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Original Article

An examination of the Antimicrobial and Anticancer Properties of *Khaya senegalensis* (Desr.) A. Juss. Bark Extracts

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ABSTRACT

Background: *Khaya senegalensis* (Desr.) A. Juss. 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. senegalensis* bark 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. senegalensis* bark powder was extracted by both solvent maceration and subcritical fluid extraction (SFE). The extracts were tested for the ability to inhibit bacterial and *G. duodenalis* growth. Inhibition of Caco-2 and HeLa cancer cells was evaluated using MTS-based colorimetric cell proliferation assays. Toxicity was evaluated using an *Artemia franciscana* nauplii bioassay and GC-MS headspace analysis was used to identify phytochemical components. **Results:** *K. senegalensis* bark extracts displayed strong inhibitory activity against bacterial triggers of several autoimmune inflammatory diseases. The growth inhibitory activity of the methanolic and subcritical extracts was particularly noteworthy against *P. mirabilis* (MIC values of 185 and 211µg/mL, respectively against the reference strains). These extracts were similarly potent growth inhibitors of *K. pneumoniae* and *A. baylyi*, and were moderate inhibitors (MIC >1000µg/mL) of *P. aeruginosa* and *S. pyogenes* growth. The methanolic and subcritical *K. senegalensis* extracts were also potent inhibitors of *G. duodenalis* (187 and 328µg/mL, respectively), as well as Caco-2 (268 and 470µg/mL, respectively) and HeLa carcinomas (155 and 174µg/mL, respectively). GC-MS analysis of the SFE extract revealed relative abundances of a variety of mono- and sesquiterpenoids. Furthermore, all *K. senegalensis* bark extracts were non-toxic in the *Artemia franciscana* toxicity assay, indicating their safety for therapeutic use. **Conclusion:** These studies validate traditional African therapeutic usage of *K. senegalensis* in the treatment of microbial infections, autoimmune inflammatory diseases and some cancers.

Key words: African mahogany, Meliaceae, Sub-critical fluid extraction, Anti bacterial activity, *Giardia duodenalis*, Anti-proliferative activity, Anti-cancer activity, Terpenoid.

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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. The traditional uses and therapeutic properties of many African plants have been well documented.[1,2] For many African plant medicines, the traditional uses have been validated via bioactivity and phytochemical studies. Several African plant-derived medicines have also found a place in allopathic medicine. For example, the anti-tumour agents vinblastine and vincristine (derived from *Catharanthus roseus*) are used in the treatment of a variety of tumours.[1] The medicinal properties of other African plant species are less well understood despite a long history of ethnobotanical usage. *Khaya senegalensis* (Desr.) A.Juss. (family Meliaceae; commonly known as African mahogany, Gambia mahogany, Senegal mahogany, Khaya wood) is a tall evergreen African tree with a wide geographical range, occurring from Central Africa to Western Africa.[3] It is native to Benin, Burkina Faso, Cameroon, Central African Republic, Chad, Côte d'Ivoire, Democratic Republic of Congo, Gabon, Gambia, Ghana, Guinea, Guinea-Bissau, Mali, Niger, Nigeria, Republic of Congo, Senegal, Sierra Leone, Sudan, South Sedan, Togo and Uganda, usually growing in high rainfall woodlands and alongside rivers.

The therapeutic value of *K. senegalensis* is recognised by multiple ethnic groupings across the regions in which it occurs for the treatment of a wide range of diseases/conditions (Table 1). Whilst the stem bark is most often cited as having therapeutic properties, multiple parts of the *K. senegalensis* tree have been used in traditional healing systems. Decoctions prepared from the stem bark were used to treat dermatitis and other skin diseases, diarrhoea and dysentery, fever, jaundice, malaria and sexually-transmitted diseases.[4,5] Bark decoctions are also useful as an anti-helminth, particularly in treating hookworm and tapeworm infestations.[4,5]

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Despite, the widespread therapeutic usage of *K. senegalensis*, there have been limited rigorous scientific studies to verify the ethnomedicinal properties of this plant. Instead, many studies have examined the phytochemistry without linking the components to bioactivity studies. Whilst several studies verifying bioactivities associated with ethnobotanical usage exist, many of these reports have focussed on the bark, and to a lesser extent, the leaf. Bark extracts have been reported to have good anticancer[6,13,14] and anti-inflammatory properties[6] are good immunostimulants[11] and are insect deterrents.[8] *K. senegalensis* bark extracts have potential in the treatment of diabetes and have been reported to have anti-hyperglycemic activity comparable to glibenclamide.[12] Furthermore, *K. senegalensis* stem bark extracts have been shown to have high antioxidant content,[6] further indicating the therapeutic potential of this species.

Several studies have also examined the antimicrobial properties of *K. senegalensis*. The anti-protozoal properties have been particularly well established. Solvent extracts of several plant parts have been reported to inhibit the growth of *Plasmodium falciparum*,[15] *Trypanosoma evansi* and *Trypanosoma brucei*[16] and several *Leishmania* spp.[11] Interestingly, we were unable to find any studies examining the effects of *K. senegalensis* extracts on the gastrointestinal protozoal parasite *Giardia duodenalis*. Several studies have also reported bacterial growth inhibitory properties for *K. senegalensis* extracts.[9,10] Both leaf and bark extracts inhibited the growth of the gram positive bacteria *Staphylococcus aureus* and *Streptococcus faecalis* but were ineffective against the gram negative bacteria, *Escherichia coli*.[9] However, the MIC values reported in that study (4000-8000µg/mL) are indicative of only low to moderate growth inhibitory activity. A different study reported antibacterial activity against a broader bacterial panel, although that study only tested a single extract concentration and did not report MIC values, making it impossible to compare the efficacy with other studies.[10]

Whilst further studies are required to fully characterise the phytochemistry of *K. senegalensis*, a number of interesting compounds have already been identified. The bark,[17,18] leaves[19] and fruit[8] contain significant quantities of a variety of limonoids (Figure 1a). The bark also contains substantial levels of khayanolides (Figure 1b)[20,21] and senagalene triterpenoids (Figure 1d)[20] Angolensates have also been reported in the fruit.[8] Furthermore, the dimeric proanthocyanidins proanthocyanidin B3 (Figure 1e) and fisetinidol-(4α, 6)-catechin (Figure 1f) were detected in *K. senegalensis* extracts [Kayser and Abreu, 2001], although that report does not state which part of the plant was investigated. Several similar compounds of these classes have been reported to have antimicrobial and anticancer therapeutic bioactivities.[22]

Despite the promising earlier studies, there has been a relative lack of recent reports into the therapeutic properties of this species. Furthermore, we were unable to find any previous studies examining the therapeutic properties and phytochemistry of *K. senegalensis* bark. The current study was undertaken to extend the earlier antibacterial studies of *K. senegalensis* by examining the growth inhibitory properties of bark extracts against a panel of bacterial triggers of autoimmune inflammatory diseases. 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. senegalensis* bark extracts was evaluated against 2 cancer cell lines, extending earlier reports of anticancer activity for *K. senegalensis* extracts. The toxicity of the *K. senegalensis* bark extracts was also determined to evaluate their usefulness as medicinal agents.

MATERIALS AND METHODS

Laboratory scale extraction

K. senegalensis bark was obtained from African Mahogany [Australia] Pty Ltd and stored at –30°C until processing. The shavings were thawed at room temperature, cut into small pieces and thoroughly dried in a Sunbeam food dehydrator. The dried pieces were subsequently ground into a coarse powder and extracted by standardised methods.[23] Briefly, an amount of 1g of powdered plant material was weighed into each of five tubes and five different extracts were prepared by adding 50mL of methanol, water, ethyl acetate, chloroform or hexane, respectively. All solvents were obtained from Ajax and were AR grade. The ground dried bark shavings were extracted individually 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 centrifugal evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10mL of deionised water.

Sub-critical fluid extraction (SFE) of *K. senegalensis* with dimethyl ether (DME)

The ground *K. senegalensis* bark was extracted by subcritical extraction techniques as previously described.[24] Briefly, the ground plant material was packed into the biomass chamber of the extraction system. The system was sealed and evacuated before the plant material in the biomass chamber was covered with compressed solvent (dimethyl ether). The compressed gas was cycled repeatedly across the plant material for 20 minutes. Subcritical DME extraction was carried out at room temperature and at a pressure of 500MPa. The solvent was recycled and stored in a solvent reservoir. After the compressed dimethyl ether gas had been removed, the material that had been extracted from the plant biomass was collected in a separate vessel.

Qualitative phytochemical studies

Phytochemical analysis of the *K. senegalensis* extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[25-27]

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Australia. Reference strains of *Acinitobacter baylyi* (ATCC33304), *Klebsiella* pneumoniae (ATCC31488), *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA. All other clinical microbial strains were obtained from the School of Natural Sciences teaching laboratory, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4°C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion method.[28-30] Briefly, 100µL of the test bacteria were grown in 10mL of fresh nutrient broth until they reached a count of approximately 10^8 cells/mL (as determined by direct microscopic determination). One hundred microliters of microbial suspension was subsequently spread onto the agar plates. The extracts were tested using 5mm sterilised filter paper discs. Discs were infused with 10µL of the test sample, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 hours before incubation with the test microbial agents. The plates were then incubated at 30°C for 24 hours and the diameters of the inhibition zones were measured in millimetres. 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 (10µg) were obtained from Oxoid Ltd., Australia and served as positive controls. Filter discs infused with 10µL of distilled water were used as negative controls.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the *K. senegalensis* extracts were determined by a modified disc diffusion method.[31,32] The plant extracts were diluted in deionised water across a concentration range of 5mg/mL to 0.1mg/mL. Discs were infused with 10µL 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.

