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The Potential of Selected South African Plants with Anti-Klebsiella Activity for the Treatment and Prevention of Ankylosing Spondylitis

I. E. Cock,[a,b*] S.F. van Vuuren[c]

[a]*Environmental Futures Research Institute and*

[b]*School of Natural Sciences, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia*

[c]*Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, Parktown, Gauteng, 2193, South Africa.*

**Author of correspondence: Dr. I. E. Cock:*

E-mail: I.Cock@griffith.edu.au

Tel: +61 7 37357637

ABSTRACT

A wide variety of herbal remedies are used in traditional African medicine to treat inflammatory disorders, including some autoimmune diseases. Thirty-four extracts from 13 South African plant species traditionally used for the treatment of inflammation were investigated for their ability to control a microbial trigger for ankylosing spondylitis (*K. pneumoniae*). Twenty six of the extracts (76.5 %) inhibited the growth of *K. pneumoniae.* Methanol and water extracts of *Ballota africana*, *Carpobrotus edulis* leaves, *Kigellia africana*, *Lippia javanica*, *Pelargonium fasiculata*, *Syzygium cordatum* (including bark), *Terminalia pruinoides* and *Terminalia sericea* were effective *K. pneumoniae* inhibitors, with MIC values < 1000 µg/ml. The roots of *Tulbaghia violaceae* and bark from *Warburgia salutaris* also demonstrated efficacy. The most potent extracts were examined by RP-HPLC and UV-Vis spectroscopy for the presence of resveratrol. Methanolic extracts of *B. africana*, *C. edulis* leaves, *L. javanica*, *T. pruinoides* and *T. sericea,* as well as aqueous *B. africana*, *T. pruinoides* and *T. sericea* extracts, displayed peaks with retention times and UV-Vis spectra consistent with the presence of resveratrol. Resveratrol was generally a minor component, indicating that resveratrol was not solely responsible for the anti-Klebsiella growth inhibitory properties. Plant extracts with *K. pneumoniae* inhibitory activity were either non-toxic, or of low toxicity in the Artemia (brine shrimp) nauplii bioassay. Their low toxicity and antibiotic bioactivity against *K. pneumoniae* indicate their potential for both preventing the onset of ankylosing spondylitis and minimising its symptoms once the disease is established.

Keywords: Ankylosing spondylitis, spondyloarthritis, *Klebsiella pneumoniae*, chronic inflammation, South African plants, resveratrol.

INTRODUCTION

Auto-immune inflammatory disorders (AIID's) are a group of debilitating conditions including rheumatoid arthritis, ankylosing spondylitis and lupus, which afflict genetically susceptible individuals. There is no common susceptibility profile for these disorders. Rheumatoid arthritis for example is most prevalent in middle aged to older women, whereas the onset of ankylosing spondylitis occurs most frequently in

younger males (Lawrence et al. 1998). There are currently no cures for any of these conditions so there is an urgent need to develop effective treatments which will not only alleviate the symptoms, but also prevent the disease onset and progression.

Ankylosing spondylitis (AS) is a form of chronic inflammatory arthritis which mainly affects the spinal joints and large synovial joints such as the sacroiliac joint in the pelvis. AS afflicts up to 0.9 % of the world's population (Rashid and Ebringer 2007), and accounts for 0.5-1.9 % of all types of spondyloarthritis (a similar prevalence to that of rheumatoid arthritis (RA)) (Braun et al. 1998). In contrast to RA which afflicts approximately three times as many women as men (Lawrence et al, 1998), AS has a significantly greater incidence in males (Rashid and Ebringer 2007). Also in contrast with RA, where onset generally occurs in middle to old age, the onset of AS predominantly occurs in the second and third decades of life (Braun and Sieper 2007; Rashid and Ebringer 2007). AS is characterized by spinal stiffness associated with inflammation and chronic back pain, which is often also referred to the buttocks and/or the back of the thigh. The disease may produce only mild to moderate symptoms in some individuals. In many sufferers it produces severe, disabling pain with substantial loss of function and mobility if not adequately treated. The chronic inflammation associated with AS often causes the formation of bridging syndesmophytes on the vertebrae due to ossification of the annulus fibrosis. These syndesmophytes may bridge the vertebrae and fuse them together, leading to the formation of 'bamboo spine'.

There is currently no known cure for AS. Current treatment strategies aim to either alleviate the symptoms (particularly pain and local tissue swelling) with analgesics and anti-inflammatory agents, and/or the use of disease modifying anti-rheumatic drugs (DMARDs). Alternatively, AS may be controlled through the use of biological therapies such as anti-tumour necrosis factor therapy (Braun et al, 2005). None of the current pharmacological treatments are ideal as prolonged usage of these drugs is often accompanied by significant side effects and toxicity (Alataha et al. 2003). In severe cases of AS, surgery to replace joints (particularly hip and knee joints) may also be required. Whilst surgical procedures are also available to repair spinal fusions, this is considered so risky that it is rarely considered except for very severe cases of spinal curvature (Braun et al. 2011). There is a need to find and develop safe,

effective drugs for the treatment of AS which will not only alleviate the symptoms, but which might also cure (or prevent) the disease. Greater understanding of the disease's etiology and progression should allow more relevant drug discovery and development.

AS is generally accepted to be an autoimmune disorder which may be triggered by specific microbial infections in genetically susceptible individuals (individuals with the MHC class 1 allele HLA-B27) (Rashid and Ebringer 2007). *K. pneumoniae* has been suggested as a possible trigger for the pathogenesis of AS based on several lines of evidence:

• Many studies have documented the presence of elevated serum levels of antibodies specific to *K. pneumoniae* in individuals suffering from AS (Ebringer et al. 2006; Blankenberg-Sprenkels et al. 1998; Chou et al. 1998; Maki-Ikola et al. 1997; Tani et al. 1997; Tiwana et al. 1997).

• Sera from rabbits immunised with HLA-B27 reacted significantly with *Klebsiella* spp. (Welsh et al. 1980).

• Sera from *Klebsiella*-immunised rabbits were able to distinguish between HLA-B27 positive lymphocytes from either AS patients or healthy controls and HLA-B27 negative lymphocytes obtained from healthy individuals (Baines et al. 1990).

• Amino acid sequence homologies have been identified between the 'QTDRED' sequence motifs present in both HLA-B27 and in the *K. pneumoniae* nitrogenase reductase enzyme (Schwimmbeck et al. 1987).

• Further peptide similarities were also identified between the 'DRED' motif in HLA-B27 and a 'DRDE' amino acid sequence in *K. pneumoniae* pullulanase enzyme, and between 'GxP' sequences in *K. pneumoniae* pullulanase and type I, III and IV collagens (which are located in the spine and large joints predominantly affected by AS) (Fielder et al. 1995).

K. pneumoniae is generally not pathogenic in most individuals unless aspirated, when it can cause severe inflammation in the respiratory tract, particularly in immune-compromised individuals. Alternatively, *K. pneumoniae* may also infect the urinary tract, biliary tract, wounds or gut lesions. Patients with invasive devices (e.g. urinary catheters or respiratory support equipment) are at an increased risk of infection from contaminated equipment. However, it is generally only when *K. pneumoniae* interacts

with the immune system (usually via gastrointestinal lesions) that it stimulates an immune response (Rashid and Ebringer 2007). *K. pneumoniae* infections can induce the production of anti-QTDRED and anti-DRED HLA-B27 antibodies as well antibodies against collagens I, III and IV. It has been postulated that these antibodies cross-react with self tissue in susceptible individuals, particularly in the spine and in large joints (Rashid and Ebringer 2007). This antibody binding activates a number of downstream processes including inflammatory cascades involving acute phase proteins, components of complement, vasoactive amines, as well as cytokines, tumour necrosis factor-α (TNF-α) and various other cytotoxic components (Rashid and Ebringer 2007). Furthermore, this antibody cross-reactivity activates further cellular processes, including those involving the action of natural killer cells (Braun and Sieper 2007). Initial infections often result in mild symptoms. Recurrent infections result in enhanced production of cross-reactive antibodies and intensification of the immunological reactions with ensuing tissue damage.

