant anti-proliferative activity, the ethyl acetate extract was less effective, resulting in approximately 62% of the untreated control levels. Neither the chloroform of hexane extracts inhibited Jeg-3 choriocarcinoma cell proliferation, with levels similar to those of the untreated control values. Inhibition of proliferation was dose dependent, with the level of inhibitory activity decreasing at lower concentrations.

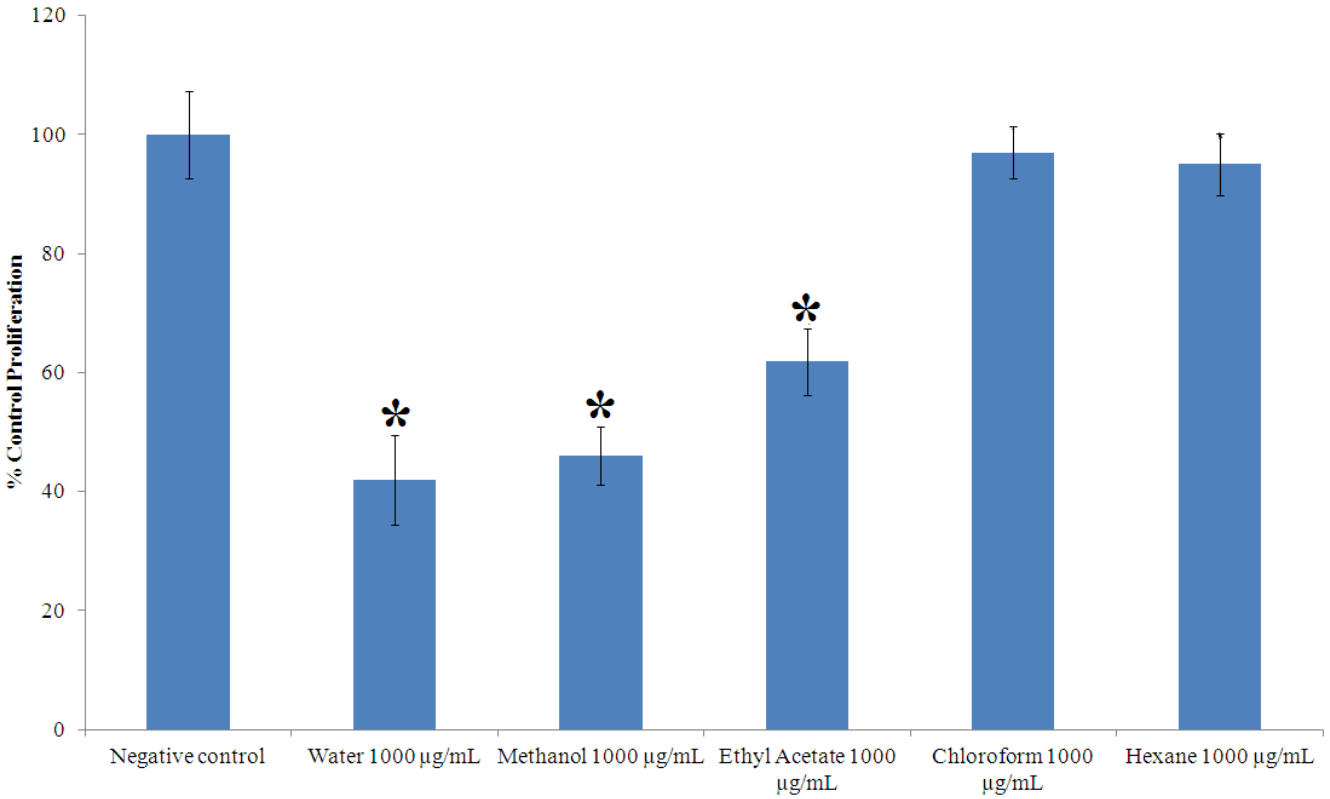

Figure 1. Anti-proliferative activity of *K. africana* fruit extracts and untreated controls against Jeg-3 cancer cell lines measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$).

The level of anti-proliferative activity was further evaluated by determining the IC50 values (Table 2) for each extract. The methanol extract was the best inhibitor of Jeg-3 cell proliferation with an IC50 of 687 µg/ml, indicating its potential for cancer therapeutic development. The aqueous extract displayed lower anti-proliferative activity (approximately 836 µg/ml). An IC50 could not be determined for the ethyl acetate extract as the level of proliferation was not <50% of the control value at any concentration tested.

Table 2: The IC50 values (µg/ml) of Jeg-3 choriocarcinoma cells and the LC50 values (µg/ml) for *Artemia franciscana* nauplii exposed to *K. africana* extracts and control solutions.

Bioassay	Solvent Extracts					Controls	
	Methanol	Water	Ethyl Acetate	Chloroform	Hexane	Negative Control	Potassium Dichromate
Jeg choriocarcinoma cells IC50	687	836	CND	-	-	-	ND
Artemia franciscana nauplii 24 h LC50	985	477	3270	-	-	-	224

Numbers indicate the mean IC50 or LC50 values of at least triplicate determinations. - indicates no significant growth inhibition/ brine shrimp mortality. CND indicates that an IC50 could not be determined as the proliferation was >50% for all concentrations tested. ND indicates the test was not performed.

Quantification of Toxicity

K. africana fruit extracts were diluted to 4000 µg/ml (to give a bioassay concentration of 2000 µg/ml) in artificial seawater for toxicity testing in the Artemia nauplii lethality bioassay. For comparison, the reference toxin potassium dichromate was also tested in the bioassay. Potassium dichromate was rapid in its induction of mortality, with mortality evident within 4 hours of exposure (unpublished results). The *K. africana* fruit extracts were slower at inducing mortality, with ≥ 12 hours needed for mortality induction. Despite the slower onset of mortality, the methanol, water and ethyl acetate extracts induced mortality significantly above that of the artificial seawater control (Figure 2). Table 2 shows the extract and control toxin concentrations required to achieve 50% mortality (LC50) at various times. As toxicity of crude plant