Inhibitory bioactivity against *Giardia duodenalis* trophozoites

Parasite culture

The reference *Giardia duodenalis* trophozoite strain (ATTC203333) used in this study was purchased from American Tissue Culture Collection, USA. *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 200IU/mL penicillin/200µg/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 (5mL) was seeded with approximately 1 x 10^5 trophozoites for each passage.

Evaluation of anti-Giardial activity

Anti-Giardial activity of the *K. senegalensis* extracts was assessed by direct enumeration of parasite numbers in the presence or absence of extract[33,34] For each test, aliquots of the trophozoite suspension (70µL) containing approximately 1 x 10^5 trophozoites were added to the wells of a 96 well plate. A volume of 30µL 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 8hours in a humidified anaerobic atmosphere. Following the 8h incubation, all tubes were placed on ice for a minimum of 10min, followed by vortexing to dislodge the adherent trophozoites from the walls of the culture vessel. The suspensions were mounted onto a Neubauer haemocytometer (Weber, UK) and the total trophozoites per mL were determined. The anti-proliferative activity of the test extracts was determined and expressed as a % of the untreated control trophozoites per mL.

Determination of IC_{50} values against Giardial trophozoites

For IC_{50} determinations, the plant extracts were tested by the direct enumeration method across a range of concentrations. The assays were 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 IC_{50} values.

Screen for anti-cancer bioactivity

Cancer cell lines

The Caco-2 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 20mM HEPES, 10mM sodium bicarbonate, 50µg/mL streptomycin, 50IU/mL penicillin, 2mM glutamine and 10% foetal calf serum (Life Technologies). The cells were maintained as monolayers in 75mL flasks at 37°C, 5% CO_2 in a humidified atmosphere until approximately 80% confluent.

Evaluation of cancer cell anti-proliferative activity

Evaluation of the anti-proliferative activity of the *K. senegalensis* extracts was as previously described[35,36] Briefly, 1mL of trypsin (Sigma) was added to the culture flasks and incubated at 37°C, 5% CO_2 for 15min to dislodge the cancer cells. The cell suspensions were then transferred to a 10mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9mL of fresh media. Aliquots of the resuspended cells (70µL, containing approximately 5000 cells) were added to the wells of a 96 well plate. A volume of 30µL 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 20µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3hours. Absorbances were recorded at 490nm 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:

Proliferation (% untreated control) = (A_{ct}/A_{cc}) x 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).

Determination of IC_{50} values against Caco-2 and HeLa carcinoma cells

For IC_{50} determinations, the plant extracts were tested by the Cell Titre 96 colourimetric method across a range of concentrations. The assays were 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 IC_{50} values.

Toxicity screening

Reference toxins for biological screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 2mg/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.[37-39] 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 34g/L distilled water were prepared prior to use. An amount of 1g of *A. franciscana* cysts were incubated in 500mL synthetic seawater under artificial light at 25°C, 2000 Lux with continuous aeration. Hatching commenced within 16-18h of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10h 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 LC_{50} determination. A volume of 400µL of seawater containing approximately 52 (mean 51.8, n = 125, SD 11.7) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 4mg/mL in seawater for toxicity testing, resulting in a 2mg/mL concentration in the bioassay. Volumes of 400µL of diluted plant extract and the reference toxins were transferred to individual wells and incubated at 25 ± 1°C under artificial light (1000

Lux). A negative control (400µL 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 dead if no movement of the appendages was observed within 10 sec. After 48h all nauplii were sacrificed and counted to determine the total number per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Non-targeted GC-MS head space analysis

Separation and quantification of phytochemical components were performed using a Shimadzu GC-2010 plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass selective detector system as previously described.[40] Briefly, the system was equipped with a Shimadzu auto-sampler AOC-5000 plus (USA) fitted with a solid phase micro-extraction fibre (SPME) handling system utilising a Supelco (USA) divinyl benzene/carbowax/polydimethylsiloxane (DVB/CAR/PDMS). Chromatographic separation was accomplished using a 5% phenyl, 95% dimethylpolysiloxane (30m x 0.25mm id x 0.25um) capillary column (Restek USA). Helium (99.999%) was employed as a carrier gas at a flow rate of 0.79mL/min. The injector temperature was set at 230°C. Sampling utilised a SPME cycle which consisted of an agitation phase at 500rpm for a period of 5sec. The fibre was exposed to the sample for 10min to allow for absorption and then desorbed in the injection port for 1min at 250°C. The initial column temperature was held at 30°C for 2min, increased to 140°C for 5 min, then increased to 270°C over a period of 3mins and held at that temperature for the duration of the analysis. The GC-MS interface was maintained at 200°C with no signal acquired for a min after injection in split-less mode. The mass spectrometer was operated in the electron ionisation mode at 70eV. The analytes were then recorded in total ion count (TIC) mode. The TIC was acquired after a min and for duration of 45mins utilising a mass range of 45 - 450m/z.

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.

Table 1: Therapeutic uses, selected disease models and the drug targets that *K. senegalensis* extracts have been studied against.

Therapeutic Use/ Disease	Target/Test System	Plant Product Tested	Comments/Phytocompounds/Mechanisms	References
Chronic oxidative disease	DPPH free radical scavenging activity	Stem bark solvent extractions	Bioactive components were not determined.	[6]
Fungal pathogens	*Botrytis cinerea*	*K. senegalensis* fruit extracts and isolated compounds	Multiple bioactive limonoids including several seneganolides.	[7]
Insect deterrant/ antifeedant properties	*Spodoptera littoralis* (African cotton leafworm) larvae	Stem bark extracts and isolated compounds	Khayanolides A, B and C, other limonoids, angolensates and triterpenoids.	[8]
Diarrhoea, dysentry	A broad panel of bacterial species associated with food poisoning, diarrhoea and dysentery.	Leaf and bark solvent extractions	Flavonoids, terpenoids, tannins, saponins	[9, 10]
Antiseptic	*Staphylococcus aureus, Streptococcus spp., E. coli, P. aeruginosa, Salmmonella* spp., *B. cereus*	Root, stem bark and leaf extracts	Polyphenolic compounds, flavonoids, saponins, terpenoids	[9, 10]
Immunostimulation	Macrophages	Solvent extractions and isolated compounds	Dimeric proanthocyanidins induce TNF-α secretion from macrophages	[11]
Inflammation	HCT-15, HT-29 and HCA-7 colorectal cancer cells	Stem bark solvent extractions	Bioactive components were not determined. COX-2 dependent and independent anti-inflammatory mechanisms were reported.	[6]
Diabetes	Wistar rats	Aqueous stem bark extract	Anti-hyperglycemic activity comparable to glibenclamide was reported.	[12]
Cancer	MCF-7 (breast cancer), SiHa (cervical cancer and Caco-2 (colorectal) cancer cell lines.	Stem bark solvent extractions	Limonoids	[13]
Colorectal cancer	HCT-15, HT-29 and HCA-7 colorectal cancer cells	Stem bark solvent extractions	Anti-proliferative and pro-apoptotic activities were reported against all cell lines.	[6, 14]
Malaria	Chloroquine resistant and chloroquine sensitive *Plasmodium falciparum* strains	Leaf, seed and stem bark solvent extracts.	Terpenoids. The bark extract was particularly potent (IC_{50} = 3µM)	[15]
Trypanosomiasis: Surra	*Trypanosoma evansi*	Stem bark, root bark and leaf extracts	Phytochemistry was not studied, although the authors postulated that flavonoids may contribute to the activity.	[16]
Trypanosomiasis: Sleeping sickness	*Trypanosoma brucei*	Stem bark extract.	Inhibits *T. brucei* proliferation *in vivo* via changes in levels of aspartate and alanine transaminases.	[16]
Leishmaniasis	*Leishmania donovani, L. infatum, L. enriettii and L. major*	Solvent extractions and isolated compounds	Dimeric proanthocyanidins were implicated via immunostimulation.	[11]

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1g of dried plant material with various solvents yielded dried plant extracts ranging from approximately 47mg to 233mg (Table 2). The methanol and the SFE extracts had the highest yields of dried extracted material (233 and 188mg, respectively). Water, ethyl acetate, chloroform and hexane extracted lower masses (approximately 130, 47, 63, and 57mg, respectively). The dried extracts were resuspended in 10mL of deionised water (containing 1% DMSO) resulting in the extract concentrations shown in Table 2. Phytochemical studies (Table 2) showed that the methanol, water and the SFE extracts contained the widest range and largest amount of phytochemicals in this study. Each of these contained high levels of total phenolics and moderate to high levels of saponins, triterpenoids and tannins. Similar classes of phytochemicals were detected in the ethyl acetate, chloroform and hexane extracts, albeit generally at lower levels.