Many antibiotics are already known to inhibit *K. pneumoniae* (e.g. aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol). However, the development of super-resistant bacterial strains has reduced their value for treating many *K. pneumoniae* infections (Ashayeri-Panah et al. 2014; Nathisuwan et al. 2012). The search is ongoing for new antimicrobials, either by 1. design and synthesis of new agents, or 2. re-searching the repertoire of natural resources for as yet unrecognised or poorly characterised antimicrobial agents. The antiseptic qualities of medicinal plants have long been recognised by many cultures. Recently there has been a revival of interest in herbal medications due to perceptions that there is often a lower incidence of adverse reactions to natural endobiotic phytochemicals compared to synthetic xenobiotic pharmaceuticals.

In a previous study examining the potential of South African plants to prevent and treat rheumatoid arthritis (Cock and van Vuuren 2014), several South African plant species were identified which have been traditionally used for the treatment of various inflammatory disorders. Therefore a study was designed to 1. examine the same panel of Southern African medicinal plant species for their *in vitro* efficacy against the bacterial trigger of AS (*K. pneumoniae*), 2. To evaluate the toxicity of the plant extracts *in vivo*.

MATERIALS AND METHODS

Ethnobotanical information

Several literature resources were utilised to identify Southern African plants with a history of usage in the treatment of rheumatism and other inflammatory disorders for a previous study (Cock and van Vuuren, 2014). The same species were also examined in this study.

Plant collection and extraction

The majority of the plant species were collected from the Walter Sisulu Botanical Gardens in Johannesburg, South Africa (SA). *Syzygium cordatum* and *Lippia javanica* were obtained from Maputaland (KwaZulu-Natal, SA) and *Agathosma betulina* was collected from Landmeterskop (Southern Cape, SA). All plants were identified by Andrew Hankey, chief botanist at the Walter Sisulu Botanical Gardens in Johannesburg, SA or respective authorities in the other provinces. Voucher specimens were prepared and are stored at the Department of Pharmacy and Pharmacology, University of Witwatersrand, Johannesburg, SA. All plant materials were air dried and ground into a fine powder.

Extracts (1 g coarsely ground plant material) was prepared by adding either 50 ml of AR grade methanol or 50 ml distilled water. Plant material was extracted in each solvent for 24 hours at 4 ^{o}C with gentle shaking. The extracts were filtered through filter paper (Whatman No. 54). The methanol extracts were subsequently allowed to dry at room temperature. The aqueous extracts were frozen at -70 ^{o}C and dried by lyophilisation. These dried extracts were then weighed and redissolved in 10 ml deionised water.

Antibacterial screening

Test micro-organisms

A reference strain of *Klebsiella pneumoniae* (ATCC 13883) was obtained from American Tissue Culture Collection (ATCC) and subcultured and maintained in nutrient broth (Oxoid Ltd, UK) at 4 ^{o} C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion method (Cock 2009; Cock and Mohanty 2011;). Briefly, 100 µl of the test bacteria were grown in 10 ml of fresh nutrient broth until they reached a count of approximately 10^8 cells/ml. One hundred microliters of this microbial suspension was spread onto nutrient agar plates.

The extracts were tested using 5 mm sterilised filter paper discs impregnated with 10 µl of the test sample, then allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 °C for 2 hours, allowing for prediffusion of the extract into the agar before incubation with the test microbial agents. The plates were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed at least in triplicate and mean values were determined. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) (Oxoid Ltd, UK) served as positive controls for antimicrobial activity. Filter discs impregnated with either 10 µl of distilled water or methanol were used as negative controls.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the plant extracts were determined by the disc diffusion MIC method (Cock and Kukkonen 2011; Vesoul and Cock 2011) across a range of different concentrations. Briefly, the plant extracts were serially diluted in deionised water across a range of concentrations. Discs were impregnated with 10 µl of the test dilutions, allowed to dry and placed onto inoculated plates. Graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity screening

Reference toxin

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Saarchem, South Africa) was prepared as a 1.6 mg/ml solution in distilled water and serially diluted in artificial seawater for use in the *A. franciscana* nauplii bioassay.

Artemia franciscana (brine shrimp) nauplii toxicity screening

A. franciscana cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Artificial seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/l distilled water were prepared prior to use. Atremia nauplii were prepared by standard methods (Sautron and Cock 2014; Winnett et al. 2014). Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay (Sirdaarta and Cock 2010; Sirdaarta and Cock 2008). Briefly, a volume of 400 µl of seawater containing approximately 37 (mean = 37.2, n = 132, SD = 11.6) nauplii were added to wells within a 48 well plate and immediately used for the bioassay. The plant extracts were first diluted to 4 mg/ml in seawater and then used at a 2 mg/ml concentration in the bioassay. A volume of 400 µl of diluted plant extract and the reference toxin were transferred to the wells and incubated at 25 ± 1 $^{\circ}$C under artificial light (1000 Lux). A negative control (400 µl seawater) was included in triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead brine shrimp counted. The nauplii were considered moribund if no movement of the appendages was observed within 10 sec. After 24 h, all nauplii were sacrificed (by the addition of 50 µl glacial acetic acid) and counted to determine the total number per well. The LC_{50} with 95 % confidence limits for each treatment was calculated using Probit analysis.

Reverse phase high performance liquid chromatography (RP-HPLC)

Gradient RP-HPLC was performed at a flow rate of 1 mg/ml using a Dionex Ultimate 3000 pump and gradient system, an Ultimate 3000 variable wavelength programmable photodiode array detector (Dionex) and a Phenomenex reverse phase C_{18} column (150 mm x 4.6 mm). The HPLC system was equipped with Chromeleon software (Dionex). The gradient was constructed as follows: 5 min elution with 5 % acetonitrile, followed by a 30 min gradient to 65 % acetonitrile. The system was then re-equilibrated to 5 % acetonitrile for subsequent chromatograms. The column temperature was maintained at 20 °C throughout. Plant extract samples (10 µl) were injected manually. Resveratrol (Sigma) was dissolved in ethanol (AR grade), diluted in deionized water and 10 µl aliquots of various concentrations were injected as a standard to calibrate the HPLC column. Deionised water (10 µl) was used as a negative control to produce the baseline curve and zero the equipment prior to each experiment.

Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments.

RESULTS

Liquid extraction yields

Extraction of 1 g of the dried plant materials with methanol and water yielded dried plant extracts with a wide range of masses, from 6 mg (*W. salutaris* bark water extract) to 284 mg (*P. obliquum* leaf methanolic extract) (Table 1). As a measure of the efficacy of the main forms in which these plants are traditionally used in ethnobotanical medicinal systems (decoctions and tinctures), methanolic and aqueous extracts were tested undiluted for their ability to inhibit the growth of *K. pneumoniae* There was no obvious trend with regards to which solvent was more efficient at extracting material from the plant samples. Methanol extracted a greater amount of material for seven of the sixteen plant samples tested (44 %), whereas water extracted the greater amount of material for eight of these samples (50 %). One source provided equal masses of extract with both water and methanol. No obvious correlation was noted between the plant part tested and the amount of extracted material. For two plant species (*Syzygium cordatum* and *Warburgia salutaris*), both bark and leaf materials were extracted. Approximately 5 times as much material was extracted from *Syzygium cordatum* bark than from its leaves by methanol. Similarly, water extracted twice as much material from the bark than from the leaves. In contrast, much more material was extracted from *Warburgia salutaris* leaves than from the bark by both methanol and water (approximately 8 fold and 2 fold higher respectively). With another plant (*Tulbaghia violaceae*), leaf and root materials were extracted and tested. Methanol extracted significantly more material from *Tulbaghia violaceae* leaves than from the roots. However, water extracted much more material from *Tulbaghia violaceae* roots than from the leaves. All dried extracts were resuspended in 10 ml of deionised water, yielding the concentrations shown in Table 1.