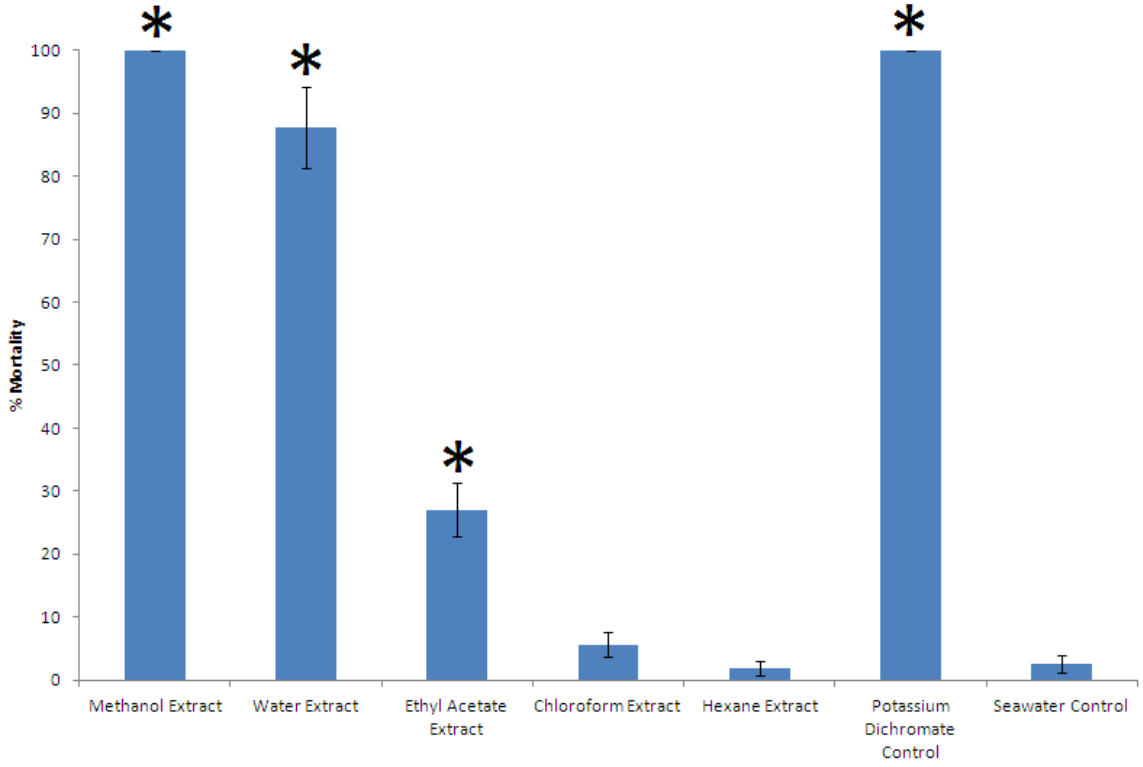

Figure 2. The lethality of *K. africana* fruit extracts (2000 µg/ml) and the potassium dichromate control (1000 µg/mL) towards *Artemia franciscana* nauplii following exposure for 24 h. All tests were performed in at least triplicate and the results are expressed as mean ± SEM. * indicates mortality that is significantly different to the seawater control at the same time point ($P < 0.05$).

extracts has previously been defined as 24 LC50 values < 1000 µg/ml,[33–36] the measured LC50 values indicate that only the water extract was significantly toxic.

HPLC-MS/MS Analysis

Two major aims of this study were to establish a HPLC-MS/MS method for the metabolic profiling of *K. africana* compounds and to use this method to qualitatively differentiate and identify a significant number of these compounds in bioactive extracts. Optimised HPLC-MS/MS parameters were developed and used to profile and compare the compound profiles from different extractions of *K. africana* fruit. The resultant total compound chromatograms for the positive ion and negative ion chromatograms are presented in Figure 3 and Figure 4 respectively. Although the negative ion chromatograms yielded significantly higher signals than those observed for the positive ion, the negative ion chromatogram had a significantly higher base peak signal to noise ratio in the total ion chromatograms.

The *K. africana* fruit methanol extract positive ion chromatogram (Figure 3a) revealed numerous overlapping peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. Nearly all of the methanol extract compounds had eluted by 10 minutes (corresponding to approximately 25% acetonitrile). Indeed, multiple peaks eluted in the first 1 minute with 5% acetonitrile. However, a large peak eluting later in the chromatogram (at approximately 33.3 min) indicates the broad spread of polarities of the compounds in this extract. The *K. africana* fruit aqueous extract (Figure 3b) also had large amounts of polar material eluting early in the chromatogram at similar elution volumes to many of the compounds in the methanol extract, although the aqueous extract had a much greater amount and size of peaks corresponding to the mid polarity compounds in the middle of the chromatogram (14.4–18 min) at approximately 34-55% acetonitrile. In contrast, the later eluting peak seen in the methanolic extract was much lower in the aqueous extract, indicating that this extract contained much less low polarity compounds.

Much fewer peaks were evident in the negative ion chromatograms (Figure 4). The methanolic (Figure 4a) and aqueous extracts (Figure 4b) negative ion chromatograms each showed several major peaks, particularly at elution times up to 10 min. Many of these elution times correspond to peaks at similar elution times in the positive

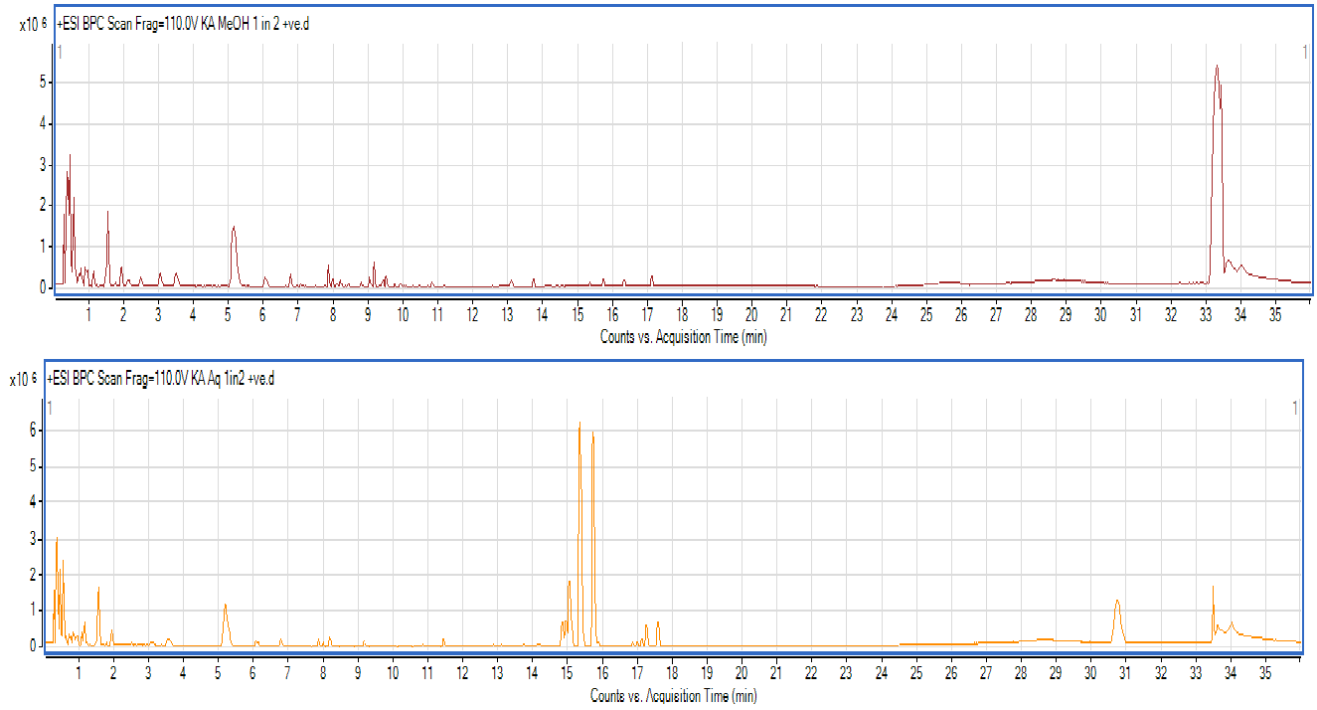

Figure 3. Positive ion RP-HPLC total compound chromatogram (TCC) of 10 µl injections of *K. africana* fruit (a) methanol extract and (b) aqueous extract.

