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Original Article

An examination of the antibacterial, antifungal, anti-Giardial and anticancer properties of *Kigelia africana* fruit extracts

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ABSTRACT: **Background:** *Kigelia africana* is a common component of the pharmacopeia's of multiple African 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 cancers. This study was undertaken to test *K. africana* fruit extracts for the ability to inhibit microbial and cancer cell growth, and thus to validate traditional African medicinal usage of this plant in treating a variety of diseases. **Materials and Methods:** *K. africana* fruit powder was extracted and tested for antimicrobial activity using modified disc diffusion and MIC methods. Inhibitory activity against the gastrointestinal protozoal parasite *Giardia duodenalis* and against CaCo2 and HeLa cancer cell lines was evaluated using colorimetric cell proliferation assays. Toxicity was evaluated using an *Artemia franciscana* nauplii bioassay. **Results:** The methanol, water and ethyl acetate *K. africana* fruit extracts displayed potent antibacterial activity. The methanol and water extracts displayed the broadest specificity, inhibiting the growth of 12 of the 18 bacteria tested (67 %) and 11 of the 18 bacteria tested (61 %) respectively. The ethyl acetate extract also displayed antibacterial activity, inhibiting the growth of 4 (22 %) of the 18 bacteria tested. These extracts were approximately equally effective against Gram-positive and Gram-negative bacteria, generally inhibiting the growth of 60–70 % of the bacteria tested. The methanol, water and ethyl acetate extracts also displayed broad spectrum antifungal activity, each inhibiting the growth of 3 of the 4 fungal species tested (75 %), including an ampicillin strain of *A. niger*. The methanol, water and ethyl acetate extracts also inhibited between 55 and 70 % of the growth of the gastrointestinal parasite *Giardia duodenalis*. These extracts also proved effective at blocking the proliferation of the colorectal cancer cell line CaCo2 to between 37 and 55 % of the untreated cell growth. The methanol extract also inhibited HeLa cervical cancer cell growth, albeit to a lesser extent (81 % of the untreated control growth), whilst the chloroform and hexane extracts stimulated HeLa cell proliferation. With the exception of the water extract, all extracts were non-toxic or of low toxicity. **Conclusion:** These studies validate traditional African therapeutic usage of *K. africana* in the treatment of several bacterial, fungal and protozoal illnesses and some cancers.

KEYWORDS: Antibacterial activity, Sausage tree, *Giardia duodenalis*, Anti-proliferative activity, Anti-cancer activity, Artemia, Toxicity.

INTRODUCTION

Plants have been used for thousands of years as medicines for treating a variety of different diseases and medical complaints by most, if not all civilisations. Phytotherapy in Asia is particularly widespread and documented. Similarly, plant based medicinal systems continue to be the primary therapeutic system in many parts of Africa. For example, *Phytolacca dodecandra* is used as a moluscicide in the control of schistosomiasis.[1] Some African plant derived medicines have also found a place in modern Western medicinal systems. The antitumour agents vinblastine and vincristine (derived from *Catharanthus roseus*) are currently used in the treatment of a variety of tumours.[2,3] The medicinal properties of other African plant species are less well understood despite a long history of ethnobotanical usage.

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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 of usage, ranging from Southern Africa, through Central Africa, to Western Africa.[4] Its use as both a therapeutic agent and as a food have 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 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.

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.[5–7] An infusion made from the ground bark and fruit is used to treat stomach problems in children.[5–7] Roots and bark are used to treat pneumonia.[5] In West Africa, leaves and twigs are used to treat wounds, dysentery, stomach and kidney disorders, snakebite, and rheumatism.[8] The fruit is used to treat constipation, gynaecological disorders, haemorrhoids, lumbago and dysentery.[8] Slices of mature baked fruits are used to ferment and flavour traditional African beer.[9] 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.

Despite, the widespread therapeutic usage of *K. africana,* there is a surprising lack of rigorous scientific studies to verify the ethnomedicinal properties of this plant. Instead, many studies have examined the phytochemistry without linking this to bioactivity studies. Whilst some studies verifying bioactivities associated with ethnobotanical usage exist, many of these reports have focussed on the bark. Extracts prepared from the bark have cytotoxic activities and have shown promising results in treating melanoma and renal carcinoma.[10] Variable solvent extracts from several parts of *K. africana* have also been shown to have anticancer activity.[11–15]

Further studies have examined the antimicrobial properties of *K. africana.* 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*.[13] 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*. a*ureus* and *Candida albicans*.[12–16] Other studies have also reported antibacterial activity for *K. africana* leaf extracts.[17,18] Of particular interest, polar *K. africana* leaf extracts have been shown to inhibit the growth of the bacterial trigger of rheumatoid arthritis.[17] Grace et al.[18] reported that extracts prepared from the *K. africana* fruit inhibited the growth of a panel of Gram positive and Gram negative bacteria. Furthermore, extracts from various parts of the *K. africana* plant have been shown to have high antioxidant content.[19,20] further indicating the therapeutic potential of this species.

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 (Figure 1a) and the coumarin kigelin (Figure 1b).[5] Several other compounds including isopinnatal (Figure 1c), kigelinone (Figure 1d), pinnatal (Figure 1e), β-sitosterol (Figure 1f), stigmasterol (Figure 1g) and vernolic acid have also been isolated from *K. africana* bark.[5, 20] 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, coumaric acid (Figure 1h), caffeic acid (Figure 1i), ethylgallic acid (Figure 1j) and chlorgenic acid.[10, 21–23] Of these, vermonoside may be of particular interest as an anti-protozoal compound due to its reported antiamoebic activity.[24]

The current study was undertaken to extend the previous antibacterial and antifungal studies of *K. africana* fruit against a more extensive microbial panel. The ability to inhibit the growth of the gastrointestinal parasite *Giardia duodenalis* was also evaluated for the first time. Furthermore, the anticancer activity of *K. africana* fruit extracts was evaluated against 2 cancer cell lines. The toxicity of *K. africana* fruit extracts was determined to evaluate their usefulness as medicinal agents.