Antibacterial activity

Bacterial growth was susceptible to inhibition by a wide range of plant extracts. Indeed, the only plant materials for which neither the methanol or water extracts inhibited *K. pneumoniae* growth were the *C. edulis* fruit, *P. viridiflorum* leaf and *W.*

salutaris bark extracts. *K. pnuemoniae* was inhibited by 26 of the 34 (77 %) extracts tested. Zones of inhibition >10 mm were considered to indicate significant antibiotic activity (Figure 1). The most effective species for the inhibition of *K. pneumoniae* growth was *S. cordatum* (based on the zones of inhibition). Indeed, methanolic and aqueous extracts of bark and leaf *S. cordatum* extracts displayed significantly more potent *K. pneumoniae* growth inhibition than either of the control antibiotics (ampicillin and chloramphenicol). The *S. cordatum* bark methanolic extract was a more potent inhibitor of *K. pneumoniae* growth than was the corresponding aqueous extract with zones of inhibition of 14.3 ± 0.6 mm and 13.0 ± 0 mm respectively. In contrast, the aqueous *S. cordatum* leaf extract was more potent than the corresponding methanolic extract based on the zones of inhibition (13.3 ± 0.6 mm and 9.4 ± 0.6 mm respectively.

T. pruinoides and *T. sericea* methanol extracts (12.7 ± 0.6 mm and 13.0 ± 0 mm respectively) also were more potent inhibitors of *K. pneumoniae* growth than were the corresponding aqueous extracts (11.6 ± 0.6 mm and 12.0 ± 1.0 mm for the *T. pruinoides* and *T. sericea* aqueous extracts respectively). The *C. edulis* leaf methanol (11.7 ± 0.6 mm) and aqueous (10.3 ± 0.6 mm) extracts and the *L. javanica* methanol extract (11.7 ± 0.6 mm) also displayed promising inhibitory activity against *K. pneumoniae*. The *A. betulina*, *B. africana*, *K. africana*, *P. fasiculata*, *T. violaceae* (particularly the root extracts) and the *W. salutaris* leaf extracts were less potent.

The relative level of antibacterial activity was further quantified by determining the MIC values for each extract against *K. pneumoniae* (Table 1). The *S. cordatum*, *T. pruinoides* and *T. sericea* extracts were again the most potent inhibitors of *K. pneumoniae* growth, with MIC values generally between 250 and 500 µg/ml (between 2.5 and 5 µg impregnated into the disc). As these are crude extracts, these MIC values compare favourably with inhibition by the ampicillin and chloramphenicol antibiotic controls (2 µg and 10 µg respectively). The *C. edulis* leaf and *L. javanica* extracts were also potent growth inhibitors, with MIC values generally below 700 µg/ml.

Interestingly, the *P. fasiculata, B. africana, T. violaceae* and *W. salutaris* methanolic and aqueous leaf extracts and the *K. africana* aqueous extract displayed potent inhibition, with MIC values generally below 700 µg/ml (7 µg impregnated into the

disc). The potent inhibitory activities of the extracts seen by MIC determination are in contrast to the lower efficacy seen in the disc diffusion screening. This may indicate that the antimicrobial compounds in these extracts may be either large or of low polarity and therefore diffuses less freely in the agar gel. The *K. africana* methanolic extract and the methanolic and aqueous extracts of *A. betulina* and *P. obliquum* also displayed significant growth inhibitory activity towards *K. pneumoniae*, but were substantially less potent, with MIC values generally >1000 µg/ml.

Quantification of toxicity

The plant extracts were serially diluted in artificial seawater for mortality determenation in the Artemia nauplii lethality bioassay. Potassium dichromate (the reference toxin) was also tested in the bioassay for comparison. Figure 2 shows the % mortality induced in this bioassay following 24 of exposure to the reference toxin or the plant extracts. Potassium dichromate rapidly induced mortality within 3 hours of exposure (results not shown). After 24 hours exposure, potassium dichromate had killed 100 % of the Artemia nauplii. At 24 hours, the only extracts to induce mortality significantly above that of the seawater control were the aqueous extracts of *P. fasiculata*, *T. violaceae* root and leaf water extracts.

To compare the toxicity of those plant extracts showing significant mortality with other toxins, the LC_{50} values of the extracts and the reference toxin were determined by testing across a range of concentrations in the Artemia nauplii bioassay. Table 1 shows the LC_{50} values of the extracts which had shown toxicity towards *A. franciscana*. Whilst the *P. fasiculata* leaf water extract initially appeared toxic in the extract mortality screening study (Figure 2), determination of its LC_{50} demonstrates that it is non-toxic as LC_{50} values of $\geq$ 1000 µg/ml have previously been defined as non-toxic in the Artemia nauplii bioassay (Ruebhart et al. 2009; Cock 2008). The *T. violaceae* root and leaf water extracts each displayed 24 hour LC50 values of approximately 800 µg/ml, which is considered to be low toxicity.

RP-HPLC analysis of extracts displaying antimicrobial activity

The *K. pneumoniae* growth inhibition and MIC studies indicated that the *B. africana*, *C. edulis* leaf, *L. javanica* leaf, *S. cordatum* bark, *S. cordatum* leaf, *T. pruinoides* leaf and *T. sericea* leaf extracts were the most promising extracts for further phytochemical characterisation. These were examined for the presence of trans–3,4,5-trihydroxy-trans-stilbene (resveratrol). Resveratrol is a potent phenolic anti-inflammatory antioxidant produced as a phytoalexin (plant antibiotic) by many diverse plant species including grapes, berries, peanuts as well as some species of pine trees, *Acacia* spp., *Terminalia* spp. (including *T. sericea*), orchids and lilies (Joseph et al, 2007). The methanolic and aqueous extracts of the plant species displaying the most potent *K. pneumoniae* growth inhibitory activity were further analysed by RP-HPLC chromatography to determine whether they contain resveratrol. However, resveratrol itself did not show *K. pneumoniae* growth inhibitory activity at levels up to 1000 µg/ml.

The *B. africana* methanol (Figure 3a) and aqueous extracts (Figure 3b) both displayed peaks at the same elution volume as pure resveratrol (Figure 3c). An examination of the UV visual spectra of these peaks showed that the spectra were consistent with that of pure resveratrol (Figure 3d), with absorption maxima at approximately 216 and 303 nm. However, resveratrol was present as only a minor component in both *B. africana* extracts.

Similarly, *C. edulis* and *L. javanica* leaf methanolic extracts displayed peaks at similar elution volumes and with similar UV visual spectra to pure resveratrol. In contrast to the *B. africana* extracts, only the *C. edulis* and *L. javanica* methanolic extracts (Figures 4a and 4c respectively) showed peaks at similar elution volumes as pure resveratrol. The UV visual spectra of these peaks were consistent with the authentic resveratrol spectra (Figure 3d). Resveratrol was not detected in either the *C. edulis* leaf aqueous extract (Figure 4b), or the *L. javanica* aqueous extract (Figure 4d). As the aqueous extracts of both species had similar MIC values as the corresponding methanolic extracts, it is unlikely that resveratrol is solely responsible for the *K. pneumoniae* inhibitory activity.

None of the *S. cordatum* extracts (Figures 5a-d) displayed chromatographic peaks consistent with authentic resveratrol. This is noteworthy as the *S. cordatum* extracts were amongst the most potent *K. pneumoniae* growth inhibitors (as judged by the zones of inhibition and MIC analyses). It is evident that resveratrol is not responsible for the direct anti-inflammatory properties, or for the ankylosing spondylitis preventative/*K. pneumoniae* inhibitory properties of *S. cordatum*. In contrast, *T. pruinoides* and *T. sericea* methanolic and aqueous extracts (Figures 6a-d) all displayed peaks at the same elution volume and with similar UV-Vis spectra to pure resveratrol. However, as with the *B. africana* (methanolic and aqueous extracts), *C. edulis* and *L. javanica* (methanolic) extracts, resveratrol was a minor component of the *Terminalia* spp. extracts, with only minor peaks evident.