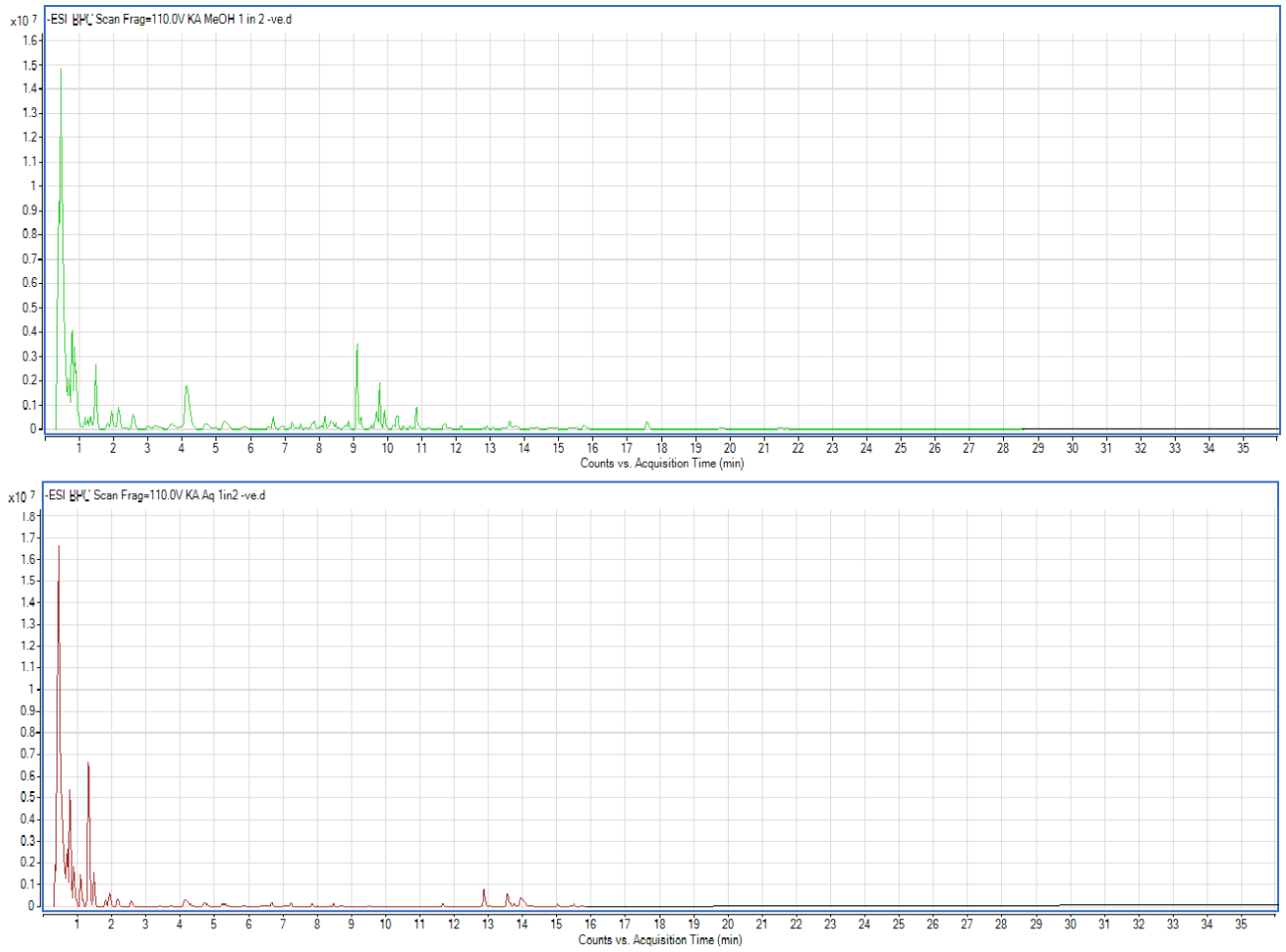

Figure 4. Negative ion RP-HPLC total compound chromatogram (TCC) of 10 µl injections of *K. africana* fruit (a) methanol extract, (b) aqueous extract.

ion chromatograms, indicating the corresponding compounds eluting at these times have functional groups that are capable of both gaining and losing electrons. Similarly, several very early eluting peaks (< 1 min) were seen in both extracts at times also corresponding to peaks in the positive ion chromatograms.

Qualitative Mass Spectral Analysis of *K. africana* Fruit Extracts

In total, 356 unique mass signals were noted for the *K. africana* fruit extracts (Table 3). Putative empirical formulas were achieved for all except 5 of these compounds. As the detection software was set to calculate molecular weights and empirical formulas of compounds containing C, H, N, O, P, S, Br, Cl and F only, it is possible that the remaining compounds contain other atoms (eg. Na, Se etc), which may account for the inability of the software to establish an empirical formula. Of the 356 unique molecular mass signals detected, approximately 227 compounds were putatively identified by comparison against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (650 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds).

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
112.0162	0.756	C5 H4 O3	3-furanoic acid	-	-
122.073	1.465	C8 H10 O	2,4,6-octatrienal		+
129.1516	2.98	C8 H19 N	Octodrine	+	
134.0226	0.382	C4 H6 O5	malic acid	-	-
138.0342	2.122	C7 H6 O3	salicyclic acid	-	-
140.0843	3.74	C8 H12 O2	2-octynoic acid		+
146.1487	1.23	C9 H6 O2	Norviburtinal	-	-
147.0684	1,799	C9 H9 N O	indol-3-carbinol	+	
148.0451	0.522	C3 H8 N4 O S	4-Amino-5-(hydroxymethyl)-1,2,4-triazolidine-3-thione	-/+	-
150.0683	1.919	C9 H10 O2	4-Ethylbenzoic acid	+	+
154.0241	1.302	C7 H6 O4	protocatechuic acid	-	-
157.1463	3.329	C9 H19 N O			+
162.0323	1,799	C9 H6 O3	o-Hydroxyphenylpyruvic acid lactone	+	
165.0428	3.857	C8 H7 N O3	N-formylanthranilic acid	+	
164.0436	6.797	C4 H8 N2 O5		-	
164.0462	1.799	C9 H8 O3	coumaric acid	+	+
165.0626	0.882	C6 H7 N5 O	1-Methylguanine		+
166.0524	3.857	C6 H6 N4 O2	3-Methylxanthine	+	
168.0794	1.918	C9 H12 O3	4-Hydroxy-3-methoxyphenethyl alcohol	+	+
169.122	0.825	C8 H15 N3 O			+
170.02	0.68	C7 H6 O5	(1S,5R)-4-Oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2-carboxylic acid		-
173.1164	0.478	C7 H15 N3 O2	Indospicine		+
174.0186	0.757	C6 H6 O6	dehydroascorbic acid (oxidised vitamin C)	-	-
176.0699	2.539	C7 H12 O5	2-Isopropylmalic acid	-	-
178.0284	3.818	C9 H6 O4	ESCULETIN	-	
180.047	4.141	C9 H8 O4	caffeic acid	-/+	-
180.982	33.541	C7 H3 N O3 S			+
182.0571	0.67	C9 H10 O4	,4-Dihydroxyphenylpropionic acid	+	
183.9735	33.487	C3 H2 Cl F5 O	Isoflurane	+	+
281.0364	1.919	C8 H11 N O10			+
184.0735	5.271	C9 H12 O4	3-Methoxy-4-hydroxyphenylglycol	+	+
186.0917	1.919	C10 H10 N4		-/+	-/+
187.0866	0.419	C8 H13 N O4	(3S,4R,5S)-5-(aminomethyl)-3,4-dihydroxycyclohex-1-ene-1-carboxylic acid	+	+
188.0394	0.527	C13 H4 N2		-	-
189.0308	4.705	C12 H3 N3		+	+
192.0256	0.431	C6 H8 O7	citric acid	-	-
192.0591	0.86	C6 H12 N2 O3 S	Cys Ala	-	-
194.0329	0.418	C9 H10 N2 O3	2-Pyridylacetylglycine	-	-