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.[25,26] 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

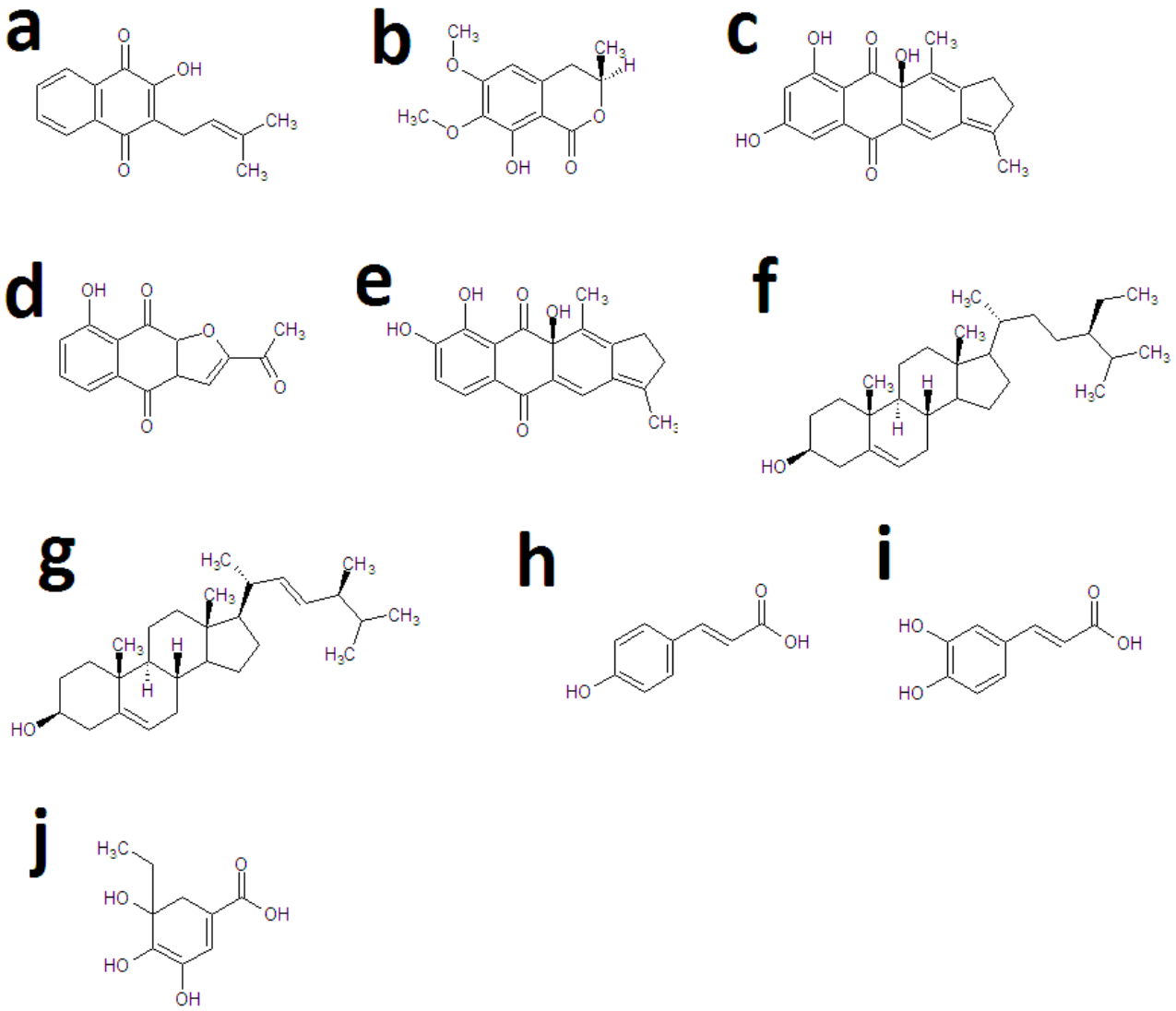

Figure 1. Chemical structures of selected molecules identified in *K. africana*: **(a)** lapachol, **(b)** kigelin, **(c)** isopinnatal, **(d)** kigelinone, **(e)** pinnatal, **(f)** β-sitosterol, **(g)** stigmasterol, **(h)** coumaric acid, **(i)** caffeic acid, **(j)** ethylgallic acid.

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.[27–29]

Antibacterial screening

Test microorganisms

All microbial strains were obtained from Michelle Mendell and Tarita Morais, Griffith University, Australia. Stock cultures of *Acintobacter bayleii*, *Aeromonas hydrophila, Alcaligenes feacalis, Bacillus cereus, Citrobacter freundii, Enterobacter aerogenes, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Penicillium chrysogenum, Proteus mirabilis, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella newport, Serratia marcescens, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pyogen*es were subcultured and maintained in nutrient broth at 4°C. The fungal species *Aspergillus nig*er, *Candida albicans* and *Penicillium chrysogenum* were subcultured and maintained in Sabouraud broth at 4°C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion method.[30–32] Briefly, 100 µl of the test bacteria or fungi were grown in 10 ml of fresh nutrient broth or Sabouraud broth respectively until they reached a count of approximately 10^8 cells/ml as determined by direct microscopic determination. One hundred microliters of microbial suspension was spread onto the appropriate agar plates. The extracts were tested using 5 mm sterilised filter paper discs. Discs were impregnated with 10 µl of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 °C for 2 hours before incubation with the test microbial agents. Plates inoculated with *Acintobacter bayleii, Alcaligenes feacalis*, *Aeromonas hydrophilia*, *Bacillus cereus*, *Candida albicans, Citrobacter freundii*, *Enterobacter aerogenes, Klebsiella pneumoniae*, *Penicillium chrysogenum, Proteus mirabilis, Pseudomonas aeruginosa, Pseudomonas fluorescens*, *Serratia marcescens*, were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Enterococcus faecalis, Escherichia coli*, *Salmonella newport*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were measured. Plates inoculated with *Aspergillius niger* were grown at room temperature for 48 hours, then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate and mean values were determined. Standard discs of ampicillin (2 µg) and nystatin (100 µg) were obtained from Oxoid Ltd. and served as positive controls for antibacterial and antifungal activity respectively. Filter discs impregnated with 10 µl of distilled water were used as negative controls.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the *K. africana* extracts were determined by the disc diffusion method across a range of doses.[33,34] The plant extracts were diluted in deionised water across a concentration range of 5 mg/ml to 0.1 mg/ml. Discs were impregnated with 10 µl of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

linhibitory bioactivity against *Giardia duodenalis* trophozoites

Parasite culture

The *Giardia duodenalis* S-2 (sheep strain 2) trophozoite strain used in this study were previously supplied by Professor Andre Buret, University of Calgary, Canada. *G. duodenalis* tropozoites were maintained and subcultured anaerobically at 37 °C in TYI-S-33 growth media supplemented with 1% bovine bile (Sigma), 10 % Serum Supreme (Cambrex Bioproducts) and 200 IU/ml penicillin/200 µg/ml streptomycin (Invitrogen, USA). Confluent mid log phase cultures were passaged every 2 days by chilling the cultures on ice for a minimum of 10 min, followed by vortexing to dislodge the adherent trophozoites from the walls of the culture vessel. Fresh culture media (5 ml) was seeded with approximately 1 x 10^5 trophozoites for each passage.