Antimicrobial activity

Aliquots (10µL) of each extract were tested in the disc diffusion assay against bacterial species associated with the induction of rheumatoid arthritis (*Proteus mirabilis*, Figure 2a; *Proteus vulgaris*, Figure 2b), ankylosing spondylitis (*Klebsiella pneumoniae*, Figure 3), multiple sclerosis (*Acinitobacter baylyi*, Figure 4a; *Pseudomonas aeruginosa*, Figure 4b) and rheumatic fever (*Streptococcus pyogenes*, Figure 5). The *K. senegalensis* bark extracts were potent inhibitors of reference and clinical strains of *P. mirabilis* (Figure 2a), with zones of inhibition up to approximately 15mm. The methanolic extract was particularly potent, with inhibition zones of 13.0 ± 1.0 and 14.7 ± 1.2 mm against the reference and clinical strains, respectively. The aqueous (9.6 ± 0.6 and 8.8 ± 0.4 mm for the reference and clinical strains respectively) and ethyl acetate (8.9 ± 0.6 and 7.3 ± 0.3mm for the reference and clinical strains, respectively) were also good inhibitors of *P. mirabilis* growth. The hexane extract also inhibited *P. mirabilis* growth, although lower efficacy (7.3 ± 0.3mm and 6.7 ± 0.3mm for the reference and clinical strains, respectively) was evident than for the methanolic, aqueous and ethyl acetate extracts. In contrast, the chloroform extract was completely devoid of *P. mirabilis* growth inhibitory activity. The *K. senegalensis* bark extracts were similarly potent growth inhibitors against *P. vulgaris* (Figure 2b). A zone of inhibition of 9.7 ± 0.6mm was recorded for the methanolic extract. Similarly, growth inhibition zones of 7.5 ± 0.5 and 6.7 ± 0.3mm were seen for the aqueous and ethyl acetate extracts, respectively. In contrast, both the chloroform and hexane extracts were completely devoid of *P. vulgaris* growth inhibitory activity.

The subcritical *K. senegalensis* bark extract was also a good inhibitor of *Proteus* spp. growth. It was a particularly potent inhibitor of *P. vulgaris* growth (9.3 ± 0.6mm; Figure 2b). Similarly, although slightly lower growth inhibitory potency was noted against *P. mirabilis,* with zones of inhibition of 8.3 ± 0.3 and 8.0 mm against *P. mirabilis* (reference and clinical strains, respectively). The subcritical extract displayed slightly lower efficacy to the small scale laboratory extraction (as judged by zone of inhibition). As *Proteus* spp. (particularly *P. mirabilis*) are triggers of rheumatoid arthritis in genetically susceptible people,[41,42] these extracts may be useful in the prevention and treatment of this disease.

A similar activity profile was evident for *K. pneumoniae* growth inhibition (Figure 3). The methanolic extract was the most potent growth inhibitor, with zones of inhibition of 9.3 ± 0.3mm (reference strain) and 8.6 ± 0.3mm (clinical strain). This compares favourably with the ampicillin control (10µg) which had 9.2 ± 0.4 (reference strain) and 8.7 ± 0.3mm (clinical strain) zones of inhibition. The aqueous, ethyl acetate chloroform, hexane and subcritical extracts also inhibited *K. pneumoniae* growth, albeit with lower efficacy (6.7 to 7.5mm zones of inhibition). As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible individuals,[43] these extracts have potential in the prevention and treatment of this disease.

The *K. senegalensis* bark extracts were also screened for growth inhibitory activity against bacterial triggers of multiple sclerosis (*Acinitobacter baylyi*, Figure 4a; *Pseudomonas aeruginosa*, Figure 4b),[44,45] The ethyl acetate extract was the most potent *A. baylyi* growth inhibitor, with zones

Table 2: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *K. senegalensis* bark extracts.

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/ml)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
M	233	23.3	+++	++	+++	-	++	++	+	-	-	-	+++	-	-
W	130	13	+++	++	+++	-	+++	++	-	-	-	+	++	-	-
E	47	4.7	+	+	-	-	+	-	-	-	-	-	+	-	-
C	63	6.3	+	+	+	-	++	+	-	-	-	-	+	-	-
H	57	5.7	+	+	-	-	++	-	-	-	-	-	+	-	-
SC	188	18.8	+++	++	+++	-	+	++	+	-	-	-	++	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SC= subcritical extract.

Table 3: Minimum bacterial growth inhibitory concentration (µg/mL) of the *K. senegalensis* bark extracts, Giardia and carcinoma anti-proliferative IC_{50} values (µg/mL) and LC_{50} values (µg/mL) in the *Artemia franciscana* nauplii bioassay.

		P. mirabilis		*P. vulgaris*	*K. pneumoniae*		*A. baylyi*		*P. aeruginosa*		*S. pyogenes*	*G. duodenalis*	Carcinoma cells		Toxicity
		Reference strain	Clinical strain	Clinical strain	Reference strain	Clinical strain	Reference strain	Clinical strain	Reference strain	Clinical strain	Clinical strain	Clinical strain	$CaCo_2$	HeLa	*A. franciscana*
Extract	M	185	250	258	167	233	608	212	1218	659	1437	187	268	155	1255
	W	345	384	524	727	628	1956	1620	2750	2385	1290	CND	CND	CND	2247
	E	1212	1426	1493	1424	1120	586	323	2313	1510	-	CND	-	-	1886
	C	-	-	-	3350	3210	2544	1975	659	375	-	-	-	-	2250
	H	986	1252	-	1870	2180	1774	1692	1718	1674	-	-	-	-	2080
	SC	211	432	446	251	387	670	588	1337	1250	1685	328	470	174	1360
Positive Controls	Met	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	18	NT	NT	NT
	Cis	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	8400	15710	NT

Numbers indicate the mean MIC, IC50 and LC50 values of triplicate determinations. - indicates no inhibition. CND indicates that an IC_{50} or LC_{50} value could not be determined as inhibition or mortality did not exceed 50% at any concentration tested. NT = not tested; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SC = subcritical extract; Met = metronidazole; Cis = cisplatin.

of inhibition of 12.0 ± 1.0 and 15.7 ± 1.3mm against the reference and clinical isolate strains, respectively. Potent growth inhibition was also determined for the methanolic extract (9.0 ± 1.0 and 10.7 ± 1.2mm for the reference and clinical isolate strains, respectively), hexane (8.3 ± 0.3 and 9.0mm for the reference and clinical isolate strains, respectively) and SFE extracts (9.5 ± 0.5 and 10.0 ± 1.0mm for the reference and clinical isolate strains, respectively). These results compare favourably to the ampicillin control (8.6 ± 0.3mm zones of inhibition against both *A. baylyi* strains). Although smaller inhibition zones (generally 7-8mm) were measured for the aqueous and chloroform extracts, these extracts were still deemed to be good *A. baylyi* growth inhibitors.

The methanolic (zones of inhibition of 14.6 ± 1.3 and 17.3 ± 1.2mm against the reference and clinical isolate strains, respectively) and chloroform *K. senegalensis* extracts (zones of inhibition of 12.0 ± 1.0 and 18.0± 1.0mm against the reference and clinical isolate strains, respectively) were also potent inhibitors of *P. aeruginosa* growth (Figure 4b). The ethyl acetate extract was a similarly potent growth inhibitor against the clinical strain (10.3 ± 0.6mm). However, it was a substantially less potent against the reference strain (8.3 ± 0.3mm), although these results are still indicative of strong growth inhibition. Whilst less potent, the aqueous, hexane and SFE extracts were also good *P. aeruginosa* growth inhibitors, with zones of inhibition generally 6.7-8.3mm. The *P. aeruginosa* growth inhibition was particularly noteworthy as both the reference and clinical *P. aeruginosa* strains are antibiotic resistant strains. Indeed, the 10µg ampicillin control used in our studies only produced 6.2 ± 0.4 and 5.5 ± 0.3mm zones of inhibition for the reference and clinical strains, respectively. This finding is supported by previous studies which have also reported these strains to be antibiotic resistant.[24,47] Thus, the *K. senegalensis* extracts are particularly potent growth inhibitors and may be useful for the inhibition of *P. aeruginosa* growth. Therefore, as both *A. baylyi* and *P. aeruginosa* can trigger multiple sclerosis in genetically susceptible individuals,[44] these extracts have potential in the prevention and treatment of this disease.

The *K. senegalensis* methanolic, aqueous and SFE extracts also inhibited *Streptococcus pyogenes* growth (Figure 5), albeit with zones of inhibition that indicate only moderate inhibitory activity. The aqueous leaf extract was the most potent growth inhibitor, with an inhibition zone of 7.3 ± 0.3mm, whilst both the methanolic and subcritical extracts gave inhibition zones substantially <7mm. However, this would be considered to be only moderate inhibition and is substantially less potent than the ampicillin control (8.3 ± 0.3mm zones of inhibition). In contrast, the ethyl acetate, chloroform and hexane extracts were completely devoid of inhibitory activity. *S. pyogenes* has been implicated in a number of diseases including rheumatic fever. Thus, the *K. senegalensis* methanolic, aqueous and subcritical extracts have some potential in the prevention and treatment of these diseases.