DISCUSSION

Traditional plant-based medicines have been used for thousands of years by indigenous populations worldwide. Many of these have a rich tradition of herbal medicine and multiple plants have been used to treat inflammation. Whilst numerous plant species (with a history of traditional use in the treatment of inflammatory diseases) have been screened *in vitro*, the vast majority of these studies have focussed on their ability to inhibit the production of cytokines and other inflammatory mediators. Very few studies have examined the ability of traditional medicinal plants to block the initiation phases of autoimmune inflammatory diseases, including AS. In a recent study we identified a variety of South African plants with traditional uses in the treatment of inflammation and examined their potential in the treatment of rheumatoid arthritis (Cock and van Vuuren 2014). This study examined the same panel of plants for their antibiotic activity against the microbial trigger (*K. pneumoniae*), another chronic inflammatory disease (AS).

Figure 7 summarises the proposed progression of AS as an inflammatory disease, from the initial pathogenic events through to the symptoms (the numbers in Figure 7 and in the text below refer to the current and/or proposed treatment targets). Gastrointestinal *K. pneumoniae* may act as a trigger for AS (Rashid and Ebringer

2007); so limiting the burden of gastrointestinal *K. pneumoniae* (1) would help prevent AS intiation and minimize its downstream effects. Gastrointestinal *K. pneumoniae* cannot interact with the immune system unless (2) epithelial lesions (or other epithelial interruptions) allow for the production of anti-*K. pneumoniae* antibodies (Rashid and Ebringer 2007). Inhibition of any agents causing lesions of the intestine (e.g. Crohn's disease) might also block AS initiation. Blocking the interaction of *K. pneumoniae* with immunological cells (3) or the subsequent immune response (4) would also reduce the production of self-reactive antibodies and also diminish the later phase of AS pathogenesis and the consequent disease symptoms. However, immunomodulatory therapy should be used with caution as inhibiting the patient's immune capability also exposes the patient to a variety of other infections. Most current AS therapies target the later phase events (5) by blocking the inflammatory cascades or at stages (6 and 7) as palliatives by decreasing the symptoms of AS (e.g. pain, swelling, heat). Drugs primarily targeting these later events effectively ease patient discomfort, although they do little to control the tissue damage (associated with the self-reactive antibodies). Targeting earlier events prior to the induction of the immune response would not only alleviate the symptoms and discomfort of AS, but would also lessen/prevent the joint damage associated with bacterial initiated chronic inflammation.

A treatment regime targeting the microbial trigger of AS is an attractive prospect as it would be expected to block/decrease production of self-reactive antibodies linked to disease onset and progression. The results presented here show the ability of a variety of plants used traditionally to treat rheumatism and other inflammatory complaints in Southern Africa to inhibit *K. pneumoniae* growth when tested in the format used in traditional medicine (i.e. decoctions and tinctures). Further quantification by MIC determination demonstrated the efficacy of these extracts as inhibitors of one possible microbial trigger of AS. Methanol and water extracts of *B. africana*, *C. edulis* leaf, *L. javanica*, *P. viridiflorum*, *S. cordatum* leaf and bark, *T. pruinoides*, *T. sericea*, *T. violaceae* roots and leaves and *W. salutaris* bark were of particular interest where the MIC values of approximately 300 µg/ml for several extracts i.e. were substantially more potent than the reference antibiotics (ampicillin and chloramphenicol). They are

thus worthy of further phytochemical and mechanistic studies. Many of the plant extracts studied here may also affect other inflammatory processes (e.g. cytokine release) and therefore may help control AS by pluripotent/multifaceted mechanisms.

Herbal remedies have long been used to alleviate rheumatic complaints in all traditional medicine systems. In many cases, the major anti-inflammatory components of these plants are available in a pure form and already available as anti-inflammatory agents. These compounds include a variety of polyphenolic compounds, of which 3,5,4'-trihydroxy-trans-stilbene (resveratrol) has received much recent attention due to its presence in many plants used in the treatment of inflammation, and due to its potent anti-inflammatory activity (Elmali et al. 2007; Wang et al. 2006). Most studies examining the role of resveratrol in the treatment of inflammatory conditions focus on its ability to act as a potent and specific inhibitor of NF-κB activation, the inducer for two major inflammatory cytokines, TFN-α and IL-1β (Elmali et al. 2007). It is possible that resveratrol may also have other anti-inflammatory effects as it also exhibits antibacterial activity against a variety of bacterial species (Paulo et al. 2010). Of the plant extracts examined in this study, *B. africana* (methanol and aqueous extracts), *C. edulis* leaf (methanol extract), *L. javanica* (methanol extract) and the methanol and aqueous extracts of both *Terminalia* species (*T. pruinoides* and *T. sericea*) contained resveratrol at comparatively low levels. In contrast, *S. cordatum* extracts did not contain discernable levels of resveratrol. Significantly, resveratrol did not inhibit the growth of *K. pneumoniae* in our test systems, even at concentrations up to 1000 µg/ml i.e. it was not an antibiotic for this micro-organism (personal unpublished results).

However, it is possible that resveratrol may still contribute to the anti-inflammatory activities of these extracts. Resveratrol glycosides may be present and target cells may metabolise these to release resveratrol. 2-[3-Hydroxy-5[(E)-2-(4-hydroxyphenyl) ethenyl] phenoxy]-6-(hydroxymethyl) oxane-3,4,5-triol (piceid) and polydatin are glycosylated stilbenes that have both been shown to block inflammation by decreasing IL-17 production in stimulated human mononuclear cells (Lanzilli et al. 2012). These glycosylated stilbenes may possibly block the later phases of inflammation by similar mechanisms. Another study (Jayatilake et al. 1993) indicated

that the antibacterial activity of resveratrol is due to a protein tyrosine kinase activity. These authors also reported that resveratrol glycosides do not have the same bacterial inhibitory activity, indicating that free hydroxyl groups on both phenyl groups are required for antibacterial activity.

Other stilbenes might be present, even if resveratrol is lacking in an extract. Indeed several stilbenes and stilbene glycosides (including resveratrol glycosides) have been identified in *T. sericea* (Joseph et al. 2008). A number of other anti-inflammatory stilbenes are found in other plant species. For example, 2,3,4,5-tetrahydroxystilbene-2-O-β-D-glucoside (TSG) inhibits inflammation by suppressing the induction of pro-inflammatory mediators by reducing NF-κB binding to DNA (Huang et al. 2013). The same study detected TSG in numerous herbs used to treat inflammation in Chinese traditional medicine. Furthermore, nine stilbene and stilbene derivatives isolated from the roots of *Cicer* spp. (chickpeas) were shown to inhibit bacterial and fungal growth (Aslam et al. 2009).

Whilst future studies may detect stilbene compounds in *S. cordatum* extracts and in other extracts with *K. pneumoniae* growth inhibitory activity, it is likely that other phytochemical classes also contribute to the anti-inflammatory properties of these extracts. Alkaloids, anthraquionones, flavonoids, polyphenolics, phytosterols, saponins, tannins and terpenes have also been linked with anti-bacterial activity in different plant species and thus may be responsible (at least in part) for the anti-*K. pneumoniae* activities reported here. The terpenes isomenthone and diosphenol as found in *A. betulina* are well known for their antimicrobial and urinary tract disinfectant properties (van Wyk et al. 2009). *S. cordatum* extracts contain pentacyclic triterpenoids, phytosterols, proanthrocyanidins and tannins (van Wyk et al. 2009), any of which could be responsible for antimicrobial and anti-inflammatory properties. *Terminalia* spp. and *W. salutaris* are also high in sesquiterpenoids (including polygodial) and tannins having antibacterial properties (van Wyk et al. 2009). Similarly, the volatile oils from *L. javanica* and *Pellargonium* spp. are rich in monoterpenoids with strong antibacterial and anti-inflammatory properties (van Wyk et al. 2009). Furthermore, the oils from *Pellargonium* spp. are rich in sesquiterpenes and alkaloids. The terpenoid and saponin extracts of *P. viridiflorum* have both antimicrobial and anti-inflammatory activity (van Wyk et al. 2009). Several *C. edulis*

and *K. africana* flavonoids have documented antimicrobial activity (van Wyk et al. 2009). Further bioactivity-guided separation studies are recommended to identify the anti-*K. pneumoniae* compounds in these plant extracts.