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
194.0613	9.572	C10 H10 O4	ferulic acid	-	
194.1167	1.143	C9 H14 N4 O	Azimexone	+	+
195.0532	1.799	C9 H9 N O4	N-acetyl-4-aminosalicylic acid	+	
196.0555	0.334	C6 H12 O7	(3S,4S,5R)-2-(Dihydroxymethyl)tetrahydro-2H-pyran-2,3,4,5-tetrol		-
196.0727	0.621	C10 H12 O4	ASARYLALDEHYDE		+
197.1211	8.175	C14 H15 N		+	+
198.0538	1.373	C9 H10 O5	ethyl gallate	-	
200.0687	0.537	C9 H12 O5	ethylgallic acid	+	+
202.0737	0.427	C11 H10 N2 O2		-	
202.0851	0.655	C9 H14 O5	Diethyl Oxalpropionate	-	-
206.0377	0.746	C14 H6 O2		-/+	-
206.0452	0.567	C7 H10 O7	2,3-O-(Oxomethylene)hexopyranose		-
210.0793	6.304	C13 H10 N2 O	Pyocyanine	+	
211.1111	6.947	C10 H17 N3 S	Pramipexole	+	
216.0631	0.584	ND			-
217.1032	0.416	C8 H15 N3 O4	Ala Ala Gly		+
219.1121	1.139	C9 H17 N O5	Pantothenic Acid	-/+	-/+
223.0973	0.481	C15 H13 N O	N-Fluorenylacetamide		+
228.1402	0.556	C12 H20 O4	Octahydro-4a,9a(2H,5aH)-oxanthrenediol	+	
229.982	0.425	C6 H2 N2 O8			+
232.0742	1.125	C13 H12 O4	Aloesone	+	
235.1195	0.695	C13 H17 N O3	Lophophorine	+	+
237.0907	0.424	C14 H11 N3 O	Mebendazole metabolite (2-Amino-5-benzoylbenzimidazole)	+	+
238.1417	2.279	C12 H14 O5	Kigelin	+	+
240.1378	0.594	C14 H16 N4	Budralazine	+	
242.1061	7.352	C11 H18 N2 O2 S	Thiopental	+	
242.2638	1.797	C15 H14 O3	Lapachol	+	+
243.1855	13.028	C13 H25 N O3	2) 1-oxa-4-azaspiro[4.5]decane-3,3-dimethanol, 8-propyl-	-	-/+
245.1012	0.453	C9 H15 N3 O5		+	+
246.1255	1.531	C15 H18 O3	Santonin	+	
248.973	1.307	C6 H3 N O10			+
249.0991	0.548	C8 H15 N3 O6	Gly Ser Ser		+
250.1654	17.467	C14 H22 N2 O2	3-Hydroxylidocaine	-	
251.1138	0.422	C13 H17 N O4	ACETYL TYROSINE ETHYL ESTER	+	+
254.1553	14.319	C15 H18 N4		-	
255.1111	1.744	C12 H17 N O5		+	+
257.1634	1.778	C13 H23 N O4	2-cyclohexylpiperidine oxalate	+	+
260.1052	4.094	C15 H16 O4	DIHYDROSPATHELIACHROMENE	+	

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
264.006	13.614	C15 H4 O5		-	
265.0574	1.064	C11 H11 N3 O3 S	5-Hydroxysulfapyridine		-/+
269.0993	0.54	C12 H11 N7 O	p-Hydroxytriamterene	+	+
267.0961	0.476	C10 H13 N5 O4	Adenosine	+	
270.2427	11.439	C14 H30 N4 O			+
271.1165	0.458	C16 H17 N O3	Normorphine		+
271.1794	6.048	C15 H21 N5		+	
273.0895	0.422	C17 H11 N3 O			+
274.1212	13.085	C16 H18 O4	HETEROPEUCENIN, METHYL ETHER	+	+
275.1364	0.517	C13 H17 N5 O2	Aditeren	+	+
277.1176	0.341	C12 H15 N5 O3	Queuine	+	+
279.1014	0.649	C9 H17 N3 O7	Ser Ser Ser		+
279.1283	0.395	C12 H17 N5 O3	7-Morpholinomethyltheophylline		+
279.1465	2.742	C15 H21 N O4	Afurolol	+	
280.0043	13.751	C8 H8 O11			-
281.1223	0.685	C18 H19 N S	Northiadene	+	
281.9037	33.564	C9 H2 N2 O S4			+
285.0848	0.601	C10 H12 F N5 O4	Fludarabine		-/+
285.2674	16.083	C17 H35 N O2	C17 Sphingosine		+
286.0144	1.92	C9 H10 N4 O3 S2	Sulfametrole		+
287.9863	14.978	C8 H4 N2 O10			-
288.2848	15.712	C21 H36			+
291.0948	0.4	C11 H17 N O8		-	+
292.1323	12.684	C16 H20 O5	TETRAHYDROTRIMETHYLHISPIDIN	+	
293.1059	7.422	C18 H15 N O3	Oxaprozin	+	
294.0853	0.786	C13 H14 N2 O6	4-(o-Carboxybenzamido)glutaramic acid	-	
294.1852	15.48	C17 H26 O4	gingerol	-	-
295.1535	5.798	C19 H21 N O2	hydroxydoxepin M2	+	
297.0922	0.692	C11 H15 N5 O3 S	5'-Methylthioadenosine	+	
297.1365	1.615	C18 H19 N O3	glaziovine	+	
298.1418	9.316	C15 H22 O6	Idebenone Metabolite (Benzenehexanoic acid, 2,5-dihydroxy-3,4-dimethoxy-6-methyl-)	+	+
298.1652	19.631	C14 H18 N8		-	
299.0672	1.341	C15 H13 N3 O2 S	fenbendazole	-	
299.2105	6.772	C17 H25 N5		+	+
300.1227	2.497	C12 H20 N4 O3 S	Desmethylranitidine	-	
301.0836	0.592	C18 H11 N3 O2			+
301.1801	1.541	C17 H23 N3 O2	N-Acetylprimaquine	+	+
302.071	9.019	C13 H10 N4 O5	Nicarbazin	+	
303.0891	0.638	C19 H13 N O3			-

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
303.1324	0.488	C14 H17 N5 O3	PIPEMIDIC ACID		+
303.1412	0.497	C17 H21 N O4	scopolamine	+	
303.2773	15.938	C17 H37 N O3			+
306.1329	1.487	C14 H18 N4 O4	Trimethoprim 3-N-oxide	+	+
306.1475	13.1	C17 H22 O5	Matricin	+	+
307.1063	8.011	C19 H17 N O S	Tolnaftate	+	
308.3551	1.3	C19 H16 O4	Kigelinone	-	-
309.1068	0.517	C11 H19 N O9	N-Acetyl-b-neuraminic acid	+	
310.1209	9.75	C19 H18 O4	Warfarin alcohol	+	
310.1287	1.001	C19 H19 Cl N2	Descarboethoxyloratadine		+
311.1376	0.743	C19 H21 N O S	1-Propanamine, N,N-dimethyl-3-(5-oxidodibenzo[b,e]thiepin-11(6H)-ylidene)-, (E)-(9CI)	+	
312.1853	21.349	C19 H24 N2 O2	Praziquantel	-	
313.0801	1.801	C13 H15 N O8			-
313.0814	0.595	C14 H11 N5 O4		-	-
314.1006	0.483	C12 H27 O P S3	S,S,S,-TRIBUTYLPHOSPHOROTRITHIOATE	-	
315.0957	0.707	C11 H17 N5 O4 S	Gly His Cys	-	-/+
315.1721	0.701	C16 H21 N5 O2	Alizapride		+
315.2044	5.183	C23 H25 N	FENDILINE	+	+
315.3144	16.938	C19 H41 N O2			+
316.1168	1.432	C15 H16 N4 O4		-	
316.1438	0.625	C22 H20 O2	4,7,10,13,16,19-Docosahexaynoic acid	+	
317.112	0.455	C14 H15 N5 O4		-	
319.9847	13.941	ND			-
322.1286	0.711	C19 H18 N2 O3	Kebuzone	+	+
324.0858	9.749	C15 H21 Br N2 O	Bromadolin	+	
324.1091	3.22	C13 H16 N4 O6	4-Amino-7-(2-C-methylpentofuranosyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxylic acid	+	
324.1427	4.698	C19 H20 N2 O3	p-Hydroxyphenylbutazone	-	-
326.1147	0.483	C16 H22 O5 S	(1s, 4R, 5S, 8S)-4,8-dimethyl-4[(phenylsulfonyl) methyl]-2,3-dioxabicyclo[3.3.1] nonan-8-ol	-	
328.1293	13.103	C19 H20 O5	DEOXYSAPPANONE B TRIMETHYL ETHER	+	
328.2295	12.865	C18 H32 O5	9,12,13-trihydroxy-10,15-octadecadienoic acid	-	-
329.1112	1.43	C12 H19 N5 O4 S	His Ala Cys		-/+
330.0954	1.969	C14 H18 O9		-	
330.1172	0.439	C17 H18 N2 O5	Nitrosonifedipine	+	
330.2433	13.545	C18 H34 O5	9,12,13-trihydroxy-10-octadecenoic acid	-	-
331.3092	15.878	C19 H41 N O3			+