Evaluation of anti-Giardial activity

To test for anti-Giardial activity, confluent cultures were chilled on ice for a minimum of 10 min, followed by vortexing to dislodge the adherent trophozoites. Aliquots of the trophozoite suspension (70 µl) containing approximately 1 x 10^5 trophozoites were added to the wells of a 96 well plate. A volume of 30 µl of the test extracts or the vehicle solvent or culture media (for the negative controls) was added to individual wells and the plates were incubated anaerobically at 37 °C for 12 hours in a humidified anaerobic atmosphere. A volume of 20 µl of CellTiter 96® AQueous One Solution Cell Proliferation Assay Reagent (Promega) was subsequently added to each well and the plates were incubated for a further 3 hours. The CellTiter 96® AQueous One Solution Cell Proliferation Assay(a) is a colourimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96® AQueous One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active (live) cells. 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:

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

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).

Screen for anti-cancer bioactivity

Cancer cell lines

The CaCo2 and HeLa carcinoma cell lines used in this study were obtained from American Type Culture Collection (Rockville, USA). 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 cancer cell anti-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 the cancer cells. The cell suspensions were 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:

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

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.[35–37] 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.

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.

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.

ANTIMICROBIAL ACTIVITY

Aliquots (10 µl) of each extract were tested in the disc diffusion assay against panels of Gram negative (Figure 2) and Gram positive bacteria (Figure 3), as well as fungi (Figure 4). The methanol and water extracts displayed broad spectrum inhibitory activity against Gram negative bacteria, inhibiting the growth of 9 (69 %) and 8 (62 %) of the 13 Gram negative species tested respectively (Figure 2). The methanol extract was generally more effective at inhibiting Gram negative bacterial growth than was the aqueous extract (as determined by zones of inhibition). The ethyl acetate extract also displayed inhibitory activity towards the Gram negative bacteria, albeit with a narrower specificity, inhibiting 3 of the 13 bacterial species (23 %). *Proteus mirabilis* was particularly susceptible, with zones of inhibition greater than 10 mm for the methanol, water and ethyl acetate extracts. The chloroform and hexane extracts were unable to inhibit any of the bacterial species tested.

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.

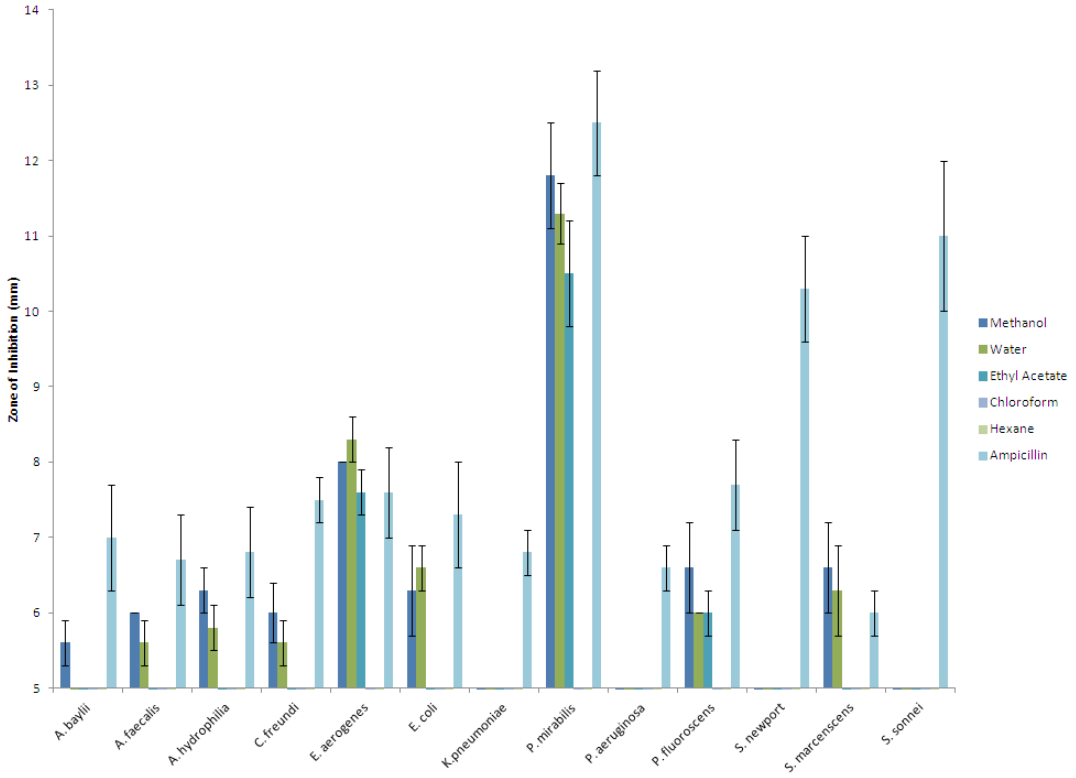

Figure 2. Antibacterial activity of *K. africana* fruit extracts and an ampicillin control (2 µg) measured as zones of inhibition (mm) against Gram negative bacteria. Results are expressed as mean ± SEM of at least triplicate determinations.