The relative level of antimicrobial activity was further evaluated by determining the MIC values (Table 3) for each extract against the bacterial 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 many MIC values against the bacterial species that they inhibited generally < 1000µg/mL (<10µg impregnated in the disc), indicating the potent antimicrobial activity of these extracts. These MIC values compare favourably with the dosages of the pure standard ampicillin which was tested using 10µg per disc. The methanol, water and SFE extracts were particularly potent with MIC values in the range 160-500µg/ml against several species. Indeed, MIC values of 185µg/mL (approximately 1.9mg in the disc; *P. mirabilis* reference strain), 167µg/mL (approximately 1.7mg in the disc; *K. pneumoniae* reference strain) and 212µg/mL (approximately 2.2mg in the disc; *A. baylyi* clinical strain), were determined for the methanolic extract. Similarly potencies were

evident for the aqueous and SFE extracts. Whilst the ethyl acetate, chloroform and hexane extracts also had broad spectrum inhibitory activity against many of the bacterial species, they generally had much lower efficacies (with some MIC values >2000µg/ml).

Anti-Giardial activity

K. senegalensis bark extracts were screened for their ability to inhibit *Giardia duodenalis* growth (Figure 6). The methanol, water, ethyl acetate and SFE extracts displayed significant inhibitory activity. The methanolic and SFE extracts were particularly potent, inhibiting 95% and 100% of the Giardial growth, respectively (compared to the untreated control). The chloroform and hexane extracts were ineffective as proliferation inhibitors, with no significant difference to the untreated control levels.

The *K. senegalensis* extracts were further tested over a range of concentrations to determine the IC_{50} 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. The

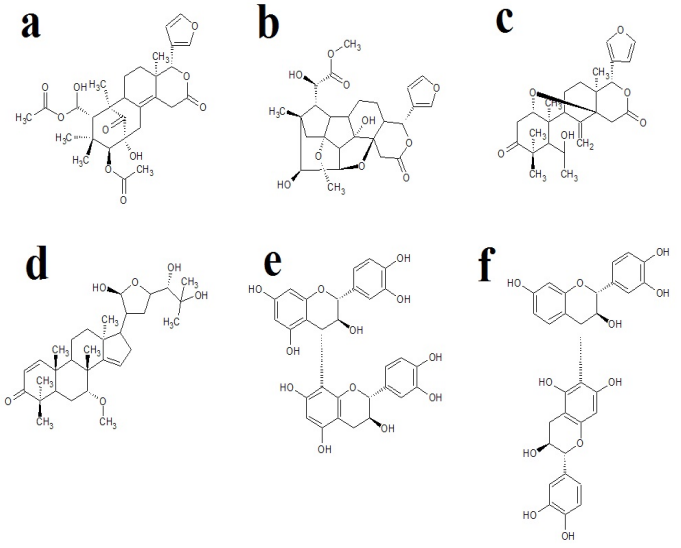

Figure 1: Chemical structures of selected molecules identified *K. senegalensis* extracts: (a) a characteristic limonoid structure (2-hydroxyseneganolide A is depicted), (b) a characteristic khayanolide structure (1-O-acetylkhayanolide B is depicted), (c) 6-hydroxy-methyl angolensate, (d) a characteristic senegalene triterpenoid structure (senegalene C is depicted), (e) proanthocyanidin B3, (f) fisetinidol-(4α, 6)-catechin.

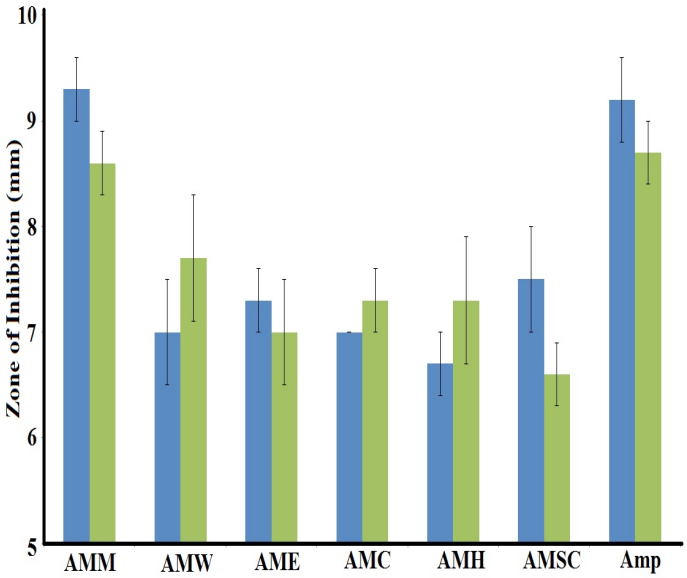

Figure 3: Antibacterial activity of *K. senegalensis* extracts measured as zones of inhibition (mm) against a bacterial trigger of ankylosing spondylitis (*Klebsiella pneumoniae*). Results are expressed as mean ± SEM of at least triplicate determinations. Blue bars represent inhibition of the reference bacterial strain. Green bars represent inhibition of the clinical bacterial strain. AMM = methanolic extract; AMW = aqueous extract; AME = ethyl acetate extract; AMC = chloroform extract; AMH = hexane extract; AMSC = SFE extract; Amp = ampicillin control (10µg).

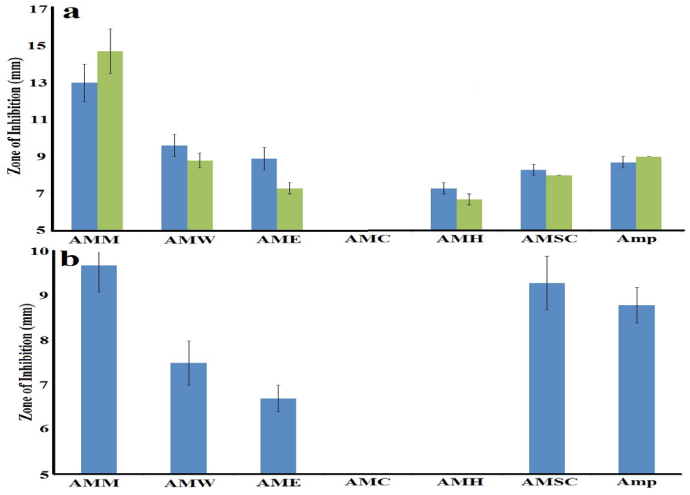

Figure 2: Antibacterial activity of the *K. senegalensis* extracts measured as zones of inhibition (mm) against bacterial triggers of rheumatoid arthritis: (a) *Proteus mirabilis*, (b) *Proteus vulgaris*. Results are expressed as mean ± SEM of at least triplicate determinations. Blue bars represent inhibition of the reference bacterial strain. Green bars represent inhibition of the clinical bacterial strain. AMM = methanolic extract; AMW = aqueous extract; AME = ethyl acetate extract; AMC = chloroform extract; AMH = hexane extract; AMSC = SFE extract; Amp = ampicillin control (10µg).

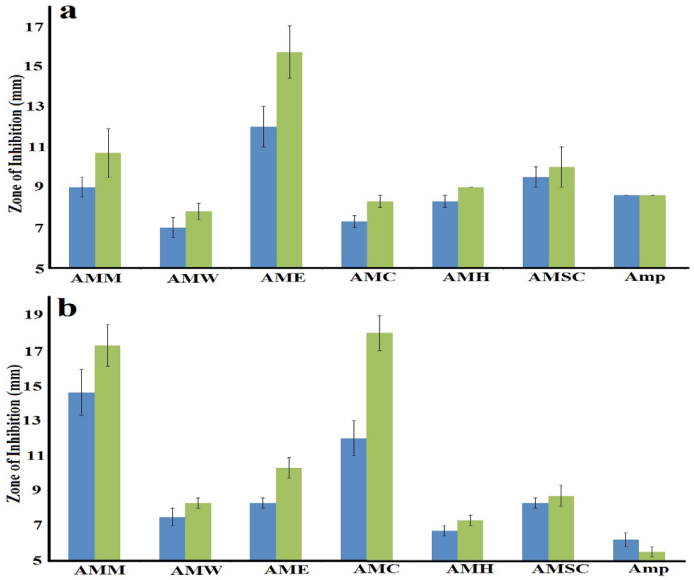

Figure 4: Antibacterial activity of *K. senegalensis* extracts measured as zones of inhibition (mm) against a bacterial trigger of multiple sclerosis: (a) *Acinito bacter baylyi*, (b) *Pseudomonas aeruginosa*. Results are expressed as mean ± SEM of at least triplicate determinations. Blue bars represent inhibition of the reference bacterial strain. Green bars represent inhibition of the clinical bacterial strain. AMM = methanolic extract; AMW = aqueous extract; AME = ethyl acetate extract; AMC = chloroform extract; AMH = hexane extract; AMSC = SFE extract; Amp = ampicillin control (10µg).

Table 4: Qualitative headspace GC-MS analysis of the SFE *K. senegalensis* bark extract, elucidation of empirical formulas and putative identification (where possible) of the compounds.