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
332.0995	0.587	C16 H16 N2 O6	3,5-Pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-, monomethyl ester		+
334.0912	0.4	C17 H19 Cl N2 O S	Chlorpromazine sulphoxide	-	
338.3534	0.472	C20 H18 O5	Pinnatal	+	+
340.1313	9.752	C20 H20 O5		+	+
341.1121	0.784	C15 H19 N O8	Diethylpropion(metabolite VIII-glucuronide)	+	
342.0986	3.529	C17 H10 N8 O		-	
342.1175	0.289	C12 H22 O11	Maltose	-	
343.1225	0.94	C15 H21 N O8	b-D-Glucopyranosiduronic acid	-	-
344.1643	0.42	C20 H24 O5	16-Hydroxy-4-carboxyretinoic acid	+	
345.106	0.372	C12 H19 N5 O5 S	Cys Ser His	-/+	+
346.1257	2.235	C15 H22 O9	Deutzioside	-/+	
347.2676	13.547	C18 H37 N O5			+
348.1433	2.149	C16 H20 N4 O5	Ser Gly Trp	-	-
349.1984	8.614	C18 H27 N3 O4	Ile Ala Phe		+
350.1942	4.87	C17 H26 N4 O4	Phe Lys Gly	-	
354.0802	0.384	C19 H15 Cl N2 O3	N-Desmethylketazolam	-	-
354.0906	6.825	C16 H18 O9	chlorogenic acid	-	
356.1126	6.411	C16 H20 O9	Gentiopicrin	-	
359.1215	1.359	C15 H21 N O9	Normetanephrine glucuronide	-/+	-
359.1727	8.447	C20 H25 N O5	C20 H25 N O5	+	
360.1059	1.756	C15 H20 O10	3-Methoxy-4-hydroxyphenylglycol glucuronide	-	
362.1221	2.556	C15 H22 O10	procumbide	-	
362.1342	0.538	C19 H22 O7	Gibberellin A8-catabolite		+
364.1371	0.658	C15 H24 O10	harpagide		-
365.1689	1.465	C22 H23 N O4	Nequinate	+	+
368.1379	1.649	C21 H16 N6 O		+	
369.9816	15.509	C13 H6 O13		-	
370.1484	0.42	C14 H26 O11	Amylose		+
370.9846	15.71	C23 H N O5			-
373.1105	0.455	C13 H19 N5 O6 S	Asp His Cys	-	
473.1658	2.324	C20 H23 N7 O7	N10-Formyltetrahydrofolic acid	-	
374.0913	9.507	C16 H15 Cl N6 O3	Desmethylzopiclone	+	+
374.1763	0.382	C21 H27 Cl N2 O2	hydroxyzine		+
374.1884	14.843	C25 H26 O3	ESTRONE BENZOATE	-	
376.1013	0.909	C15 H20 O11		-	-
376.1368	2.089	C17 H20 N4 O6	Vitamin B2 (riboflavin)	-	
378.0866	0.317	C17 H18 N2 O6 S	carbenicillin	-	
378.1035	0.51	C22 H18 O6	METHYL ROBUSTONE	-	

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
378.1896	8.465	C18 H26 N4 O5	Asn Phe Val	-/+	-
380.0649	0.339	C19 H12 N2 O7		+	+
380.1681	3.366	C17 H24 N4 O6	Gln Phe Ser	+	-
384.1193	1.467	C14 H20 N6 O5 S	S-Adenosylhomocysteine	-	-
386.173	15.489	C15 H26 N6 O4 S	Lys Cys His	+	
388.1073	9.556	C17 H17 Cl N6 O3	Zopiclone	+	
388.1365	3.707	C17 H24 O10	Secologanin		-
388.1597	0.374	C20 H24 N2 O6	Nisoldipine	+	+
390.1164	1.967	C16 H22 O11	Monotropein	-	-
392.1308	1.492	C23 H20 O6	DEHYDROROTENONE	-	
392.1324	5.257	C14 H24 N4 O7 S	Met Glu Asn	-	-
392.1445	0.592	C20 H24 O8	b-D-Glucopyranosiduronic acid, 3-(6-hydroxy-2-naphthalenyl)-1-methylpropyl	+	
394.1476	1.463	C17 H22 N4 O7	Phe Asn Asp		-
396.1328	1.645	C18 H24 N2 O6 S	Glibornuride M2 (p-carboxyglibornuride)	+	
396.159	0.501	C23 H24 O6	ROTENONIC ACID	+	
396.1998	4.844	C18 H28 N4 O6	Lys Ser Tyr	-/+	
398.1729	0.87	C19 H22 N6 O4	Trp His Gly	+	+
401.1787	0.502	C21 H24 F N3 O4	MOXIFLOXACIN	+	
402.1732	0.466	C22 H27 Cl N2 O3	Lorajmine		+
404.2183	15.613	C23 H32 O6	STROPHANTHIDIN	+	
408.1212	0.4	C23 H20 O7	ICHTHYNONE	-	
408.1945	25.642	C22 H29 F O6	6-Hydroxydexamethasone	+	+
409.165	1.895	C19 H27 N3 O5 S	Met Pro Tyr	+	
410.1428	0.566	C17 H22 N4 O8	Tyr Asn Asp	-/+	
412.1494	13.934	C18 H24 N2 O9	N-Carboxytocainide glucuronide	+	+
412.1936	0.762	C18 H28 N4 O7	Deoxypyridinoline		+
412.6961	14.053	C29 H48 O	Stigmasterol	-	
414.7052	16.329	C29 H50 O	β - Sitosterol	+	+
415.222	7.846	C21 H29 N5 O4	Phe Leu His	+	+
416.1765	0.518	C24 H29 Cl O4	Cyproterone acetate	+	
418.0814	8.308	C23 H10 N6 O3		-	
421.2464	15.615	C19 H31 N7 O4	Mopidamol	+	
422.2166	10.78	C20 H30 N4 O6	Gln Tyr Leu	-	
426.137	0.928	C22 H28 Cl2 O4	Mometasone	-	
426.1443	1.703	C22 H22 N2 O7	Anhydrotetracycline	+	
428.1848	0.535	C24 H28 O7	RHODOMYRTOXIN B	+	
428.1919	1.098	C18 H28 N4 O8	Pyridinoline	+	
429.239	9.152	C22 H31 N5 O4	Pro Trp Lys	-/+	
431.1127	8.093	C24 H13 N7 O2		+	