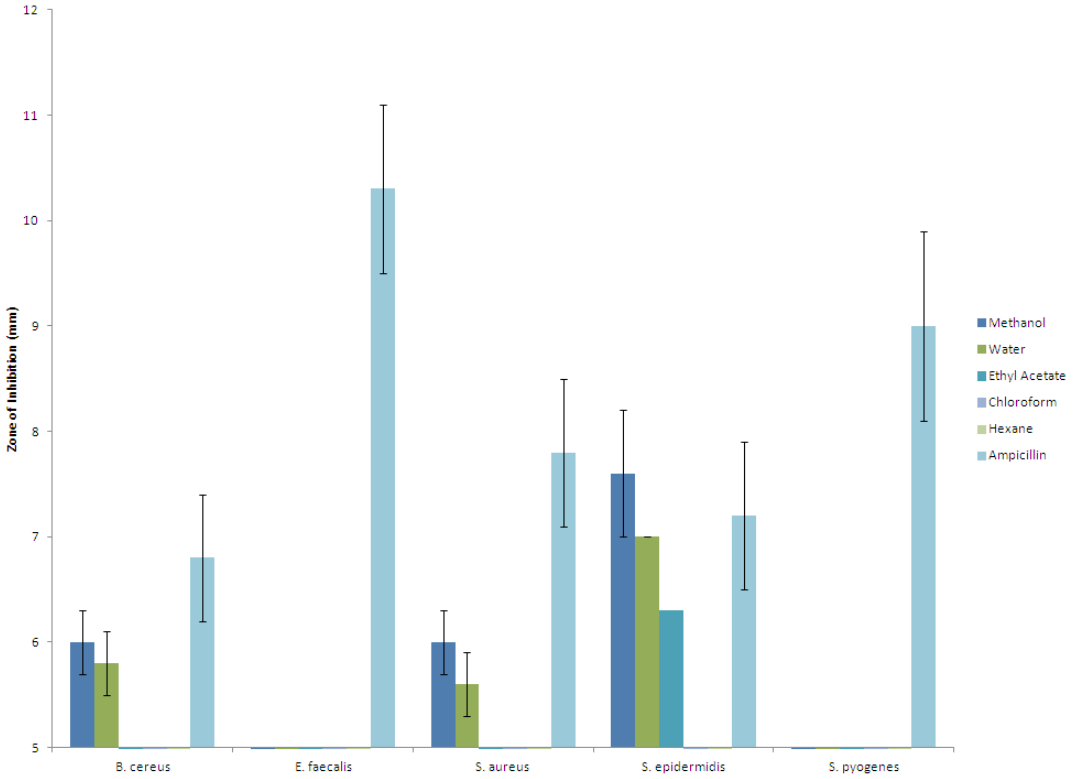

Figure 3. Antibacterial activity of *K. africana* fruit extracts and an ampicillin control (2 µg) measured as zones of inhibition (mm) against Gram positive bacteria. Results are expressed as mean ± SEM of at least triplicate determinations.

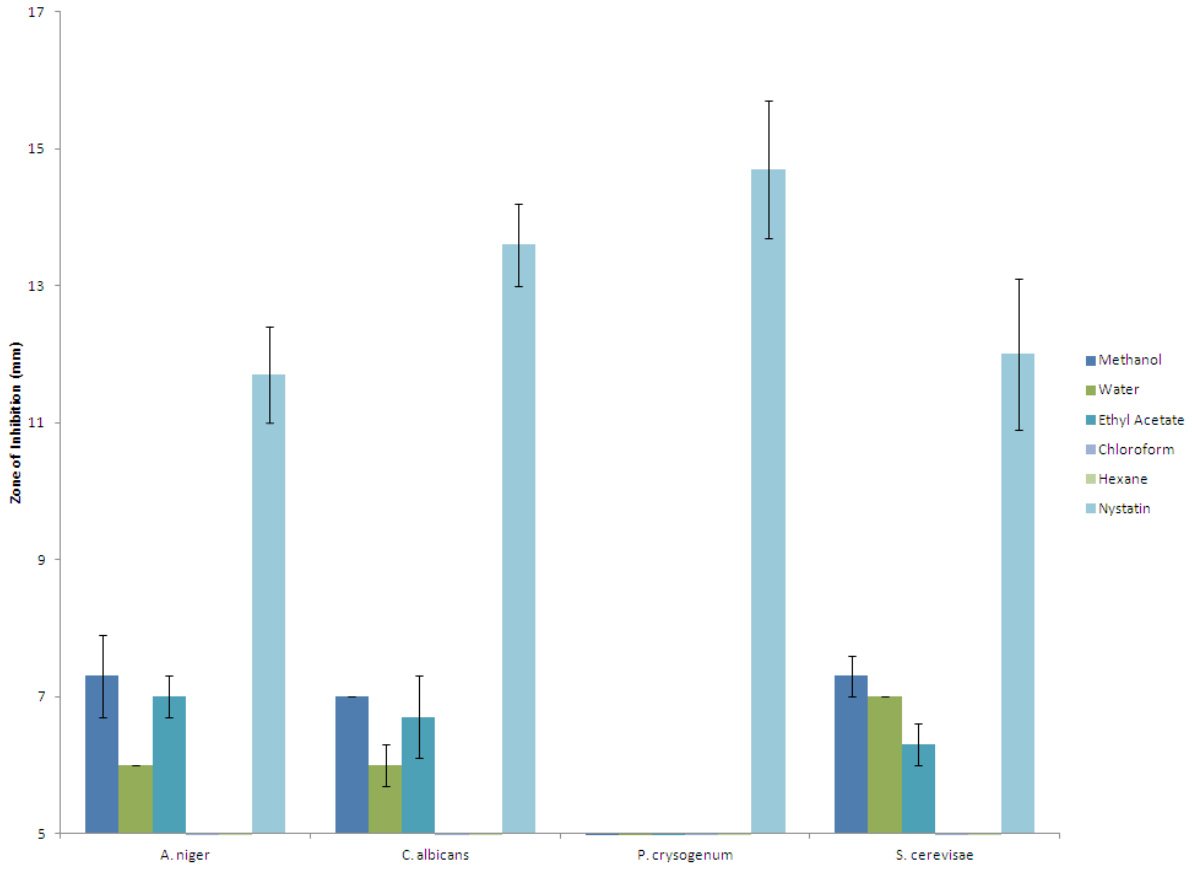

Figure 4. Antifungal activity of *K. africana* fruit extracts and a nystatin control (100 µg) measured as zones of inhibition (mm). Results are expressed as mean ± SEM of at least triplicate determinations.