Retention Time (min)	Molecular Mass	Empirical Formula	Area (%)	Putative Identification
10.54	151	$C_8H_9NO_2$	0.17	Oxime-, methoxy-phenyl-
10.825	130	$C_7H_{14}O_2$	0.19	Hexanoic acid, methyl ester
10.99	178	$C_8H_{18}O_4$	0.23	2,5-Dimethylhexane-2,5-dihydroperoxide
11.125	122	$C_8H_{10}O$	0.15	Oxepine, 2,7-dimethyl-
11.385	128	$C_8H_{16}O$	0.36	2-Heptanone, 4-methyl-
11.695	100	$C_6H_{12}O$	0.06	Furan, tetrahydro-2,5-dimethyl-
11.935			0.22	
12.425	144	$C_8H_{16}O_2$	1.31	Formic acid, heptyl ester
12.66	128	$C_8H_{16}O$	0.73	1-Octen-3-ol
12.815	170	$C_{10}H_{18}O_2$	0.69	2-Hydroxy-1,8-cineole
13.05	136	$C_{10}H_{16}$	4.11	β-Pinene
13.105			0.35	
13.21			0.91	
13.46			3.34	
13.87	136	$C_{10}H_{16}$	0.37	Terpinolene
14.03	134	$C_{10}H_{14}$	0.4	o-Cymene
14.295	154	$C_{10}H_{18}O$	20.43	1,8-Cineole
14.48	136	$C_{10}H_{16}$	0.91	α-Pinene
14.875	136	$C_{10}H_{16}$	1.79	Pseudolimonene
15.125	168	$C_{19}H_{16}O_2$	0.74	2H-Pyran-2-one, tetrahydro-6-(2-penteny
15.215			0.06	
15.33	156	$C_9H_{16}O_2$	0.08	Butanoic acid, 4-pentenyl ester
15.675	242	$C_{13}H_{22}O_4$	4.91	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate
15.66	120	C_8H_8O	0.01	Benzaldehyde, 2-methyl-
15.9	130	$C_7H_{14}O_2$	0.66	Heptanoic acid
16.05	170	$C_{10}H_{18}O_2$	3.2	Linalool oxide
16.16	136	$C_7H_8N_2O$	0.49	Benzoic acid, hydrazide
16.635	154	$C_{10}H_{18}O$	30.34	Linalool
16.83			0.39	
17.325	136	$C_{10}H_{16}$	0.4	2,4,6-Octatriene, 2,6-dimethyl-, (E,E)-
17.475	168	$C_{11}H_{20}O$	0.28	Linalool, methyl ether
17.575	152	$C_{10}H_{16}O$	0.33	Pinocarveol
17.72			0.42	
17.875	128	$C_7H_{12}O_2$	0.14	2(3H)-Furanone, dihydro-5-propyl-
18.15			0.33	
18.405	154	$C_{10}H_{18}O$	0.52	δ-Terpineol
18.48	170	$C_{10}H_{18}O_2$	0.19	trans-Linalool 3,7-oxide
18.605			0.58	
18.78	154	$C_{10}H_{18}O$	1.88	1-Terpinen-4-ol
19.175	154	$C_{10}H_{18}O$	4.25	α-Terpineol
19.24	150	$C_{10}H_{14}O$	0.03	α-Thujenal
19.47	246	$C_{18}H_{30}$	0.11	Benzene, (2,3-dimethyldecyl)-
19.785			0.33	
19.895	128	$C_8H_{16}O$	0.03	3-Heptanone, 2-methyl-
19.975	168	$C_{11}H_{20}O$	0.05	4-(2-Methoxypropan-2-yl)-1-methylcyclohex-1-ene

20.135	168	$C_{11}H_{20}O$	0.04	2,6-Octadiene, 1-methoxy-3,7-dimethyl-,
20.285	196	$C_{12}H_{20}O_2$	0.09	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate,
20.445	152	$C_9H_{12}O_2$	0.07	3,4-Dimethoxytoluene
20.56	152	$C_{10}H_{16}O$	0.09	Neral
20.67	113	$C_6H_{11}NO$	0.05	Caprolactam
21.065	152	$C_{10}H_{16}O$	2.2	Piperitone
21.2			0.09	
21.42	158	$C_9H_{18}O_2$	0.51	Pelargic acid
21.9			0.18	
22.545	168	$C_9H_{12}O_3$	0.06	Methylsyringol
22.78			0.14	
23.41			0.09	
23.52	154	$C_8H_{10}O_3$	0.02	Pyrogallol 1,3-dimethyl ether
23.745	164	$C_{10}H_{12}O_2$	0.47	Eugenol
23.83	142	$C_8H_{14}O_2$	0.09	2(3H)-Furanone, 5-butyldihydro-
23.925	284	$C_{18}H_{36}O_2$	0.04	Stearic acid
24.245	216	$C_{12}H_{24}O_3$	0.12	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester
24.515	180	$C_{12}H_{20}O$	0.02	-(2,6,6-Trimethyl-1-cyclohexen-1-yl)propanal
25.06	178	$C_{11}H_{14}O_2$	0.32	Methyleugenol
25.48			0.17	
26.74	220	$C_{15}H_{24}O$	0.09	(+)-Cycloisolongifol-5-ol
26.885			0.14	
28.145			0.33	
28.98	206	$C_{14}H_{22}O$	0.59	,5-Di-tert-butylphenol
29.195			0.16	
29.56	202	$C_{15}H_{22}$	0.17	(-)-Calamenene
29.96	220	$C_{15}H_{25}O$	0.13	Italicene ether
30.09	200	$C_{15}H_{20}$	0.05	α.-Dehydro-ar-himachalene
30.27	200	$C_{15}H_{20}$	0.2	α.-Calacorene
30.43	220	$C_{15}H_{24}O$	0.25	Longifolenaldehyde
30.555	208	$C_{12}H_{16}O_3$	0.08	Elemicin
31.08	222	$C_{15}H_{26}O$	0.08	(-)-Globulol
31.345	220	$C_{15}H_{24}O$	1.16	(-)-Spathulenol
31.88			0.32	
31.975	222	$C_{15}H_{26}O$	0.07	Ledol
32.435	200	$C_{15}H_{20}$	0.11	α-Corocalene
32.665	222	$C_{15}H_{26}O$	0.11	γ-Eudesmol
32.875	218	$C_{15}H_{22}O$	0.61	2H-Cyclopropa[a]naphthalen-2-one, 1,1a,4,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-
32.955	204	$C_{15}H_{24}O$	0.08	Copaene
33.31			0.18	
33.405	222	$C_{15}H_{26}O$	0.06	Guai-1(10)-en-11-ol
33.52	198	$C_{15}H_{18}$	0.13	Cadalene
34.5	204	$C_{15}H_{24}$	0.04	γ-Neoclovene
34.82	238	$C_{15}H_{26}O_2$	0.16	Isocalamenediol
35.06	218	$C_{15}H_{22}O$	0.61	Cyperenone
36.505	278	$C_{16}H_{22}O_4$	0.09	Phthalic acid, diisobutyl ester
37.675	278	$C_{16}H_{22}O_4$	0.01	Dibutyl phthalate

The % area is expressed as a % of the total area under all chromatographic peaks.

methanolic extract and the SFE extract were the most potent anti-proliferative agents, with IC_{50} values of 184 and 328µg/mL respectively. Whilst the aqueous and ethyl acetate extracts inhibited *G. duodenalis* proliferation, we were unable to determine IC_{50} values as the inhibition did not exceed 50% at any concentration tested.

Inhibition of cancer cell proliferation

The *K. senegalensis* extracts were tested against 2 cancer cell lines (Caco-2 colorectal carcinoma cells, Figure 7; HeLa cervical cancer cells, Figure 8) to determine their ability to inhibit cancer cell growth. The methanol, water and SFE extracts displayed potent inhibitory activity against Caco-2 cells (Figure 7). Indeed, Caco-2 cellular proliferation was inhibited to

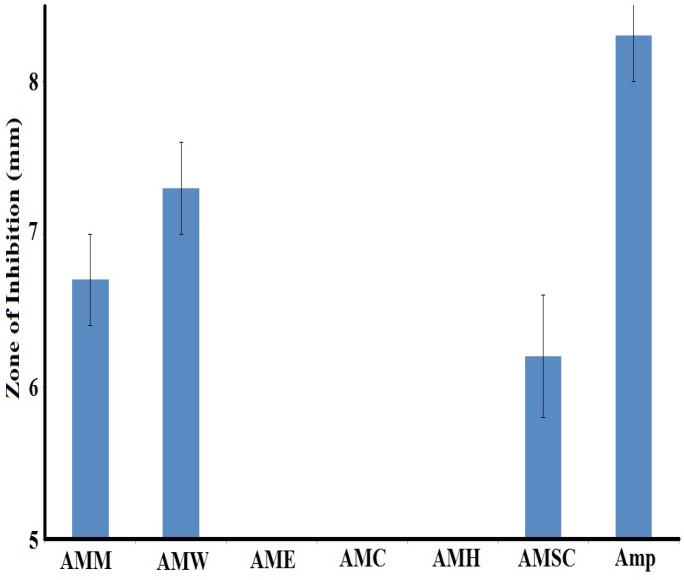

Figure 5: Antibacterial activity of *K. senegalensis* extracts measured as zones of inhibition (mm) against a bacterial trigger of rheumatic fever (*Streptococcus pyogenes*). Results are expressed as mean ± SEM of at least triplicate determinations. AMM = methanolic extract; AMW = aqueous extract; AME = ethyl acetate extract; AMC = chloroform extract; AMH = hexane extract; AMSC = SFE extract; Amp = ampicillin control (10µg).