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
431.232	17.116	C20 H29 N7 O4	Trp Arg Ala	+	
432.097	8.784	C21 H20 O10	vitexin	+	
432.163	5.097	C21 H28 N4 O6	Val Trp Glu	-	-
433.1672	0.475	C19 H23 N5 O7	Trp Asn Asp	+	+
434.1585	10.426	C19 H30 O9 S	Idebenone Metabolite (Benzenedecanoic acid, 2-hydroxy-3,4-dimethoxy-6-methyl-5-(sulfooxy)-)	+	
439.2419	10.832	C23 H37 N O5 S	Leukotriene E4	+	+
440.09	0.607	C26 H16 O7			-
440.0918	0.354	C14 H20 N2 O14			-
440.3265	4.337	C29 H44 O3	1alpha,25-dihydroxy-26,27-dimethyl-22,22,23,23-tetradehydrovitamin D3 / 1alpha,25-dihydroxy-26,27-di	+	+
441.0955	0.317	C24 H15 N3 O6		-	
442.136	0.539	C22 H22 N2 O8	12-Dehydrotetracycline	-	-
443.134	0.962	C16 H21 N5 O10	Zidovudine glucuronide		+
444.1779	0.493	C31 H24 O3	Difenacoum	+	
444.1982	4.109	C22 H28 N4 O6	Mitoxantrone	-	
446.1123	9.907	C24 H19 Cl N4 O3	Nicafenine	+	
488.1524	2.904	C19 H16 N14 O3		-	
453.1936	0.396	C13 H27 N9 O9		+	+
456.1867	0.515	C24 H28 N2 O7	8-hydroxymianserin glucuronide		+
458.1269	0.344	C17 H23 N4 O9 P	FMNH	-	
458.1788	4.119	C22 H26 N4 O7	Tyr Tyr Asn		-
459.1638	0.53	C22 H25 N3 O8	Asp Tyr Tyr	+	+
460.128	10.046	C22 H27 Cl F2 O4 S	Cloticasone	+	
460.1471	0.521	C22 H24 N2 O9	Oxytetracycline	-	-
460.1945	3.502	C19 H20 N14 O		-/+	
362.1334	0.532	C19 H22 O7	Gibberellin A8-catabolite	+	
462.1738	2.556	C21 H26 N4 O8	Trp Glu Glu	-	-
464.1233	8.392	C22 H24 O11	viscumside	+	
464.187	6.825	C17 H24 N10 O6		-	
467.2057	0.432	C25 H29 N3 O6		+	
468.2235	10.749	C20 H32 N6 O5 S	Met Tyr Arg	-	
471.1578	0.455	C22 H24 F3 N O7	b-D-Glucopyranuronic acid	-	
471.1858	0.512	C25 H29 N O8	hydroxydoxepin M3-glucuronide		+
472.1806	0.502	C20 H24 N8 O6	5-Formiminotetrahydrofolic acid		+
473.164	2.372	C20 H23 N7 O7	N10-Formyltetrahydrofolic acid	-/+	-/+
473.1989	1.285	C21 H27 N7 O6	Ketotrexate	+	
473.2245	8.406	C19 H27 N11 O4		+	
474.143	10.093	C26 H22 N2 O7		+	
474.2109	7.099	C22 H30 N6 O4 S	SILDENAFIL	+	

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
478.1324	0.317	C17 H14 N14 O4		-	
479.199	2.53	C26 H29 N3 O6	Nicardipine	+	
480.147	0.928	C17 H16 N14 O4	Asn Thr Phe	-	
484.3531	2.215	C31 H48 O4	(22S)-1alpha,25-dihydroxy-22-ethoxy-26,27-dimethyl-23,24-tetradehydro-20-epivitamin D3 / (22S)-1alph	+	+
491.174	1.437	C31 H25 N O5		-/+	-/+
494.1632	1.453	C18 H18 N14 O4		-	-
494.1632	4.273	C18 H18 N14 O4			-
496.0801	0.39	C16 H20 N2 O16		+	
502.1852	9.195	C27 H31 Cl O7	3-CHLORO-8beta-HYDROXYCARAPIN, 3,8-HEMIACETAL	+	
504.1499	5.639	C23 H20 N8 O6		-/+	
508.4588	9.864	C22 H28 O12	Specioside	-	
510.176	9.63	C26 H22 N8 O4		-	
516.132	0.447	C25 H26 Cl2 N4 O4	Ketoconazole Metabolite (1-Piperazinecarboxaldehyde, 4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-	-	-
518.1792	10.637	C24 H18 N14 O		+	
518.2364	7.803	C25 H34 N4 O8	1-methyl-N-(9-methyl-9-azabicyclo[3.3.1]non-3-yl)-7-(sulfooxy)-, endo- glucuronide	-	
520.1803	8.014	C23 H28 N4 O10		-	
522.2106	8.708	C24 H22 N14 O		-	
524.1466	9.034	C15 H24 N8 O13		-	
524.1749	6.649	C19 H20 N14 O5		-	-
524.491	10.214	C24 H28 O13	Verminoside	-	
526.17	8.463	C37 H22 N2 O2		-	
524.2378	8.716	C23 H24 N16			-
530.1487	0.621	C26 H28 Cl2 N4 O4	Ketoconazole	-	
530.4	31.338	C40 H50	Anhydroeschscholtzxanthin		+
535.227	9.371	C21 H25 N15 O3		+	
538.571	10.214	C25 H30 O13	Minecoside	-/+	
538.2045	8.188	C27 H35 Cl O9	12R-acetoxy-punaglandin 3	-	
540.1832	9.373	C32 H28 O8	IRIGENIN, DIBENZYL ETHER	+	
542.1634	5.694	C22 H18 N14 O4		-/+	+
644.2323	7.415	C28 H24 N18 O2		-	
548.1567	0.731	C28 H20 N8 O5		-	
552.2214	9.602	C28 H32 N4 O8		-	
554.1477	0.648	C19 H18 N14 O7		-	
560.1567	6.96	C27 H28 O13	Deoxydaunorubicinol aglycone 13-O-b-glucuronide	+	
560.1949	0.344	C21 H36 O17		-	