The methanol and water extracts also inhibited the majority of the Gram positive species tested. Each inhibited the growth of 3 of the 5 species (60 %). In contrast, the ethyl acetate extract inhibited only a single Gram positive bacterial species (*S. epidermidis*) (20 %). It is noteworthy that whilst growth inhibition was detected for several Gram positive bacteria, the zones of inhibition indicated that this inhibition was not particularly strong against any of these bacteria. None of the Gram positive bacteria were inhibited by the chloroform or hexane extracts.

Similarly broad growth inhibitory activity was detected for the methanol, water and ethyl acetate extracts against the fungal species. Indeed, each of these extracts inhibited the growth of 3 of the 4 fungi tested (75 %). Only *P. crysogenum* was resistant to these extracts. The inhibition of *A. niger* was particularly noteworthy as this is a strain which has previously proven resistant to several antibiotics and many other plant extracts which inhibit the growth of other microbes.[38–40] Generally, the methanol extract was the strongest growth inhibitor (as judged by zones of inhibition). In contrast to bacterial inhibition, the ethyl acetate extract was more effective than the aqueous extract against 2 of the 3 fungal species inhibited. However, the zones of inhibition were relatively small, indicating that growth inhibition was not particularly strong for any extract against any of the fungi.

The relative level of antimicrobial activity was further evaluated by determining the MIC values (Table 2) for each extract against the bacterial and fungal species which were shown to be susceptible by disc diffusion assays. Most of the extracts were effective at inhibiting microbial growth at low concentrations, with MIC values against the bacterial species that they inhibited generally < 2000 µg/mL (< 20 µg impregnated in the disc), indicating the potent antimicrobial activity of these extracts. These MIC values compare favourably with the dosages of the pure standards ampicillin and nystatin which was tested using 2 µg and 100 µg per disc respectively. The methanol and water extracts were particularly potent, achieving MIC values in the range 84–250 µg/ml against several species. Whilst the methanol, water and ethyl acetate extracts also had broad spectrum inhibitory activity against the fungal species, they generally had much lower efficacies (with some MIC values > 2000 µg/ml).

Table 2: Minimun inhibitory concentrations (µg/ml) of *K. africana* fruit extracts against susceptible microbial species.

	Gram-Negative Bacteria									Gram-Positive Bacteria			Fungi		
	A. baylii	*A. faecalis*	*A. hydrophilia*	*C. freundi*	*E. aerogenes*	*E. coli*	*P. mirabilis*	*P. fluorescens*	*S. marcenscens*	*B. cereus*	*S. aureus*	*S. epidermidis*	*A. niger*	*C. albicans*	*S. cerevisae*
Methanol	1874	1247	141.7	1814	236	176.3	181.4	187.4	1572	1189	2487	445.4	1238	841.2	989.7
Water	–	1786	1288	2060	173.7	84.7	206.2	206.3	2118	1974	4333	847.9	2487	2060	2768
Ethyl Acetate	–	–	–	–	423.6	–	623.3	687.3	–	–	–	1655	1463	1278	1744

Numbers indicate the mean MIC values of at least triplicate determinations. - indicates no growth inhibition.

ANTI-GIARDIAL ACTIVITY

K. africana fruit extracts were screened for their ability to inhibit *Giardia duodenalis* growth (Figure 5). The methanol, water and ethyl acetate extracts displayed significant inhibitory activity. The water extract was particularly potent, inhibiting approximately 70 % of the Giardial growth (compared to the untreated control). Even at the lowest concentration tested (10 µg/ml) the trophozoite growth was still only approximately 65 % of the untreated negative control culture (unpublished results). The methanol and ethyl acetate extracts were also very effective at inhibiting trophozoite growth (to approximately 45 % of the growth of the negative controls). The chloroform and hexane extracts were ineffective as proliferation inhibitors, with no significant difference to the untreated control levels.

K. africana extracts were further tested over a range of concentrations to determine the IC50 values (Table 3) for each extract against *G. duodenalis*. Inhibition of trophozoite growth was dose-dependent, with the level of inhibitory activity decreasing at lower concentrations. Interestingly, whilst the level of inhibition decreased for the methanol, water and ethyl acetate extracts in a dose-dependent manner, all concentrations still significantly inhibited *G. duodenalis* trophozoite growth at the lowest concentration tested (10 µg/ml). The water extract was a particularly good inhibitor of *G. duodenalis* proliferation, with an IC50 of 13.7 µg/ml. The methanol and ethyl acetate extracts, whilst less potent, also displayed good anti-Giardial activity (at approximately 125.2 and 143.5 µg/ml respectively).

Inhibition of cancer cell proliferation

The *K. africana* extracts were tested against 2 cancer cell lines (CaCo2 colorectal carcinoma cells, Figure 6; HeLa cervical cancer cells, Figure 7) to determine their ability to inhibit cancer cell growth. The methanol, water and ethyl acetate extracts displayed potent inhibitory activity against CaCo2 cells, with proliferation inhibited to as low as 37 % of the untreated control cell growth for the aqueous extracts (Figure 6). The methanol and ethyl acetate extracts were also effective at inhibiting CaCo2 proliferation (to approximately 42 % and 55 % of untreated cell proliferation respectively). In contrast, the chloroform and hexane extracts had no effect on CaCo2 cell proliferation. Inhibition of proliferation by the methanol, water and ethyl acetate extracts was dose dependent, with the level of inhibitory activity decreasing at lower concentrations. Interestingly, whilst the level of inhibition decreased for all extracts, all still significantly inhibited CaCo2 proliferation at the lowest concentration tested (1.5 µg/ml).