The findings reported here also demonstrate that the majority of extracts tested were non-toxic in the *Artemia* nauplii bioassay. Only the *P. fasiculata* leaf water extract, and the *T. violaceae* root and leaf water extracts induced mortality significantly above that of the negative control. Determination of the LC_{50} values indicated that only *T. violaceae* extracts displayed LC_{50} values below 1000 µg/ml. Therefore all the other active plant extracts can be considered as non-toxic. Compounds with an LC_{50} of greater than 1000 µg/ml towards Artemia nauplii have been previously defined as being non-toxic (Ruebhart et al. 2009). Even the *T. violaceae* root and leaf water extracts (with LC_{50} values > 700 µg/ml) are considered low in toxicity. It was therefore determined that all extracts tested here were either non-toxic or of low toxicity.

CONCLUSIONS

The antimicrobial efficacies of these plants against the pathogenic microbe *K. pneumoniae* help validate the usage of these particular plants in traditional South African medicinal systems, especially to treat autoimmune inflammatory disorders including ankylosing spondylitis and related inflammatory conditions. They also indicate that the phytochemistry and pharmacology of these plants certainly deserve further study, particularly for any other activities associated with the treatment of ankylosing spondylitis (e.g. inhibition of cytokine production). Further toxicity studies using human cell lines are needed to verify the acceptability of the toxicity reported herein should further clinical trials be considered.

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Figure 1: Antibacterial activity of aqueous and methanolic plant extracts measured as zones of inhibition (mm) against *K. pneumoniae*. Inhibition zones are represented as the means of at least triplicate experiments ± SEM. W = water extract, M = methanolic extract, B = bark, L = leaf. Ampicillin (2 µg) and chloramphenicol (10 µg) controls were included for comparison.

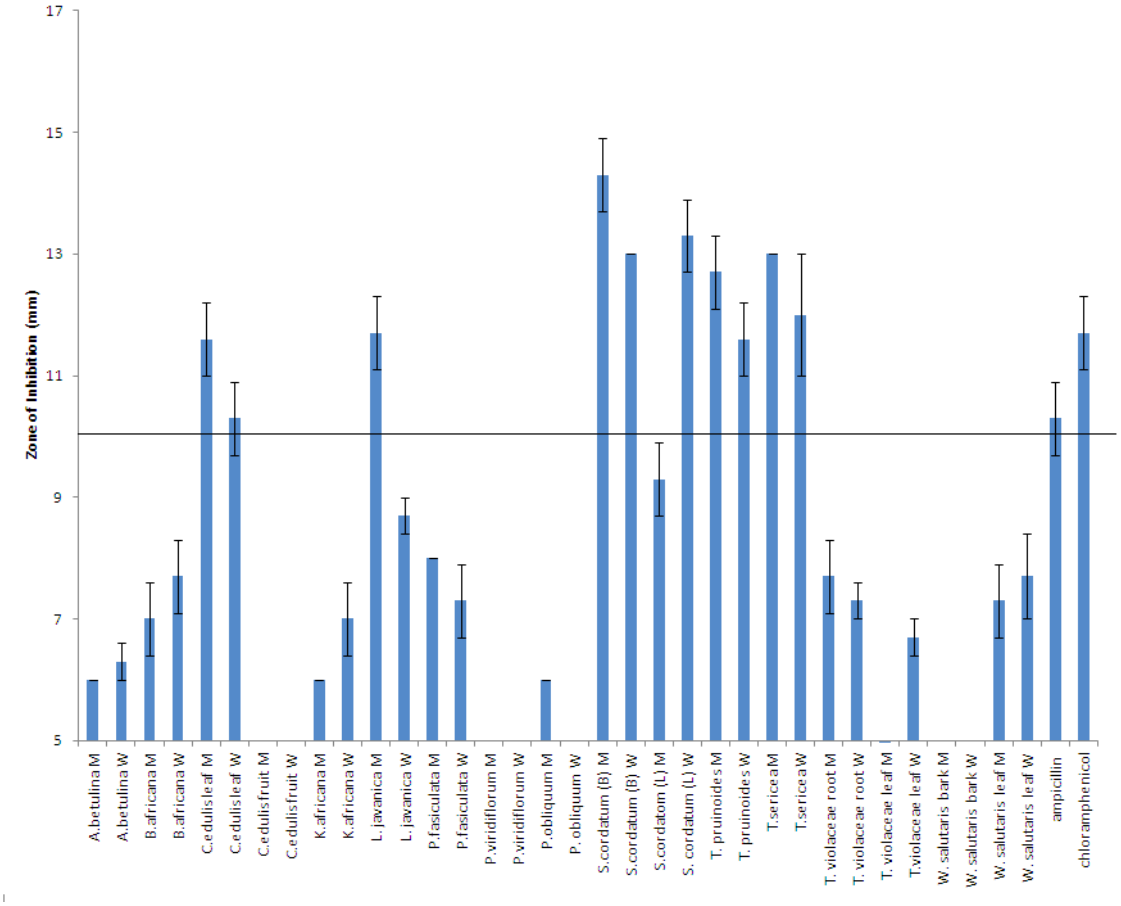

Figure 2: The lethality of plant extracts (2000 µg/mL) and potassium dichromate control (1000 µg/mL) towards Artemia nauplii after 24 hours exposure. All bioassays were performed in triplicate and the means ± SEM are shown here. 1 = *A. betulina* leaf methanolic extract; 2 = *A. betulina* leaf water extract; 3 = *B. africana* leaf methanolic extract; 4 = *B. africana* leaf water extract; 5 = *C. edulis* leaf methanolic extract; 6 = *C. edulis* leaf water extract; 7 = *K. africana* leaf methanolic extract; 8 = *K. africana* leaf methanolic extract; 9 = *L. javanica* leaf methanolic extract; 10 = *L. javanica* leaf water extract; 11 = *P. fasiculata* leaf methanolic extract; 12 = *P. fasiculata* leaf water extract; 13 = *P. viridiflorum* leaf methanolic extract; 14 = *P. viridiflorum* leaf water extract; 15 = *P. obliquum* leaf methanolic extract; 16 = *P. obliquum* leaf water extract; 17 = *S. cordatum* bark methanolic extract; 18 = *S. cordatum* bark water extract; 19 = *S. cordatum* leaf methanolic extract; 20 = *S. cordatum* leaf water extract; 21 = *T. pruinoides* leaf methanolic extract; 22 = *T. pruinoides* leaf water extract; 23 = *T. sericea* leaf methanolic extract; 24 = *T. sericea* leaf water extract; 25 = *T. violaceae* root methanolic extract; 26 = *T. violaceae* root water extract; 27 = *T. violaceae* leaf methanolic extract; 28 = *T. violaceae* leaf water extract; 29 = *W. salutaris* bark methanolic extract; 30 = *W. salutaris* bark water extract; 31 = *W. salutaris* leaf methanolic extract; 32 = *W. salutaris* leaf water extract; 33 = potassium dichromate control; 34 = deionised water control.