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
562.1458	0.468	C27 H18 N10 O5		+	
562.2024	8.495	C24 H30 N6 O10		+	
566.2017	9.572	C28 H30 N4 O9		-	
567.2299	9.682	C24 H29 N11 O6		+	
569.2475	11.638	C25 H27 N15 O2		+	
572.2223	0.534	C38 H28 N4 O2			+
574.2035	11.631	C26 H26 N10 O6		+	
580.2162	9.864	C26 H24 N14 O3		-	
583.263	12.863	C26 H29 N15 O2		+	
584.2106	7.59	C27 H36 O14	scaevoloside	-	
586.1804	0.455	ND			-
586.2257	7.834	C25 H26 N14 O4		-	-
588.2187	12.863	C24 H20 N20		+	
595.2189	0.506	C32 H25 N11 O2		+	+
596.2117	9.149	C27 H20 N18		-	
597.2336	0.413	C31 H31 N7 O6		+	+
598.2253	11.642	C28 H38 O14	katecateroside	-	-
598.3841	7.985	C32 H50 N6 O5		+	+
599.2577	11.696	C26 H29 N15 O3		+	
604.2127	11.7	C24 H20 N20 O		+	
606.2072	12.55	C29 H30 N6 O9		+	+
608.1754	0.483	C28 H32 O15	drosmin	-	
612.1773	33.327	C28 H28 N4 O12	(3Z)-3-[(4-Nitrophenyl)hydrazono]-1-(2,3,4,6-tetra-O-acetylhexopyranosyl)-1,3-dihydro-2H-indol-2-one	+	
615.1605	33.331	C31 H21 N9 O6		+	
622.1856	0.4	C36 H30 O10	DIOONFLAVONE	-	
622.1927	7.678	C29 H34 O15	PECTOLINARIN	-	
625.2189	0.455	C29 H34 F3 N3 O7 S	Flupenthixol-O-glucuronide	-	
628.2362	11.689	C27 H28 N14 O5		-	-
638.222	10.16	C29 H22 N18 O		-	
640.2002	8.335	C27 H24 N14 O6		-	-
641.2068	33.351	C25 H23 N17 O5		+	
642.2072	0.537	C44 H26 N4 O2			-
646.2466	8.739	C27 H30 N14 O6		-	
652.2009	7.991	C28 H24 N14 O6		-	
653.2144	0.372	C32 H40 Br N5 O5	Bromocriptine	-	
654.2178	9.149	C31 H34 N4 O12		-	
658.1721	8.038	C25 H26 N10 O12		-	
659.2206	0.455	C45 H29 N3 O3		-	
679.4733	30.765	C30 H53 N19			+

Continue...

Table 3: Qualitative HPLC-MS/MS analysis of the K. africana extracts, elucidation of empirical formulas and putative identification (where possible) of the compound.

Molecular Mass	Retention Time	Empirical Formula	Putative Identification	M	W
682.2447	9.63	C26 H26 N20 O4		-	
684.2275	0.4	C49 H32 O4		-	
686.2037	0.521	C40 H22 N12 O			-
702.2399	0.531	C25 H42 N4 O19	Neu5Acalpha2-6Galbeta1-4Glcbeta-Sp	+	
704.2165	7.149	C28 H28 N14 O9		-	
705.4889	30.76	C32 H55 N19			+
716.211	0.4	C38 H36 O14		-	
720.2635	10.603	C35 H44 O16	Azadirachtin A	-	
735.5357	30.76	C34 H61 N19			+
740.2157	0.344	C30 H32 N10 O13		-	
746.2783	11.172	C32 H26 N24		-	-
776.2896	10.834	C28 H48 N4 O21	Galbeta1-4GlcNAcbeta1-3Galbeta1-4Glcbeta-Sp-4[Fucalpha1-3]GlcNAcbeta-Sp	-	-
785.527	30.76	C42 H76 N O10 P	GPSer(18:1(9Z)/18:2(9Z,12Z))[U]		+
830.2704	5.833	ND			-
862.4246	15.346	C46 H62 N4 O12	32-Hydroxyrifabutin		+
908.3338	9.511	ND			-
938.347	9.667	C34 H58 N4 O26	Galbeta1-3GalNAcbeta1-3Galalpha1-4Galbeta1-4Glcbeta-Sp	-	-

ND indicates that an empirical formula could not be accurately determined. + and - indicates the mass spectral mode that the molecule was detected in. The compounds detection in the various extracts is highlighted. M = methanolic extract; W = water extract;

The methanol extracts had greater phytochemical diversity than the aqueous extracts, with 281 and 179 mass signals detected respectively. Significantly more compounds were detected solely in the negative mode (186 of the 356 compounds detected; 52.2%) than in the positive mode (142 compounds; 39.9%) or in both modes (28 compounds; 7.9%). One hundred and three compounds were determined to be present both the methanol and aqueous extracts. Of these, 13 were identified as peptides/amino acids and a further 25 were unable to be identified. Amongst those compounds that were identified in both the methanol and aqueous extracts, the naphthoquinone lapachol (Figure 5a) and the dihydroioscoumarin kigelin (Figure 5b) were present as major components. Several other compounds including kigelinone (Figure 5d), pinnatal (Figure 5e) (and/or isopinnatal (Figure 5c)), β-sitosterol (Figure 5f), (Figure 5g), coumaric acid (Figure 5h), caffeic acid (Figure 5i), ethylgallic acid (Figure 5j) and norviburtinal (Figure 5m) were also detected in both extracts. Chlorogenic acid (Figure 5k), verminoside (Figure 5l) and ferulic acid (Figure 5n) were detected only in the methanolic *K. africana* fruit extract.

DISCUSSION

The aim of plant metabolomics is to identify the complete complement of metabolites in a plant sample as rapidly as possible and without bias.[37] This can be a difficult, time consuming task, requiring a range of analytical methodologies to achieve an extensive examination of the plants metabolome. The diversity of chemical properties and the wide concentration ranges of these compounds makes this difficult as the method used not only needs to identify a vast amount of compounds in a single sample, but also needs to be robust and reproducible enough to allow samples to be reliably compared.[38]

Early metabolomic studies have focused mainly on gas chromatography - mass spectroscopy (GC-MS) based