The *K. africana* extracts were also shown to affect the proliferation of HeLa cells, although only the methanol extract inhibited cell proliferation (Figure 7). Furthermore, the inhibition was of lower efficacy than was evident with the CaCo2 cells. The methanol extract inhibited HeLa cell proliferation to approximately 81 % of the untreated control cell growth. In contrast to the CaCo2 study, neither the water nor the ethyl acetate extracts had proliferation rates significantly different to the untreated cell proliferation. Of note, exposure to the chloroform and hexane extracts resulted in significant proliferation of HeLa cells compared to the untreated controls, with cell proliferation levels of approximately 118 and 107 %

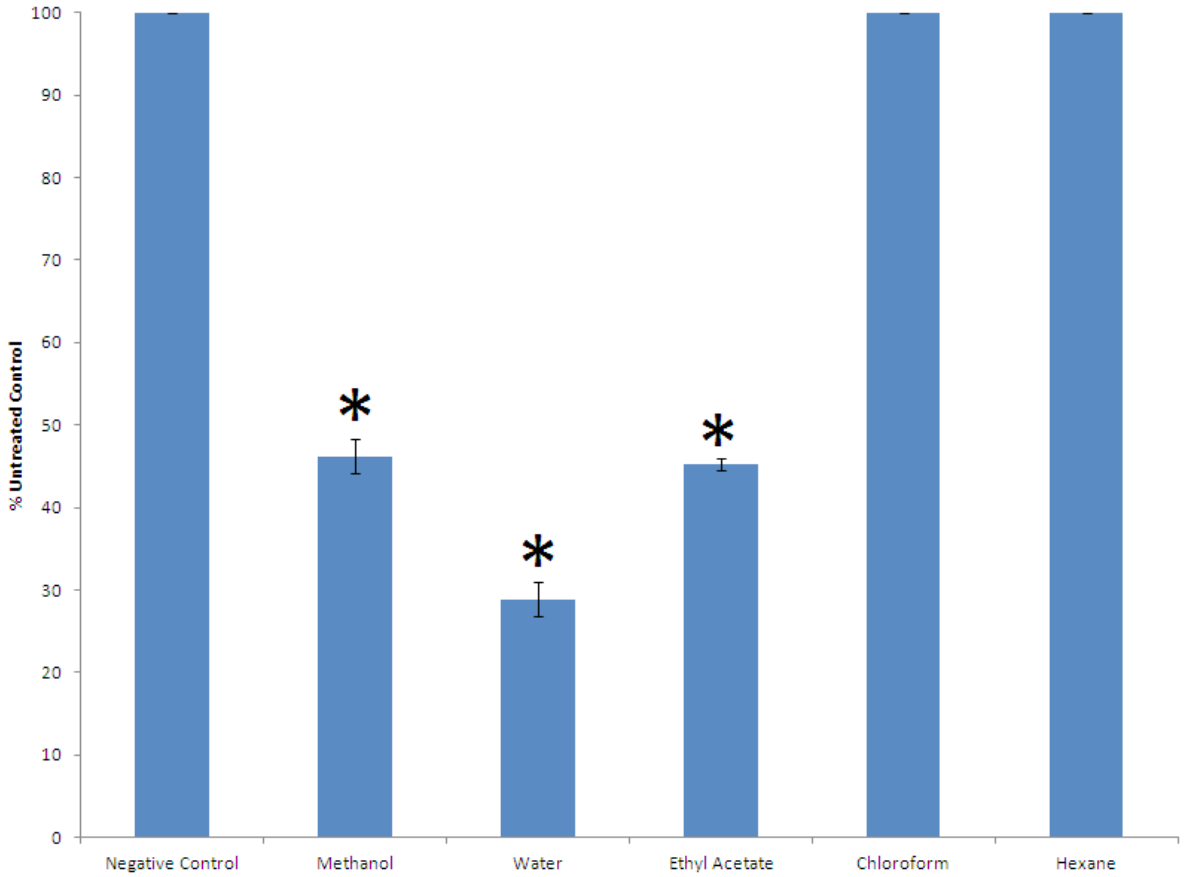

Figure 5. Inhibitory activity of *K. africana* fruit extracts against *Giardia duodenalis* trophozoites measured as a percentage the untreated control. Results are expressed as mean ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$).

Table 3: The IC50 values (µg/ml) of *G. duodenalis* and cancer cell lines, and the LC50 values (µg/ml) for *Artemia franciscana nauplii* exposed to *K. africana* nut extracts and control solutions.

Bioassay	Solvent Extracts					Controls	
	Methanol	Water	Ethyl Acetate	Chloroform	Hexane	Negative Control	Potassium Dichromate
Giardia duodenalis IC50	125.2	13.7	143.5	WND	WND	–	ND
CaCo2 cells IC50	1550	1672	1537	–	–	–	ND
HeLa cells IC50	1521	–	–	PA	PA	–	ND
Artemia franciscana nauplii 24 h LC50	985	477	1470	–	–	–	224

Numbers indicate the mean IC50 or LC50 values of at least triplicate determinations. - indicates no significant growth inhibition/ brine shrimp mortality. PA indicates that proliferative rather than inhibitory activity was seen. Therefore an IC50 value was not able to be determined. WND indicates an IC50 was not determined as the amount of live Giardia did not reach ≤50 % at any concentration tested. ND indicates the test was not performed.

respectively. Inhibition of proliferation by the methanol extract was dose dependent, with the level of inhibitory activity decreasing at lower concentrations. The dose dependent proliferation of the chloroform and hexane extracts was not evaluated in this study.