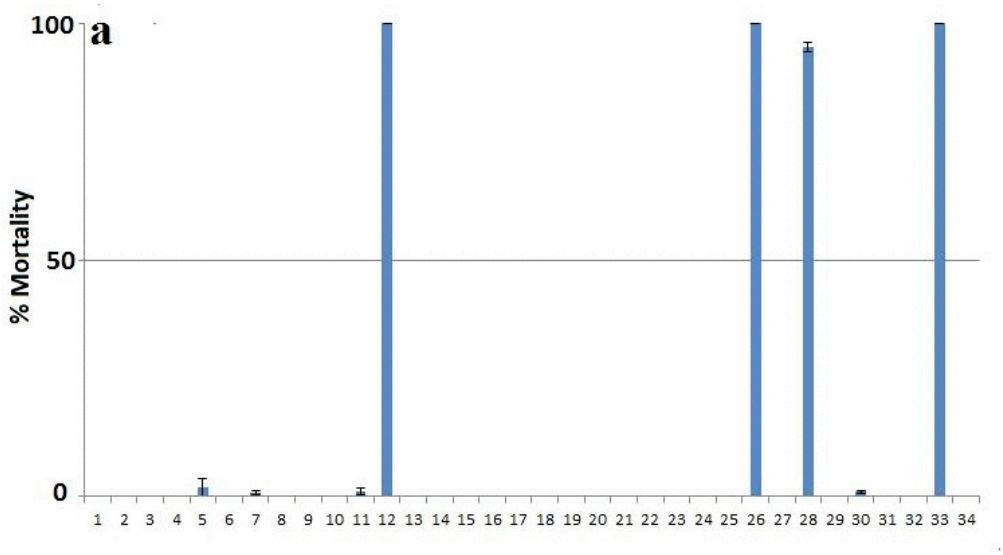

Figure 3: RP-HPLC chromatogram of 10 µl injections of (a) *B. africana* methanolic extract, (b) *B. africana* aqueous extract, (c) resveratrol standard and (d) a representative resveratrol UV/Vis spectrum. Extracts were dried and resuspended in deionised water. Absorbance was recorded at 305 nm and the arrows indicate the resveratrol peaks.

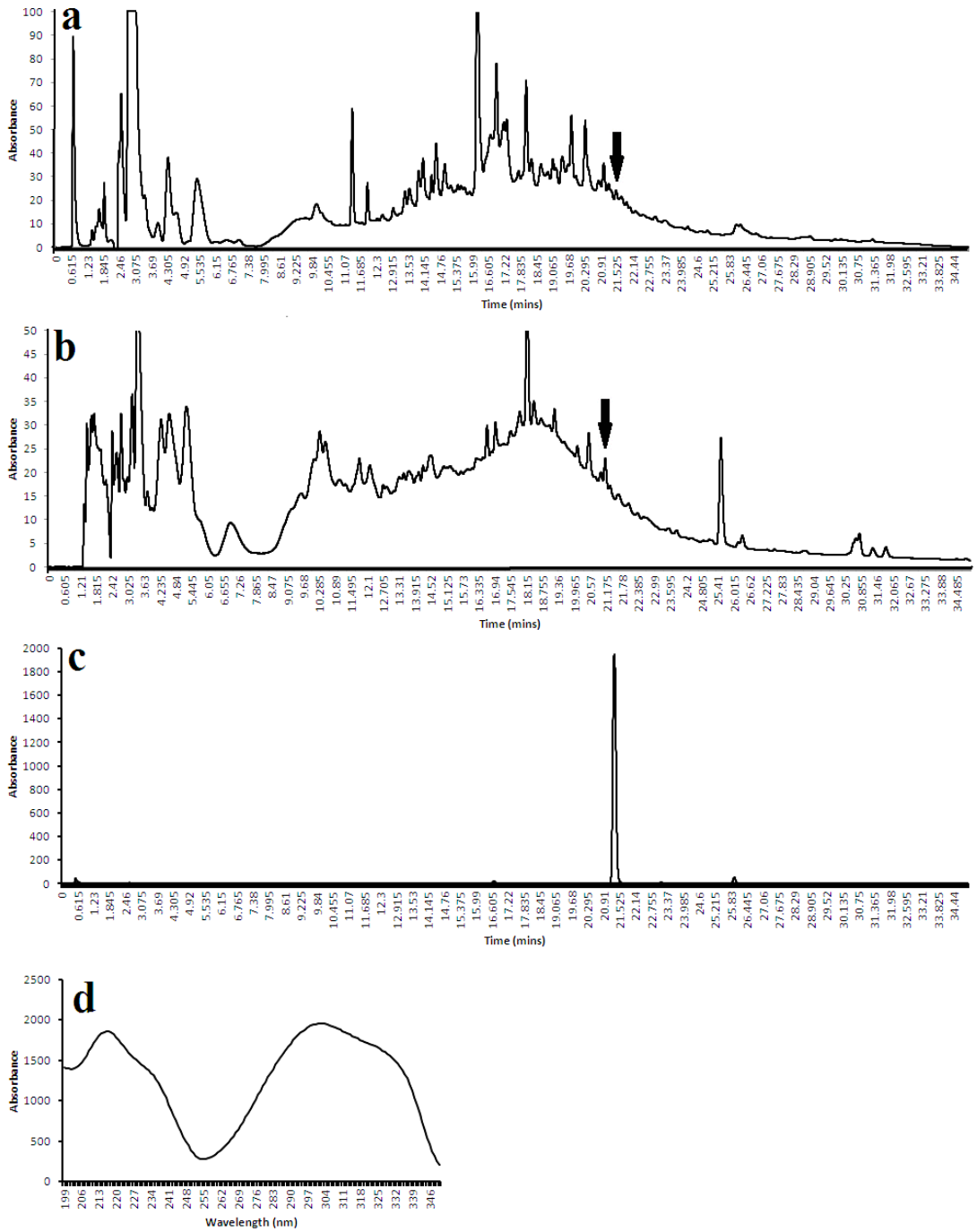

Figure 4: RP-HPLC chromatogram of 10 µl injections of (a) *C. edulis* leaf methanolic extract, (b) *C. edulis* leaf aqueous extract, (c) *L. javanica* methanolic extract, (d) *L. javanica* aqueous extract. Extracts were dried and resuspended in deionised water. Absorbance was recorded at 305 nm and the arrows indicate the resveratrol peaks.