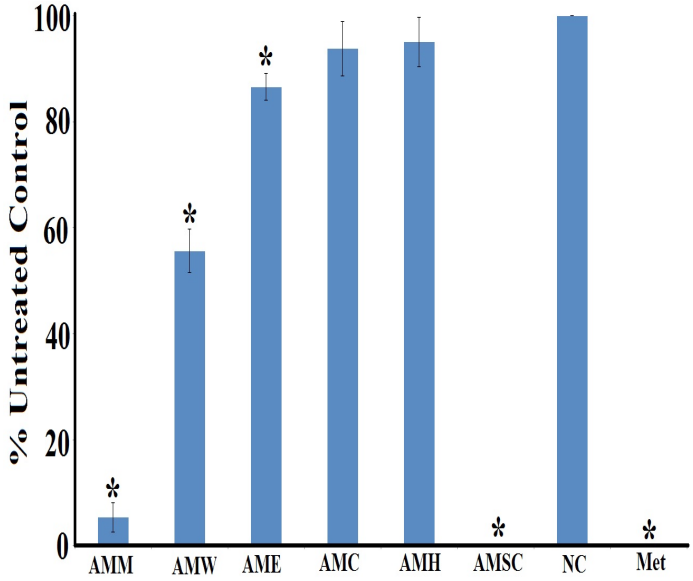

Figure 6: Inhibitory activity of *K. senegalensis* extracts against *Giardia duodenalis* trophozoites measured as a percentage the untreated control. Results are expressed as mean ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). AMM = methanolic extract; AMW = aqueous extract; AME = ethyl acetate extract; AMC = chloroform extract; AMH = hexane extract; AMSC = SFE extract; NC = negative control; PC = metronidazole control (50µg/mL).

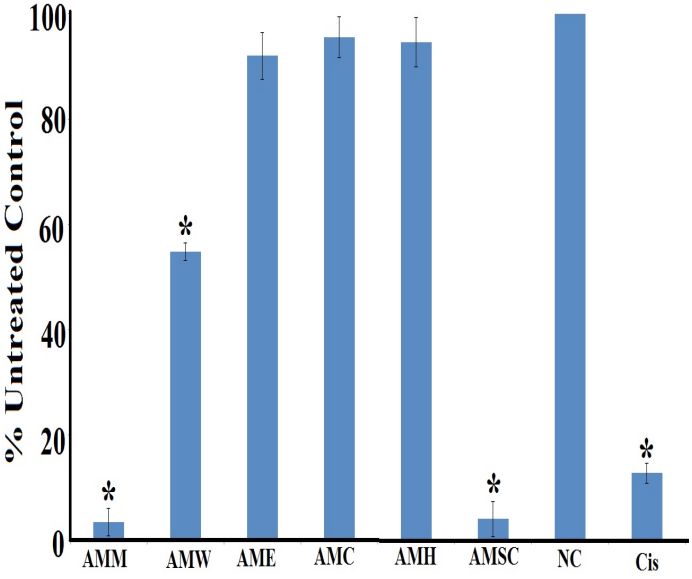

Figure 7: Anti-proliferative activity of *K. senegalensis* extracts and untreated controls against Caco-2 carcinoma cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). AMM = methanolic extract; AMW = aqueous extract; AME = ethyl acetate extract; AMC = chloroform extract; AMH = hexane extract; AMSC = SFE extract; NC = negative control; Cis = cisplatin control (50mg/mL).

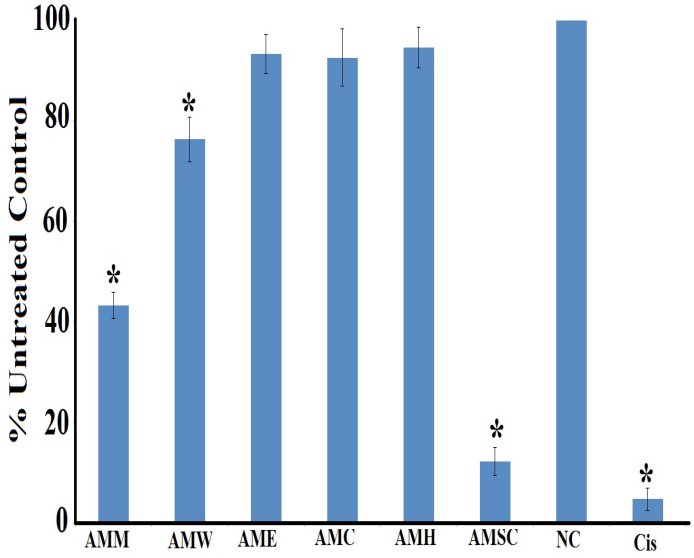

Figure 8: Anti-proliferative activity of *K. senegalensis* extracts and untreated controls against HeLa carcinoma cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). AMM = methanolic extract; AMW = aqueous extract; AME = ethyl acetate extract; AMC = chloroform extract; AMH = hexane extract; AMSC = SFE extract; NC = negative control; Cis = cisplatin control (50mg/mL).

approximately 4% of the untreated control cell growth by the methanolic and subcritical extracts (Figure 7). The aqueous extracts were also effective at inhibiting Caco-2 proliferation (to approximately 55% of untreated cell proliferation). In contrast, the ethyl acetate, chloroform and hexane extracts did not significantly affect Caco-2 cell proliferation. Inhibition of proliferation by the methanol, water and subcritical extracts was dose dependent, with the level of inhibitory activity decreasing at lower concentrations.

The *K. senegalensis* extracts also inhibited the proliferation of HeLa cells (Figure 8), albeit with lower efficacy than was evident with the Caco-2 cells. The SFE *K. senegalensis* extract was the most potent inhibitor of HeLa cell proliferation, inhibiting to approximately 12% of the untreated control cell growth. In contrast, the methanolic and aqueous extracts inhibited HeLa cell proliferation to approximately 43 and 76% of the untreated control cell growth respectively. Inhibition of proliferation by the methanol extract was dose-dependent, with the level of inhibitory activity decreasing at lower concentrations.

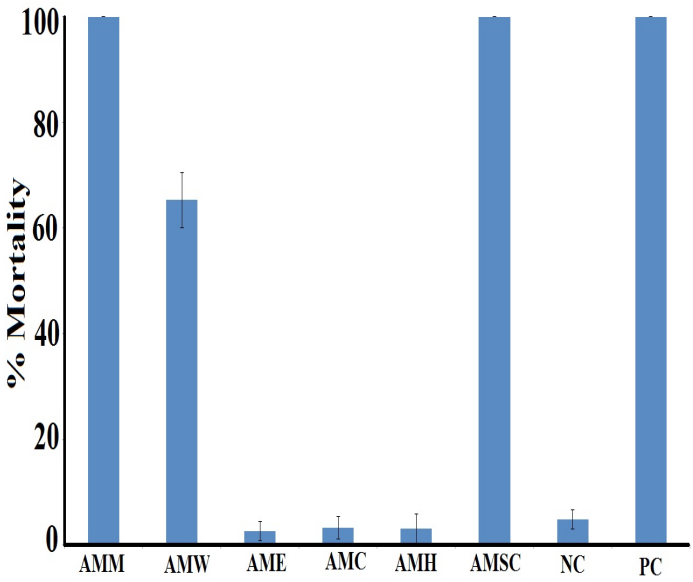

Figure 9: The lethality of *K. senegalensis* extracts and potassium dichromate (1000µg/mL) and artificial seawater controls towards *Artemia franciscana* nauplii after 24 hours exposure. Results are expressed as mean ± SEM of at least triplicate determinations. AMM = methanolic extract; AMW = aqueous extract; AME = ethyl acetate extract; AMC = chloroform extract; AMH = hexane extract; AMSC = SFE extract; NC = negative (seawater) control; PC = potassium dichromate control (1000µg/mL).

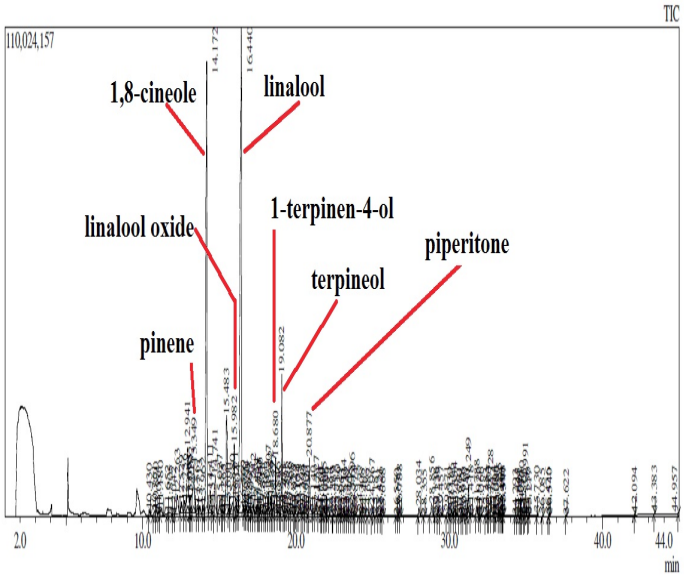

Figure 10: Head space gas chromatogram of a 0.5µL injection of the *K. senegalensis* bark SFE extract. The extract was dried and resuspended in methanol. Major phytochemical components are identified in the chromatogram.

