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A comparison of the antimicrobial activity and *in vitro* toxicity of a medicinally useful biotype of invasive *Chromolaena odorata* (Asteraceae) with a biotype not used in traditional medicine

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ABSTRACT

Two biotypes of the invasive species, *Chromolaena odorata* are known to be present in Africa, *viz.* the Asian/West African biotype (AWAB) and the southern African biotype (SAB). Although the phytochemistry, ethnomedicinal and ethnopharmacological relevance of the AWAB has been elucidated, the SAB plants have received little or no attention. This study investigated and compared the phytochemistry and pharmacological activities of two biotypes of *C. odorata* (AWAB and SAB). Antimicrobial activities of leaf extracts of the two biotypes and three different growth stages of the SAB were evaluated against several bacterial and fungal strains using a serial microdilution assay. Phytochemicals were determined through standard methods of analysis. Toxicity of the extracts of the different growth stages of the SAB was determined using the colorimetric MTT assay, while the mutagenicity assay was performed using the Ames test. The AWAB had the overall best antibacterial activity, while the SAB showed better antifungal activity. Results showed that young and mature non-flowering extracts of the SAB were the most active. AWAB contained the highest amount of phenolics and flavonoids while SAB contained the highest amount of tannins. Extracts of young SAB plants showed a low level of cytotoxicity and none of the extracts of the three growth stages were mutagenic. This is the first report suggesting that the SAB of *C. odorata* can be exploited as a source of medicine similar to the AWAB, in combating antimicrobial infections and other health problems.

1. Introduction

Chromolaena odorata (L.) R.M. King & H. Rob. (Asteraceae) is an invasive perennial shrub native to the Americas (McFadyen, 1989). Since its introduction into West Africa in 1937 (Ivens, 1974; Uyi et al., 2014) and into southern Africa in 1947 (Zachariades et al., 2011), the plant has spread into different parts of the continent. Two biotypes are known to be invasive in Africa, *viz.* the widespread Asian/West African biotype (AWAB) which originated from Trinidad and Tobago, and the southern African biotype (SAB) traced to be of Cuban or Jamaican origin (Paterson and Zachariades, 2013). *Chromolaena odorata* has become an economic burden especially because of the negative effects it has on agriculture, biodiversity and livelihoods. The plant competes effectively with native plants and becomes dominant because of its ability to compete for water, nutrients, light and space forming shade over other species. This may lead to extinction of local plant species, thereby reducing plant biodiversity. Other species around it are often eliminated and this may be due to the allelopathic properties and novel biochemical "weapons" possessed by this plant (Callaway and Ridenour, 2004).

Despite its negative effects, the AWAB of *C. odorata* is seen by locals in West Africa and in some parts in Central Africa and south Asia as a source of medicine. The locals exploit the plant for the treatment of coughs and colds, skin infections, dysentery, wounds, toothache, malaria, stomach problems, diarrhoea, stomach ulcers, and also bacterial and fungal infections, possibly because of the presence of phenolics, flavonoids, tannins and saponins (Akinmoladun et al., 2007; Panda et al., 2010; Anyasor et al., 2011; Vijayaraghavan et al., 2013). The species is said to have anthelmintic, antioxidant, analgesic, antipyretic, antispasmodic, anti-inflammatory, antimicrobial, antimalarial, and wound healing properties (Omokhua et al., 2016 and references therein).

Due to the morphological and genetic differences between the two biotypes (Paterson and Zachariades, 2013), it is possible that their phytochemistry and pharmacological potentials may differ. To our knowledge, no studies exist on the phytochemistry of the SAB plants. Although Naidoo et al. (2011) screened SAB plants for antibacterial and antifungal properties, no comparison was made with AWAB plants to decipher which of the biotypes possesses better medicinal potential. Therefore the objectives of this study were several fold: (i) to determine

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and compare the antimicrobial activities of both biotypes of *C. odorata* using the microdilution assay against bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*) and a yeast fungus (*Candida albicans*); and (ii) to investigate possible phytochemical content and to ascertain if phytochemicals present in the AWAB are also present in the SAB. Due to the fact that phytochemicals may differ between growth stages of plants (Hol, 2011), phytochemical investigation and antimicrobial screening were done using different growth stages of SAB *C. odorata*. A further objective of this study was to conduct cytotoxicity and mutagenicity tests on leaf extracts of different growth stages of the SAB plants.