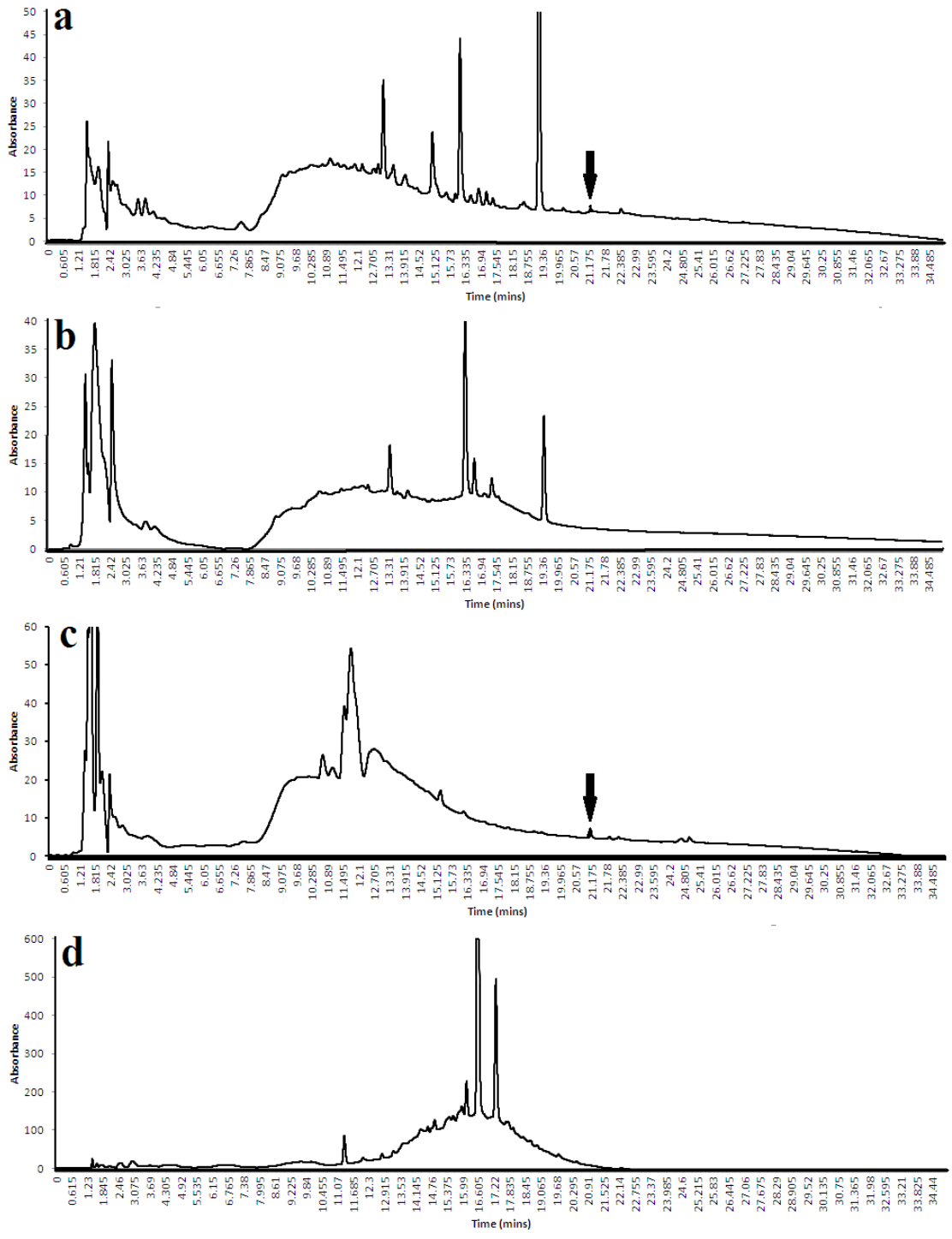

Figure 5: RP-HPLC chromatogram of 10 µl injections of (a) *S. cordatum* bark methanolic extract, (b) *S. cordatum* bark aqueous extract, (c) *S. cordatum* leaf methanolic extract, (d) *S. cordatum* leaf aqueous extract. Extracts were dried and resuspended in deionised water. Absorbance was recorded at 305 nm.

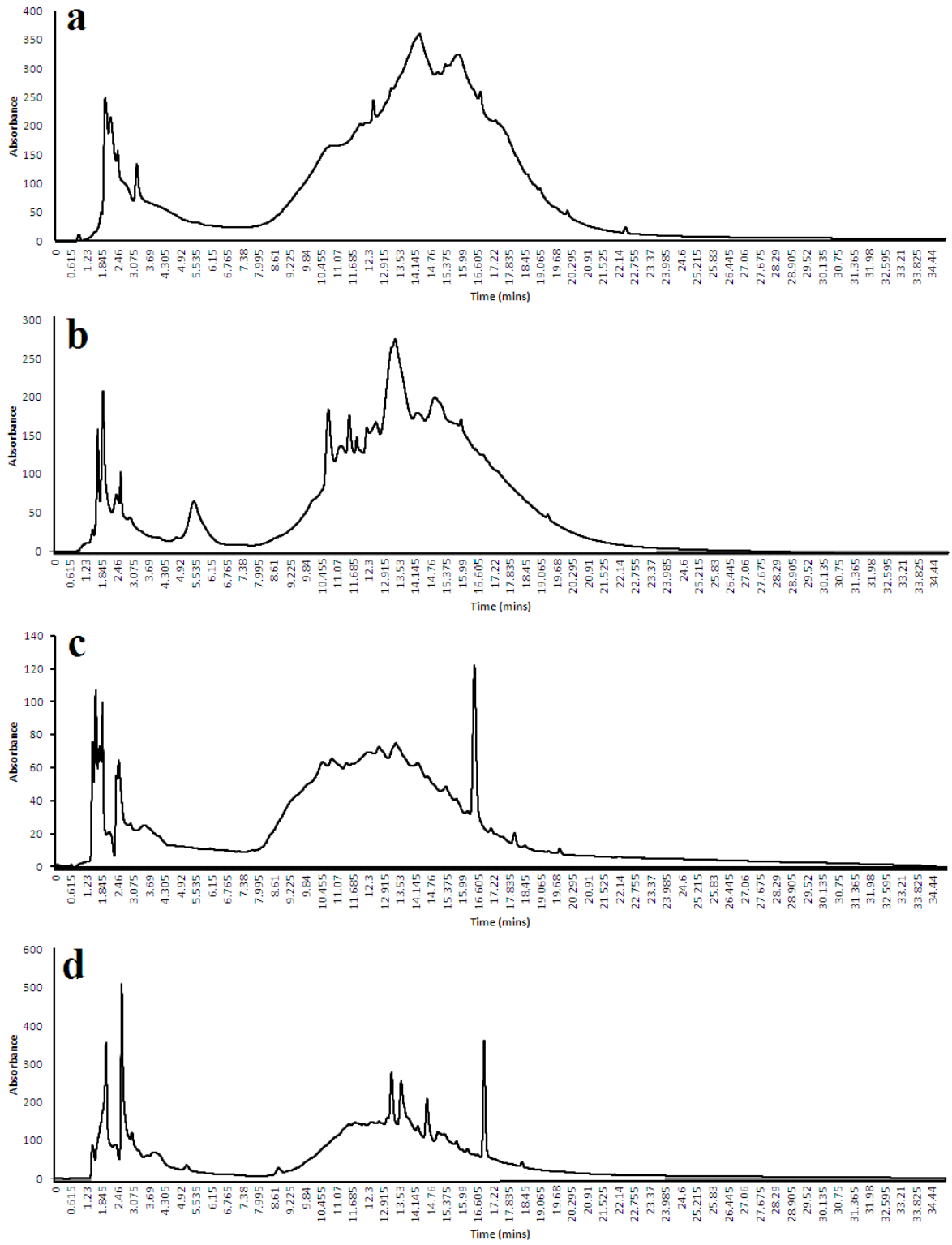

Figure 6: RP-HPLC chromatogram of 10 µl injections of (a) *T. pruinoides* methanolic extract, (b) *T. pruinoides* aqueous extract, (c) *T. sericea* methanolic extract, (d) *T. sericea* aqueous extract. Extracts were dried and resuspended in deionised water. Absorbance was recorded at 305 nm and the arrows indicate the resveratrol peaks.