The *K. senegalensis* extracts were further tested over a range of concentrations to determine the IC_{50} values (Table 3) for each extract against Caco-2 and HeLa proliferation. Inhibition of proliferation was dose-dependent for all extracts, with the level of inhibitory activity decreasing at lower concentrations (Table 3). The methanolic and SFE *K. senegalensis* extracts were particularly potent HeLa anti-proliferative agents, with IC_{50} values of 155 and 174µg/mL, respectively. These extracts were similarly potent inhibitors of Caco-2 proliferation, with IC_{50} values calculated of 268 and 470µg/mL, respectively.

Quantification of toxicity

K. senegalensis bark extracts were diluted to 4000µg/mL (to give a bioassay concentration of 2000µg/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. senegalensis* extracts were slower at inducing mortality, with ≥12 hours needed for mortality induction. Despite the slower onset of mortality, the methanol, water and SFE extracts induced mortality significantly above that of the artificial seawater control (Figure 9). Table 3 shows the extract and control toxin concentrations required to achieve 50% mortality (LC_{50}) at various times. All of the extractions also had LC_{50} values substantially >1000 µg/mL. As toxicity of crude plant extracts has previously been defined as 24 LC_{50} values <1000µg/mL,[37] all of the *K. senegalensis* extracts were deemed to be non-toxic.

Non-targeted GC-MS headspace analysis of the subcritical *K. senegalensis* extract

As the SFE *K. senegalensis* extract had potent bacterial growth inhibitory efficacy, anti-Giardial activity and carcinoma cell anti-proliferative activity (as determined by MIC and IC_{50}; Table 3), it was deemed the most promising extract for further phytochemical analysis. Optimised GC-MS parameters were developed and used to examine the phytochemical composition of this extract. The resultant gas chromatograms are presented in Figure 10. Major peaks were evident in the SFE extract at approximately 14.2, 15.5, 15.9, 16.4, 19.1 and 20.1min. Several smaller peaks were also evident throughout all stages of the chromatogram. In total, 92 unique mass signals were noted for the SFE *K. senegalensis* extract (Table 4). Putative empirical formulas and identifications were achieved for 72 (78%) of these compounds by comparison with the database.

DISCUSSION

Plant remedies are becoming increasingly sought after in the treatment of a myriad of diseases and disorders due both to their perception of greater safety than synthetic drugs, and the failure of current drug regimens to effectively treat many diseases. This is especially true for the autoimmune inflammatory diseases. The current treatments utilising disease modifying anti-rheumatic drugs (DMARDs) to alleviate the symptoms of these diseases and/or alter the disease progression are not entirely effective and have been associated with numerous adverse effects.[51] Furthermore, many of the current treatments are aimed at treating the symptoms without addressing the underlying causes and patho-

genic mechanisms. Therefore, whilst these treatments may alleviate pain, redness, swelling, *etc*., they do not address the tissue degeneration which occurs as a consequence of the disease etiology. Furthermore, all of these drugs are used as treatments and there are currently no preventative therapy options. A better understanding of the mechanisms for initiation and progression of the autoimmune inflammatory diseases is important for developing new drugs to target specific processes and thus more effectively treat autoimmune inflammatory disease. A major focus of this study was to screen *K. senegalensis* extracts for the ability to inhibit the growth of bacterial triggers of some autoimmune inflammatory diseases.

Our findings support previous studies which have also reported antibacterial properties for *K. senegalensis* extracts from other plant parts.[12,13,16,18] The *K. senegalensis* bark extracts screened in our study were particularly potent inhibitors of *P. mirabilis* and *P. vulgaris* growth, with MIC values of several extracts substantially <500µg/mL. This activity is noteworthy as *P. mirabilis* has been implicated in urinary tract infections (UTI's) and the induction of rheumatoid arthritis (RA).[41,42] Thus, the *K. senegalensis* bark extracts have the potential to 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. Furthermore, as these are crude extracts containing a number of known bioactive components, it is possible that they may also affect other phases of the rheumatoid arthritis disease process (e.g. regulation of cytokine production, immunomodulation, *etc*.) and thus may have pleuripotent effects. Further studies are required to test the effect of the extract on these other phases of the disease progression. If other therapeutic effects are subsequently detected, the *K. senegalensis* bark extracts may be a particularly attractive option for chronic sufferers of this disease, to block its onset as well as treating its symptoms once it is initiated.

The *K. senegalensis* bark extracts were similarly potent inhibitors of *K. pneumoniae* growth, with MIC values as low as 167µg/mL (methanolic extract against the reference strain), indicating that it may also be useful in the prevention of ankylosing spondylitis. Whilst ankylosing spondylitis affects different tissue than rheumatoid arthritis, it has a similar multiple phased progression.[43] The *K. senegalensis* bark extracts may therefore also have further effects on other phases of ankylosing spondylitis disease. Indeed, it is possible that the extract may also modulate cytokine production and therefore also block later inflammatory disease events, although this has yet to be tested for our extracts. The *K. senegalensis* bark extracts were also good inhibitors of *A. baylyi* and *P. aeruginosa* growth, with MIC values of several extracts <1000µg/mL. These extracts may therefore be useful in the prevention and treatment of multiple sclerosis. Indeed, MIC values of 212 and 659µg/mL were determined for the methanolic extract against clinical strains of *A. baylyi* and *P. aeruginosa,* respectively. In contrast, the *K. senegalensis* extracts were substantially less potent inhibitors of *S. pyogenes* growth. Indeed, only the methanolic, aqueous and subcritical extracts inhibited *S. pyogenes* growth to any extent. Furthermore, the MIC values for each of these extracts were substantially >1000µg/mL, indicating moderate inhibitory activity. As *S. pyogenes* can cause a variety of diseases including streptococcal pharyngitis, impetigo and rheumatic heart disease, depending on which tissue it infects, these extracts may be useful in the treatment and prevention of these diseases.

Noteably, the therapeutic properties of an extract in the treatment of autoimmune diseases may be of greater efficacy as synergistic actions may exist between various therapeutic mechanisms (antibacterial, anti-inflammatory, antioxidant, immune-stimulatory, *etc*.), providing combined effects on these complex diseases. Thus, whilst the bacterial growth inhibitory studies reported here indicate the potential of the *K. senegalensis* extracts in the treatment and prevention of these autoimmune diseases, the activity may be more profound due to combinatorial effects and further studies are required to examine the effects of these extracts on later phases of these diseases. Furthermore, this study has only tested these extracts against some microbial triggers of 4 autoimmune diseases (rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever). The microbial triggers for several other autoimmune inflammatory disorders are also known. For example, *Borrelia burgdorferi* is linked with Lyme disease.[52] Whilst microbial triggers have also been postulated for lupus, the specific causative agents are yet to be identified. Similarly, members of the Enterobacteriaceae family are associated with Graves' disease and Kawasaki syndrome. *Mycoplasma pneumoniae* is associated with several demyelinating diseases[53] It would be interesting to extend our studies to also screen for the ability of the extracts to block these microbial triggers of autoimmune diseases.

GC-MS headspace analysis of the SFE *K. senegalensis* extract detected a number of interesting compounds, including a wide diversity of terpenoids. Monoterpenoids were particularly prevalent, with 2-hydroxy-1,8-cineole (Figure 11a), β-pinene (Figure 11b), terpinolene (Figure 11c), 1,8-cineole (Figure 11d), α-pinene (Figure 11e), pseudolimonene (Figure 11f), linalool oxide (Figure 11g), linalool (Figure 11h), linalool methyl ether (Figure 11i), pinocarveol (Figure 11j), δ-terpineol (Figure 11k), 1-terpinen-4-ol (Figure 11l), α-terpineol (Figure 11m), α-thujenal (Figure 11n), neral (Figure 11o), piperitone (Figure 11p), eugenol (Figure 11q) and methyleugenol (Figure 11r) putatively identified by comparison to a commercial database. Monoterpenes have been reported to exert a wide variety of biological effects including antibacterial, antifungal, anti-inflammatory and anti-tumour activities[54] and therefore are likely to contribute to the growth inhibitory activity against the bacterial triggers of the autoimmune diseases reported here. Indeed, many of the monoterpenoids putatively identified in our study have been previously reported to have potent broad spectrum antibacterial activity[54] Further studies have reported that a wide variety of monoterpenoids inhibit the growth of an extensive panel of pathogenic and food spoilage bacteria.[55] Interestingly, several of these monoterpenoids have also been reported to suppress NF-κ B signaling (the major regulator of inflammatory diseases).[56-59] Thus, the monoterpenoid components may have a pleuripotent mechanism in blocking the autoimmune inflammatory diseases and relieving its symptoms by acting on both the initiator and downstream inflammatory stages of the disease.