2. Materials and methods

2.1. Study species

Chromolaena odorata is a scrambling perennial shrub native to the Americas from southern USA to northern Argentina (Gautier, 1992). In its invasive range, *C. odorata* grows in a wide range of vegetation types such as forest margins, grasslands, roadsides, agricultural lands, and disturbed forests posing a significant threat to agriculture, biodiversity, and livelihoods (see reviews in Zachariades et al., 2009; Omokhua et al., 2016). The weed is intolerant of deep shade, but performs well in partial shade and full-sun conditions (Zachariades et al., 2009). In an open land situation, the shrub can grow 2–3 m in height, but it can reach up to 5–10 m when supported by other vegetation. Flowering, which is often prolific, peaks in December to January in the northern hemisphere and in June to July in the southern hemisphere (Zachariades et al., 2009). The southern African biotype of *C. odorata*, which originated from Cuba or Jamaica, is morphologically and genetically distinct from the more widespread biotype (Asian/West African biotype) invading Asia, Oceania, and West-, East-, and Central Africa (Paterson and Zachariades, 2013).

2.2. Plant collection and sample preparation

Stem cuttings of the AWAB *C. odorata* were collected from the Agricultural Research Council - Plant Protection Research Institute (ARC-PPRI), Cedara (22° 38′ 36.25“ N, 120° 36′ 12.36 E), near Pietermaritzburg on 19 February 2014. The stem cuttings of the SAB were collected from an open field within the vicinity of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe (29° 70′ S, 31° 05′ E), near Durban, South Africa on the same day. All cuttings were initially planted in a mist bed in vermiculite with rooting hormone (Seradix™ No. 1) for four weeks before they were later planted in nursery pots (25 cm diameter). All plants benefited from the same potting medium (Umgeni sand: Gromor Potting Medium™ 1:1), fertilizer (Plantacote™) and watering regimes. The plants were maintained in a shade house at the Botanical Gardens of the University of KwaZulu-Natal, Pietermaritzburg and plants were watered daily using automatic drip irrigation. During the growth of the plants, 40 pots from the AWAB plants tagged mature non-flowering (AMNF) were set to be used for the experiment. While for the SAB, 40 potted plants were tagged as “young plants (SY)”, another 40 plants were tagged as “mature flowering plants (SMF)” and a final 40 were tagged as “mature non-flowering plants (SMNF)”. Each plant category was used for antimicrobial screening at their appropriate stage of development. The SY leaves were harvested in May, the SMF in July while the SMNF and AMNF were harvested in September 2014. Two voucher specimens (Omokhua 01 and Omokhua 02) were prepared for the AWAB and the SAB and were deposited in the Bews Herbarium (NU), University of KwaZulu-Natal. The leaves of all plants were carefully harvested at their allocated stages of growth and dried in an oven at 55 °C for 72 h, ground, and the powders stored in dark airtight containers at room temperature.

2.3. Preparation of plant extracts for antimicrobial, cytotoxicity and mutagenicity assays

Two grams of the powdered samples of the AWAB and SAB biotypes (AMNF and SMNF) were weighed into 50 ml conical flasks and extracted using 20 ml of redistilled 70% ethanol (EtOH). Two gram aliquots from SY, SMF and SMNF of the SAB were also weighed into 50 ml conical flasks and extracted separately using redistilled 70% EtOH, 50%, 70% methanol (MeOH), petroleum ether (PE), dichloromethane (DCM) and distilled water. All mixtures were sonicated in a sonication bath on ice for 25 min. The extracts were filtered under vacuum through filter paper (Whatman No. 1) and the filtrates were poured into weighed glass pill vials. The organic extracts were placed under a stream of air at room temperature and allowed to dry, while the water extracts were placed in glass jars and freeze-dried. The dried extracts were kept in the dark at 10 °C until required for the experiments.

2.4. Preparation of extracts for phytochemical analyses

From the ground plant material, 0.1 g was weighed into 50 ml conical flasks, 10 ml of 50% methanol were added and the flasks were sonicated in a sonication bath for 25 min. The mixtures were filtered through filter paper (Whatman No. 1) under a vacuum pressure pump, poured into pill vials and immediately used for the assays. This was done to prevent deterioration and decomposition of the metabolites in the plant samples.

2.5. Antimicrobial screening

2.5.1. Preparation of microbial stock cultures

Bacterial and fungal stock strains used for the assay were cultured in Mueller-Hinton (MH) agar (Merck, Germany) and Yeast Malt (YM) agar (Becton Dickson, USA) respectively, sterilized by autoclaving and poured into petri dishes and allowed to gel. The plates were allowed to cool at 4 °C overnight and the stock bacterial and fungal strains were streaked and sub-cultured on the plates. The inoculated plates were incubated for 24 h at 37 °C to allow the colonies to develop. Bacterial and fungal growth was controlled by storing the plates at 4 °C until required for bioassays.