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

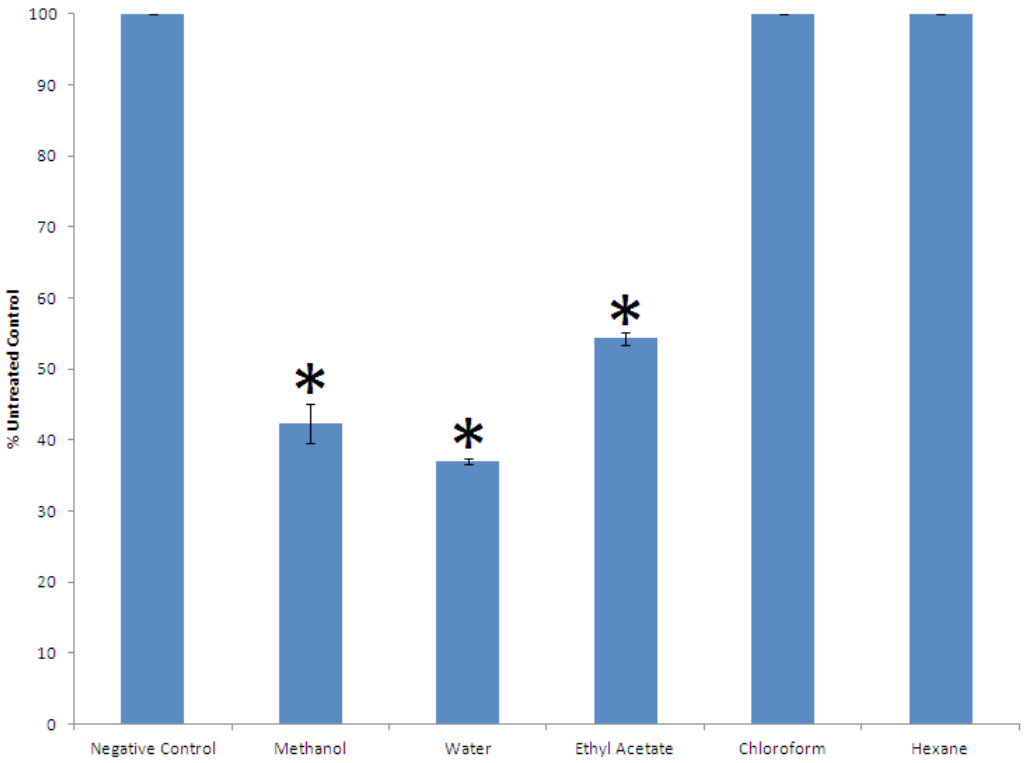

Figure 6. Anti-proliferative activity of *K. africana* fruit extracts and untreated controls against CaCo2 cancer cell lines measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$).

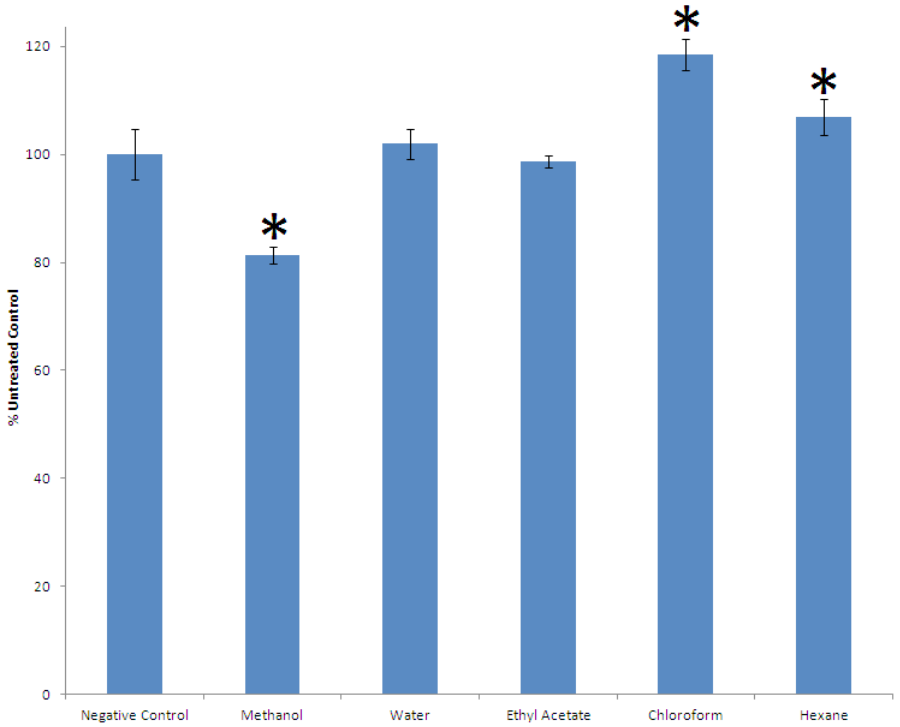

Figure 7. Anti-proliferative activity of *K. africana* fruit extracts and untreated controls against HeLa cancer cell lines measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$).

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 8). Table 3 shows the extract and control toxin concentrations required to achieve 50 % mortality (LC50) at various times. As toxicity of crude plant extracts has previously been defined as 24 LC50 values < 1000 µg/ml.[35,36] the measured LC50 values indicate that only the water extract was significantly toxic.

DISCUSSION

K. africana fruit has been used in a wide variety of traditional African medicinal systems to treat a wide variety of diseases and medical conditions including wounds, kidney and stomach disorders and dysentery.[7,8] The current study was undertaken to examine the antimicrobial and anticancer activities of various *K. africana* fruit extracts, and their toxicity. The ability of *K. africana* fruit extracts to inhibit the growth of both Gram-positive and Gram-negative bacteria is in agreement with previous reports of the antibacterial activity of other plants that have a history of medicinal usage in traditional healing systems.[41–44] The current study shows Gram-positive and Gram-negative bacteria to be approximately equally susceptible to the *K. africana* fruit extracts. This is in contrast to previous studies which have reported a greater susceptibility of Gram-positive bacteria towards solvent extracts for South American,[45] African,[46] and Australian[47] plant extracts. Results within this laboratory[43,48–50] have also confirmed the greater susceptibility of Gram-positive bacteria towards other plant extracts, although other examples of plants having a greater effect on Gram negative bacteria have also been reported.[27–29,51,52]

The *K. africana* fruit extracts were particularly potent inhibitors of *P. mirabilis*. This activity is noteworthy as *P. mirabilis* has been implicated in urinary tract infections (UTI's) and the induction of rheumatoid arthritis (RA)[17]. Thus, *K. africana* fruit extract treatment has the potential to