Several sesquiterpenoids were also detected in the SFE *K. senegalensis* extract. Comparison to a commercial database resulted in putative identification of cycloisolongifol-5-ol (Figure 12a), italicene ether (Figure 12b), α-dehydro-ar-himachalene (Figure 12c), longifolenaldehyde (Figure 12d), globulol (Figure 12e), spathulenol (Figure 12f), ledol (Figure 12g), γ-eudesmol (Figure 12h), guai-1(10)-en-11-ol (Figure 12i), isocalamenediol (Figure 12j), cyperenone (Figure 12k), phthalic acid, diisobutyl ester (Figure 12l) and dibutyl phthalate (Figure 12m). Previous studies have reported bacterial growth inhibitory activities for many sesquiterpenoids against a wide panel of pathogenic bacteria, with MIC values as low as 4µg/mL reported.[60-62] Similar compounds were also detected in the SFE *K. senegalensis* extract. Thus, sesquiterpenoids are also likely to contribute to the growth inhibitory activity determined in our study.

Potent growth inhibition of the food/water borne gastrointestinal parasite *Giardia duodenalis* was also noted for the *K. senegalensis* bark 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.[48] 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 im-

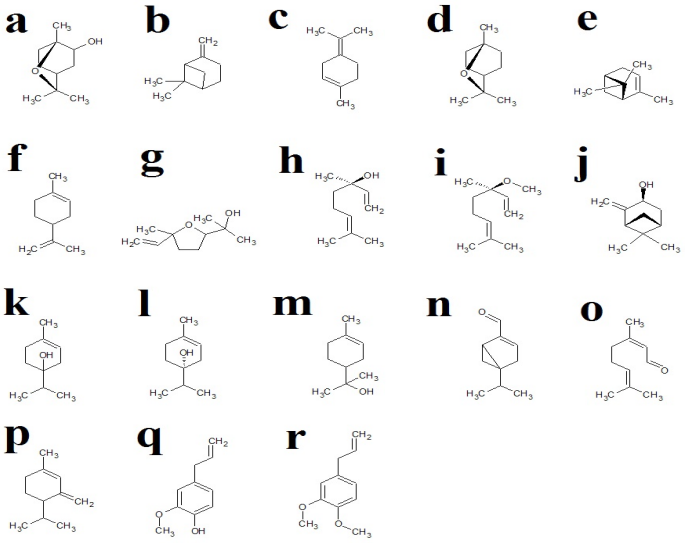

Figure 11: Monoterpenoid components of the SFE *K. senegalensis* extract: (a) 2-hydroxy-1,8-cineole, (b) β-pinene, (c) terpinolene, (d) 1,8-cineole, (e) α-pinene, (f) pseudolimonene, (g) linalool oxide, (h) linalool, (i) linalool, methyl ether, (j) pinocarveol, (k) δ-terpineol, (l) 1-terpinen-4-ol, (m) α-terpineol, (n) α-thujenal, (o) neral, (p) piperitone, (q) eugenol, (r) methyleugenol.

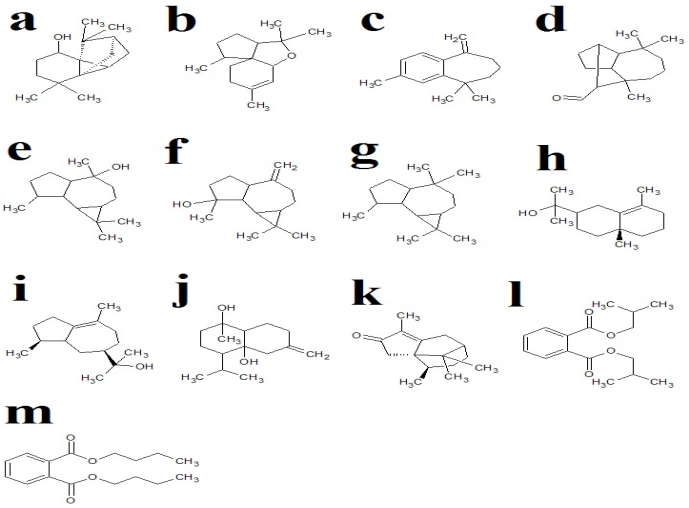

Figure 12: Sesquiterpenoid components of the SFE *K. senegalensis* extract: (a) cycloisolongifol-5-ol, (b) italicene ether, (c) α-dehydro-ar-himachalene, (d) longifolenaldehyde, (e) globulol, (f) spathulenol, (g) ledol, (h) γ-eudesmol, (i) guai-1(10)-en-11-ol, (j) isocalamenediol, (k) cyperenone, (l) phthalic acid, diisobutyl ester, (m) di-butyl phthalate.

idazole derivatives. None of these drugs is ideal as they produce unpleasant side effects including nausea, vertigo, vomiting, diarrhoea and hallucinations.[33,48] Furthermore, increasing reports of the failure of current treatments to address this disease indicates a developing drug resistance of several Giardia species.[48] 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 metronidazole.[33] Our studies demonstrate that *K. senegalensis* bark also possesses anti-Giardial activity. Whilst, further studies are required to identify the active component(s), previous studies have identified several similar terpenoids in plants[49] and marine sponges[50] which inhibit the growth of parasitic protozoans. The former of these studies is of particular note as it correlates trypanocidal activity with similar sesquiterpenoid lactones as those detected in our study.

Anti-proliferative activity against Caco-2 and HeLa carcinoma cell lines cells was noted for the *K. senegalensis* bark extracts (especially for the methanolic and SFE extracts), with IC_{50} values generally 150-500µg/mL. These findings support and extend previous studies examining the anticancer effects of other *K. senegalensis* extracts against different carcinoma cell lines. *K. senegalensis* stem bark solvent extracts significantly inhibited the growth of HCT-15, HT-29 and HCA-7 colorectal carcinoma cells.[6,14] This inhibition is comparable to the inhibition of the Caco-2 colorectal cells in our study. Furthermore, that study also examined the inhibitory mechanism and determined that the bark extracts had both antiproliferative and pro-apoptotic activities. Similarly, the growth of SiHa (cervical cancer cells) was inhibited by *K. senegalensis* stem bark extracts,[13] paralleling the HeLa cervical carcinoma proliferation results reported in our study. The previous study also examined the phytochemistry of the bark extracts and correlated the antiproliferative activity with limonoids. Interestingly, similar limonoids were not detected in the *K. senegalensis* bark extracts examined in our study. However, the GC-MS headspace analysis employed for phytochemical characterisation in our studies utilised a 45-450m/z mass range cut off. Thus, larger limonoid triterpenoid molecules would not have been detected under these conditions and may still be present. Our qualitative studies did detect relatively high triterpenoid levels, particularly in the most potent methanolic, aqueous and subcritical extracts. It is therefore likely that these extracts may contain substantial levels of similar limonoids, khayanolides and other triterpenoids as previously reported for the bark extracts.[13] Phytochemical isolation and identification studies using different analytical methods are required to confirm this.

Whilst this study provides insight into the phytochemical compositions of the *K. senegalensis* extracts, it is noteworthy that no single technique will detect and identify all compounds responsible for any therapeutic property in an extract. Our study utilised a GC-MS headspace technique to examine the extracts. This technique was chosen as many previous studies have highlighted the presence of terpenoid components in extracts produced using other *K. senegalensis* extracts (Table 1; Figure 1). As detection of these volatile, relatively nonpolar compounds is suited to GC-MS headspace analysis, this was deemed to be an appropriate analytical tool for this study. However, these extracts are likely to contain many more polar compounds that were not detected in this study. Thus, further studies using LC-MS analysis are required to further characterise the higher polarity components of the *K. senegalensis* bark extracts.

The results of this study indicate that the *K. senegalensis* bark 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. None of the *K. senegalensis* extracts displayed significant toxicity towards *A. franciscana*. Indeed, LC_{50} values substantially in excess of 1000µg/mL were measured for all extracts (extracts with LC_{50} values >1000 µg/mL in the *Artemia franciscana* nauplii bioassay are defined as being non-toxic).[37] Further evaluation of the antimicrobial and anticancer properties of these extracts is warranted. Likewise, bioactivity driven purification studies are needed to examine the mechanisms of action of the bioactive 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.

CONCLUSION

The results of this study partially validate the traditional usage of *K. senegalensis* extracts in multiple traditional African medicinal systems to

treat bacterial and protozoal diseases as well as some cancers. Bioactivity driven purifications of the active components and an examination of the mechanisms of action of these agents is warranted.

HIGHLIGHTS OF PAPER

• Methanolic and SFE *K. senegalensis* extracts were potent inhibitors of Proteus spp. growth (MICs approximately 200µg/mL).

• These extracts were also potent inhibitors of *K. pneumoniae* (MICs 170-400 µg/mL) and A. baylyi (MICs 200-700µg/mL).

• The methanolic and SFE extracts were moderate inhibitors of *P. aeruginosa* and *S. pyogenes* (MICs >1000µg/mL).

• The methanolic and SFE extracts were potent inhibitors of *Giardia duodenalis* proliferation (IC_{50} 187 and 328µg/mL, respectively).

• Proliferation of Caco-2 and HeLa carcinoma cells were inhibited by the methanolic and SFE *K. senegalensis* extracts with IC_{50} values 150-470µg/mL.

• All *K. senegalensis* bark extracts were non-toxic in the Artemia nauplii assay.

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CONFLICT OF INTEREST

None

ABBREVIATIONS USED

DMSO: Dimethyl sulfoxide; IC_{50}: The concentration required to achieve 50 % effect; LC_{50}: The concentration required to achieve 50 % mortality; MIC: minimum inhibitory concentration.

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