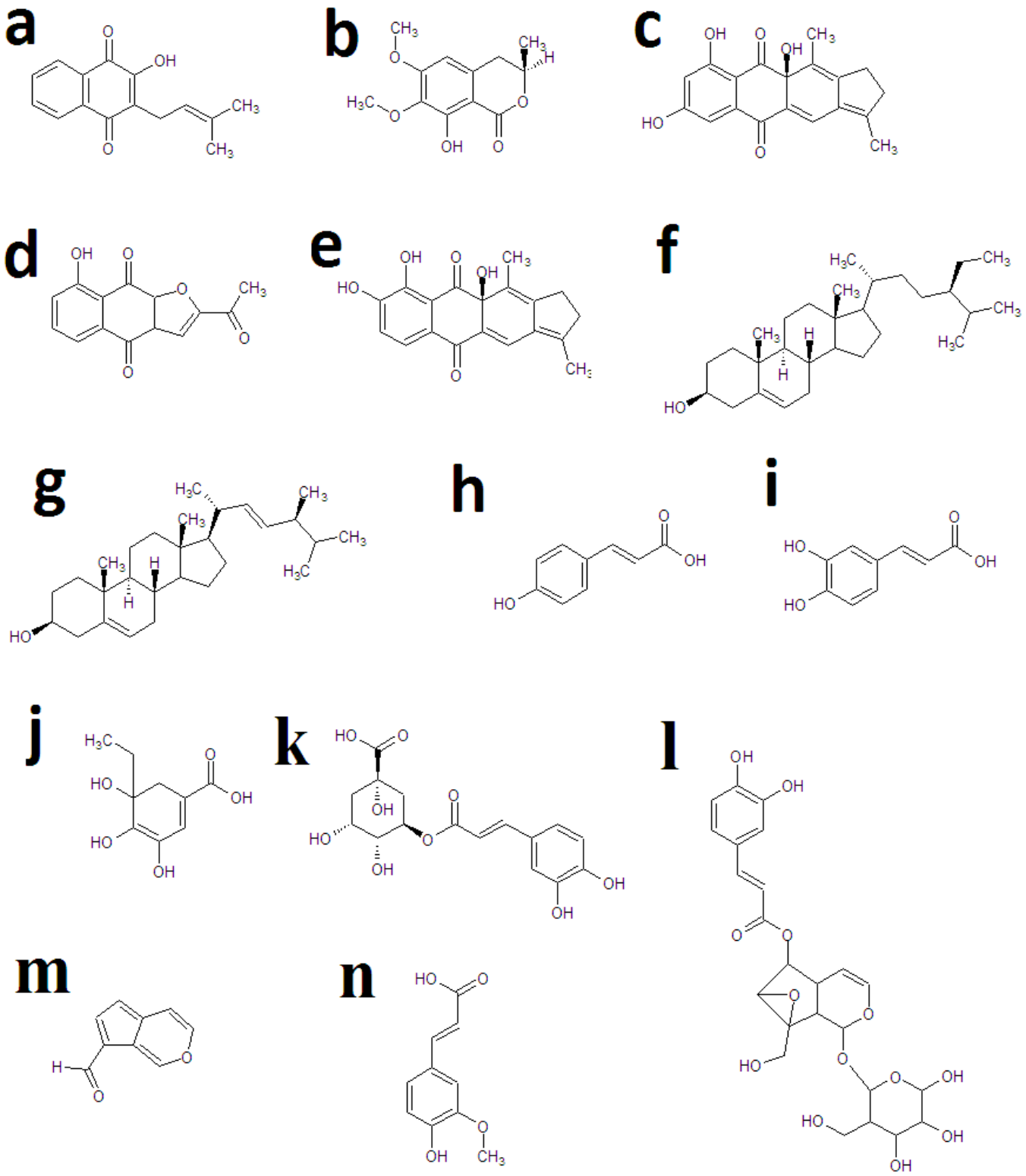

Figure 5. Chemical structures of selected molecules identified in *K. africana*: (a) lapachol, (b) kigelin, (c) isopinnatal, (d) kigelinone, (e) pinnatal, (f) β-sitosterol, (g) stigmasterol, (h) coumaric acid, (i) caffeic acid, (j) ethylgallic acid, (k) chlorogenic acid, (l) verminoside, (m) norviburtinal and (n) ferulic acid .

approaches. However, this method is limited in the types of compounds which may be separated and detected. GC-MS is generally used for the detection of low polarity compounds. Thus, a large amount of compounds of medium polarity or high polarity are often not detected by GC-MS metabolomic analysis. Liquid chromatography – mass spectroscopy (LC-MS) is better suited for the analysis of compounds of medium and high polarity. Coupling LC with high mass accuracy spectroscopy techniques using both mild ionisation and electrospray ionisation (ESI) can generate large amounts of useful information for compound identification and metabolomic analysis. Using these methods, molecular ions can be detected and their empirical formulas accurately determined and compared to databases. Furthermore, coupling this with ESI analysis also allows for the detection and characterisation of characteristic fragments, allowing for rapid identification of unknown compounds in a crude extract.

The current study demonstrated potent anti-proliferative activity in various *K. africana* solvent extracts against Jeg-3 choriocarcinoma carcinoma cells. Our studies support previous reports of anticancer activity towards other cell lines. Aqueous *K. africana* root bark extracts have been reported to inhibit the growth of melanoma cells and renal carcinoma cells.[13,17] Cytotoxic activity for *K. africana* fruit extracts has also been reported against a melanoma and 2 breast cancer cell lines.[18] That study also identified several *K. africana* phytochemicals including demethylkigelin, kigelin, ferulic acid and 2-(1-hydroxyethyl)-naphtho[2,3-b] furan-4,9-dione as inhibiting cancer cell proliferation.[18] The compound 2-(1-hydroxyethyl)-naphtho[2,3-b] furan-4,9-dione was reported to be a particularly good anticancer agent. Significant anti-proliferative activity against CaCo2 and HeLa carcinoma cell lines cells was also noted for the *K. africana* fruit extracts.[12]

Interestingly, only the *K. africana* water extract displayed significant toxicity towards *A. franciscana*, with an LC50 of 477 μg/ml. Whilst otherwise quite hardy, Artemia nauplii have been shown in our laboratory to be sensitive to pH changes (unpublished results). Extract constituents such as ascorbic acid, gallic acid and other tannins would be expected to decrease the pH of the test solution and may account for any apparent toxicity. As the water extract would be expected to contain significant quantities of ascorbic acid and tannins, it is perhaps not surprising that this extract displayed significant toxicity in this assay. Further toxicity studies using human cell lines are needed to further determine the suitability of the *K. africana* fruit extracts for medicinal purposes.

An important consideration of any metabolomic technique is that it will not detect all compounds in a complex mixture, but instead will only detect a portion of the compounds in the solvent. This is not necessarily a problem when a directed/biased study is undertaken to detect a particular compound or class of compounds and the separation and detection conditions can be optimised for the study. However, when the aim of the study is metabolomic profiling rather than metabolomic fingerprinting, the technique conditions must be chosen and optimised to separate and detect the largest amount of compounds, with the broadest possible physical and chemical characteristics. Generally, HPLC-MS/MS is a good choice for such metabolomic profiling studies as it detects a larger amount of compounds of varying polarities than the other commonly used techniques. However, this method is limited to studies of the mid-highly polar compounds and is not as useful for studies aimed at highly non-polar compounds. For these compounds, GC-MS analysis may provide further information.

In order to putatively identify as many compounds as possible in the extracts, the obtained spectra were compared with commercially available electron mass impact libraries. Furthermore, a library was prepared using approximately 600 compounds that are present in detectable amounts in a variety of plant species. When these libraries were screened against our extracts, 227 compounds of the 356 detected mass signals (63.8%) were putatively identified. However, there were still a large number of compounds that were not identified by comparison to any of the libraries we used in our studies. These compounds will require significant further efforts before a structure and putative identification can be assigned.

For further metabolomic profiling studies, perhaps HPLC-MS/MS coupled with GC-MS would give a more complete phytochemical profile. of more interest for our studies, the inability to detect all compounds by a single technique may mean that the bioactive compounds are missed. Furthermore, mass spectral techniques are generally not capable on their own to differentiate between structural isomers. Thus, comparison to the databases used in this study may have mistakenly identified an incorrect isomeric form of some compounds. Despite the promising results of our study, much work needs to be completed for a complete understanding of the anticancer properties of *K. africana*. Studies comparing the metabolomic profiles of our extracts with anti-cancer properties with non-proliferative extracts produced using other solvents may allow us to narrow the focus of compounds for further separation studies. This approach has

been successfully used previously with other species to narrow the focus of possible bioactive compounds. In one study examining the anti-viral activity of *Scaevola spinescens*, a comparison of the metabolomic profiles of solvents of varying polarities was able to highlight 2 compounds from 239 detected mass signals as possibly contributing to this activity.[23] Of these 2 compounds, 1 had been previously been reported to have anti-viral activity, validating this approach.

Narrowing the scope of extract compounds in a directed way such as comparing metabolomic profiles between various extracts would be expected to speed up the purification process and greatly improve the chances of isolating bioactive components. Further purification studies would allow isolated compounds to be tested for anti-cancer activity and for mechanistic studies to be undertaken. However, it must also be noted that whilst this metabolomic profiling approach may provide us with promising leads, it is likely that these are not the only anti-cancer compounds in these extracts. Other compounds with anti-cancer activity may be missed by this approach. The methanolic extract for example contained 281 unique mass signals (as well as the possibility of other undetected compounds), several of which may represent other anti-cancer compounds. It is possible that some of these may also contribute to the anti-cancer activity of that extract and further studies need to test this possibility.

CONCLUSION

Our results confirm that non-targeted HPLC separation coupled with high resolution time of flight MS/MS is a useful tool in metabolomic profiling of *K. africana* extracts. In this study, we used this technology to putatively identify a significant number of *K. africana* compounds and to compare the metabolomic profiles of the different solvent extracts

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