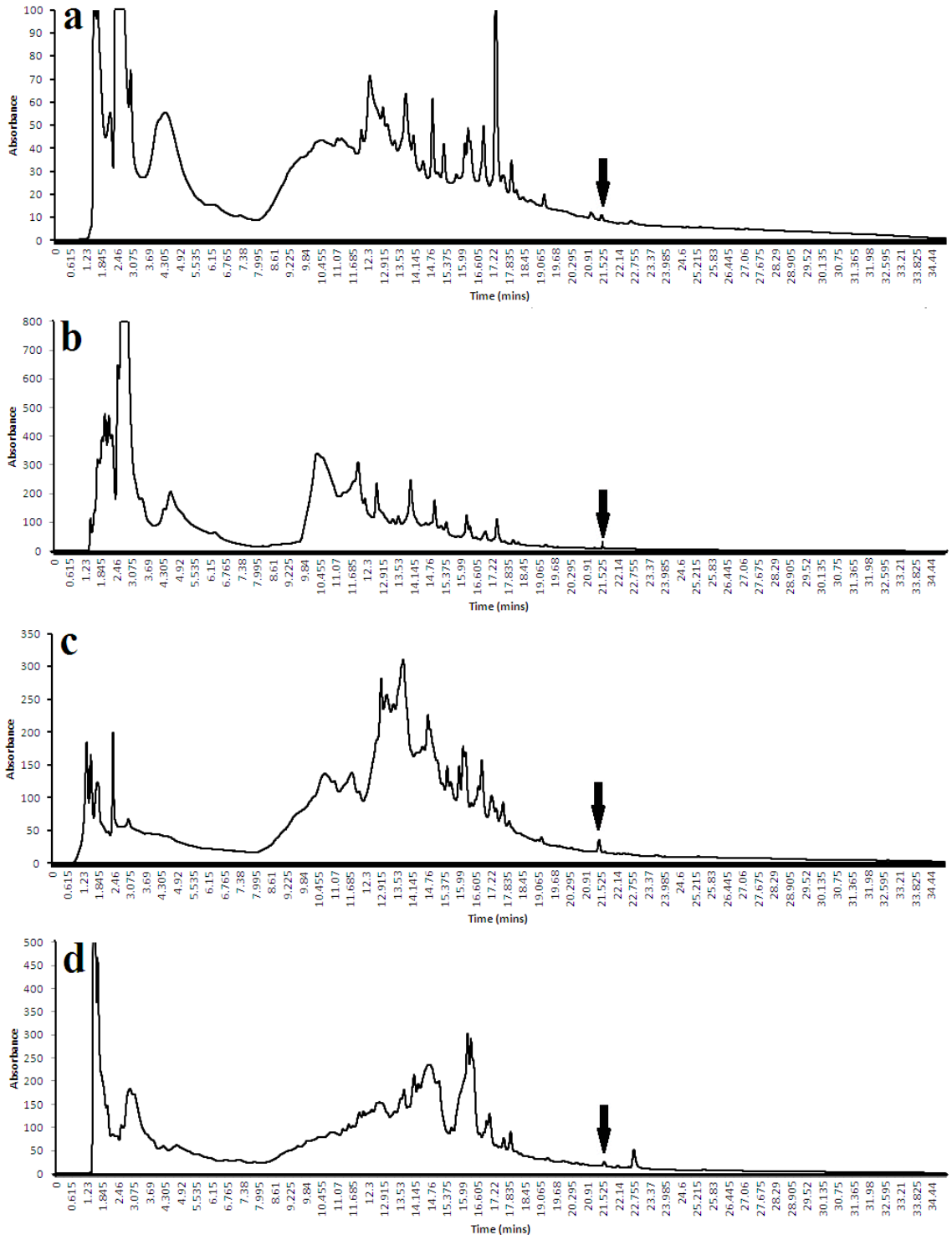

Figure 7: Schematic representation of the main proposed events leading to the establishment and progression of AS. Only major events are shown. Numbers refer to current and/or proposed targets for the prevention and treatment of AS.

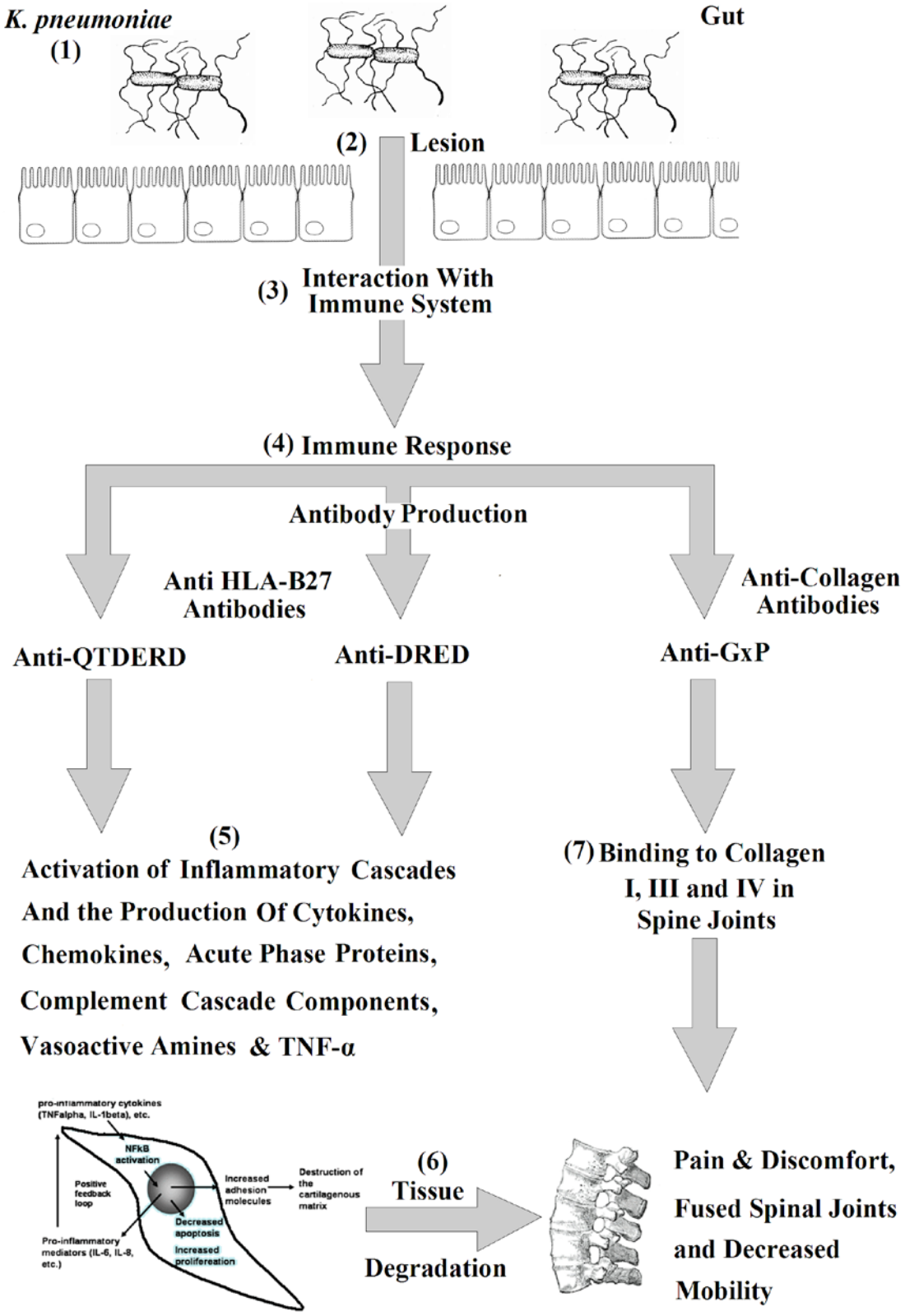

Table 1: Extract yields, minimum inhibitory concentrations (µg/ml) against *K. pneumoniae* and 24 h LC50 values (µg/ml) of South African plant extracts in the Artemia nauplii toxicity bioassay.

Species	Plant Part	Solvent	Extract (mg)	Extract Concentraction (mg/ml)	*K. pneumoniae* MIC (µg/ml)	24 h LC50 (µg/ml)
Agathosma betulina	leaves	methanol	160	16	1876	-
	leaves	water	210	21	2387	-
Ballota africana	leaves	methanol	120	12	438	-
	leaves	water	90	9	379	-
Carpobrotus edulis	leaves	methanol	273	27.3	324	-
	leaves	water	248	24.8	558	-
Carpobrotus edulis	leaves	methanol	154	15.4	-	-
	leaves	water	87	8.7	-	-
Kigelia africana	leaves	methanol	40	4	965	-
	leaves	water	40	4	663	-
Lippia javanica	leaves	methanol	122	12.2	538	-
	leaves	water	46	4.6	654	-
Pelargonium fasiculata	leaves	methanol	96	9.6	374	-
	leaves	water	151	15.1	432	1438
Ptaeroxylon obliquum	leaves	methanol	284	28.4	1977	-
	leaves	water	250	25	-	-
Syzygium cordatum	bark	methanol	270	27	312	-
	bark	water	220	22	441	-
Syzygium cordatum	leaves	methanol	50	5	387	-
	leaves	water	110	11	335	-
Terminalia pruinoides	leaves	methanol	76	7.6	432	-
	leaves	water	137	13.7	531	-
Terminalia sericea	leaves	methanol	220	22	254	-
	leaves	water	210	21	318	-
Tulbaghia violaceae	roots	methanol	20	2	526	-
	roots	water	140	14	613	772
Tulbaghia violaceae	leaves	methanol	150	15	-	-
	leaves	water	50	5	277	817
Warburgia salutaris	bark	methanol	11	11	624	-
	bark	water	6	6	677	-
Warburgia salutaris	leaves	methanol	82	8.2	-	-
	leaves	water	101	10.1	-	-
Potassium Dichromate						92.3

Numbers indicate the mean MIC or LC50 values of at least triplicate determinations. - indicates no growth inhibition or mortality.