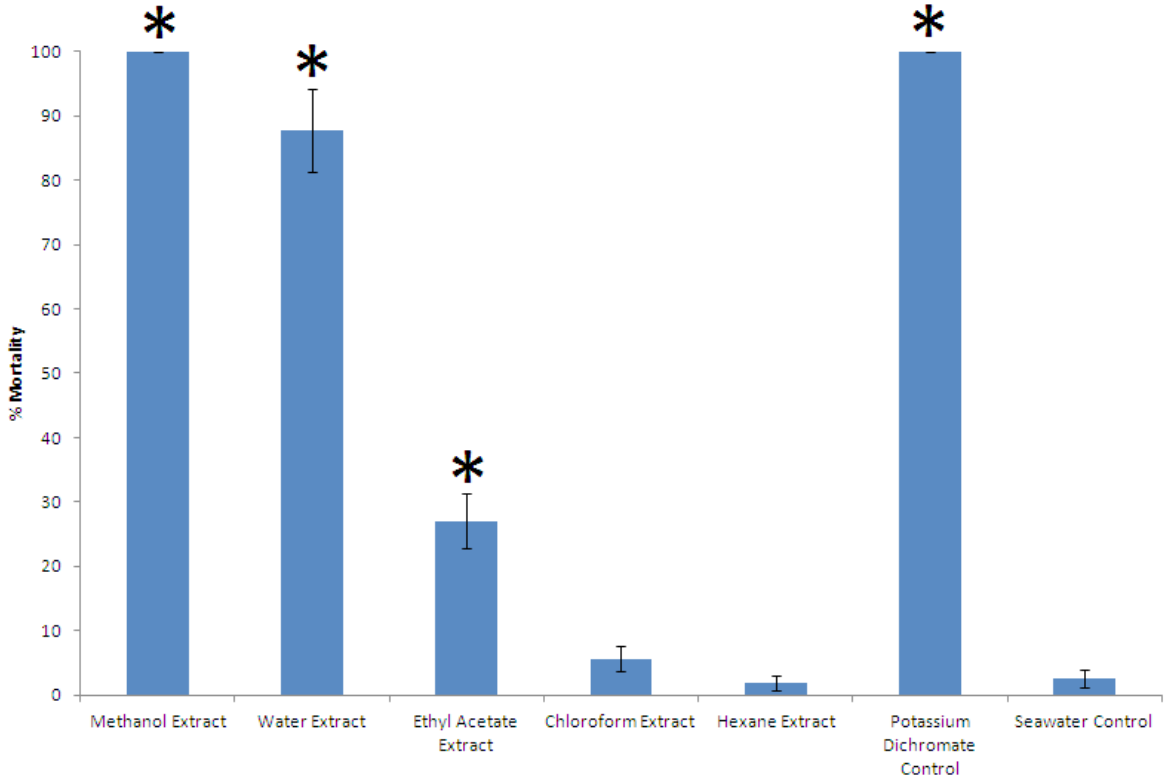

Figure 8. The lethality of *K. africana* fruit extracts (2000 µg/ml) and potassium dichromate control (1000 µg/ml) towards *Artemia franciscana* nauplii after 24 hours exposure. Results are expressed as mean ± SEM of at least triplicate determinations.

block RA before the induction of the immune response and inflammation, thus not only blocking the late phase symptoms, but also the tissue damage associated with RA. Our findings support previous studies which have also reported antibacterial properties for *K. africana* extracts from other plant parts.[12,13,16–18] Furthermore, our study found that the *K. africana* solvent extracts had broad spectrum antifungal activity. This supports previous studies which have reported that the *K. africana* stem bark extracts have potent antifungal properties.[12]

Similarly, potent growth inhibition of the food/water borne gastrointestinal parasite *Giardia duodenalis* was also noted for the *K. africana* fruit extracts in our study. Giardial infection (giardiasis) is a re-emerging disease which afflicts large numbers of individuals worldwide, with higher incidence in countries with poorer socio-economic conditions, inadequate sanitary conditions, untreated water supplies and poor diet.[53] Whilst generally not fatal, giardiasis results in debilitating symptoms including bloating, diarrhoea, excess gas, loss of appetite, loose and watery stool, stomach cramps and haematuria. Currently, there are only a narrow range of drugs effective against giardiasis, including quinalones and imidazole derivatives. None of these drugs is ideal as they produce unpleasant side effects including nausea, vertigo, vomiting, diarrhoea and hallucinations.[53,54] Furthermore, increasing reports of the failure of current treatments to address this disease indicates a developing drug resistance of several Giardia species.[54] Recent studies have highlighted the potential of plant medicines and have demonstrated that some plant components are very effective inhibitors of *Giardia duodenalis* growth with similar potency to the gold standard drug metranidazole.[53] Our studies demonstrates that *K. africana* fruit also possesses anti-Giardial activity. Whilst, further studies are required to identify the active component(s), previous studies have identified vermonoside as being inhibitory against amoebic protozoans. It is possible that this compound is also contributing to the anti-Giardial activity seen in our study.

Anti-proliferative activity against CaCo2 and HeLa carcinoma cell lines cells was noted for the *K. africana* fruit extracts (especially for the methanol water and ethyl acetate extracts), with IC50 values generally approximately 1500 μg/ml. These findings support and extend previous studies examining the anticancer effects of *K. africana* extracts against other cell lines. Aqueous root bark extracts significantly inhibit the growth of melanoma cells and renal carcinoma cells[10–20] Recent studies have demonstrated cytotoxic activity for *K. africana* fruit extracts against a melanoma and 2 breast cancer cell lines.[55] 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.[55] Of these, 2-(1-hydroxyethyl)-naphtho[2,3-b] furan-4,9-dione was a particularly potent cytotoxic agent. Our study did not identify the active components of the cytotoxic extracts. Further isolation and identification studies focussing on the furonaphthoquinones are required. Of further interest in our studies, the chloroform and hexane extracts 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.

The findings reported here also show that 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.

The results of this study indicate that the *K. africana* fruit extracts examined in this report are worthy of further study due to their antibacterial and anti-Giardial activities and ability to block cancer cell proliferation. Furthermore, as extracts with LC50 values greater than 1000 μg/ml in the Artemia nauplii bioassay have been defined as being non-toxic,[35–36] only the *K. africana* water extract displayed significant toxicity (with a 24 h LC50 of 477μg/ml). Further evaluation of the antimicrobial and anticancer properties of these extracts is warranted. Likewise, further bioactivity driven purification are needed to examine the mechanisms of action of these agents. Whilst the extracts examined in this report have potential as antimicrobial and anticancer agents, caution is needed before they can be applied to medicinal purposes due to the moderate toxicity seen for the aqueous extract.

CONCLUSION

The results of this study partially validate the traditional usage of *K. africana* fruit extracts in multiple traditional

African medicinal systems to treat microbial diseases and cancer, and indicate that they are worthy of further study. Bioactivity driven purifications of the active components and an examination of the mechanisms of action of these agents is required.

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