2.5.2. In vitro antibacterial bioassay

All leaf extracts of the two biotypes (AMNF, SMNF) and that of the three different growth stages (SY, SMF and SMNF) of the SAB *C. odorata*, were tested for antibacterial activity through determination of the minimum inhibitory concentration (MIC) using a serial microdilution bioassay in 96-well microplates (Eloff, 1998). *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 13883) and *Salmonella typhimurium* (ATCC 700720) cultured overnight (20 h) were prepared by inoculating a single colony of each bacterial species in 5 ml sterilized Mueller-Hilton (MH) broth in sterile McCartney bottles and incubated at 37 °C in a water bath with an orbital shaker. The absorbance of each overnight culture was measured at a wavelength of 600 nm using a UV–visible spectrophotometer with a starting absorbance of 0.001. The overnight bacterial cultures were diluted with 19.8 ml of sterile MH broth in McCartney bottles and used in the screening. One hundred microliters of sterile water were added to each well of a 96-well microplate. From the re-suspended plant extracts (25 mg/ml in 70% EtOH for the organic extracts and water for the water extracts), 100 μl were added to the first well of the microplates (row A) and serially diluted two-fold downwards (column 1–12: A to H). Subsequently, 100 μl of the bacterial culture were added to each well of the microplates. Similarly, 100 μl of neomycin used as the positive control were two-fold serially diluted for use in the assay. Sterile water, 70% EtOH and bacteria-free MH broth were used as the negative controls. The final concentration of

the extracts and positive controls in the wells of the microplates ranged from 0.04 to 6.25 mg/ml and 0.04 to 6.25 μg/ml respectively. Parafilm was used to cover the microplates to reduce evaporation and prevent contamination of the experiments and the plates were incubated at 37 °C for 24 h. To the incubated microplate wells, 50 μl of p-iodonitrotetrazolium (INT) were added to indicate the minimum inhibitory concentration (MIC) and the plates were re-incubated at 37 °C for 1 h. Bacterial growth in the wells was indicated by a pink-red colour. According to Eloff (1998), active micro-organisms reduce the colourless INT to a pink red colour. Clear wells with no colour change show that the bacterial growth was inhibited by the extracts and the concentrations of the last clear well were recorded as the MIC value. The experiment was carried out three times with two replicates.

2.5.3. In vitro antifungal bioassay

The antifungal activity of extracts of both *C. odorata* biotypes (AMNF and SMNF) and the three growth stages (SY, SMF and SMNF) of the SAB was evaluated by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) using the microdilution bioassay in 96-well microplates described by Eloff (1998) and modified by Masoko et al. (2007). The overnight (20 h) fungal culture was prepared by inoculating a single colony of *Candida albicans* from the cultured YM plate into 5 ml sterilized YM broth in a sterile McCartney bottle which was then incubated at 37 °C in a water bath with an orbital shaker. Overnight fungal culture (400 μl) was diluted with 4 ml of sterile saline solution (0.85% NaCl) in a sterile McCartney bottle. Absorbance of the mixture was determined using a UV–visible spectrophotometer at 530 nm. The absorbance was adjusted with sterile saline solution to match that of a 0.5 McFarland standard solution at a range of 0.25 to 0.28. From the prepared culture a 1:100 dilution with sterile YM broth (10 μl of cultured *C. albicans* to 10,000 μl of YM broth) was prepared to obtain a concentration of 5×10^5 CFU/ml. One hundred microliters of sterile water were added to each well of a 96-well microplate. From re-suspended organic plant extracts (25 mg/ml) in 70% EtOH and water extracts in water, 100 μl were added to the first well of the microplates (row A) and serially diluted two-fold downwards (column 1–12: A to H). Following this, 100 μl of the prepared culture was added to each well of the microplates. Similarly, 100 μl of amphotericin B (initial concentration 0.25 mg/ml) used as the positive control were two-fold serially diluted. Sterile water, 70% EtOH, fungal-free YM broth and *C. albicans* were used as the negative controls. The microplates were covered with parafilm to reduce evaporation and prevent contamination of the experiments and incubated at 37 °C for 24 h. After incubation, the MIC was determined by adding 50 μl of 0.02 mg/ml INT as an indicator for fungal growth and the plates were incubated at 37 °C for 48 h. Fungal growth in the wells was indicated by a pink-red colour and clear wells with no colour change indicated antifungal activity by the extracts. The concentration of the last clear well was recorded as the MIC values. To determine the minimum fungicidal concentration (MFC) values, 50 μl of YM broth was added to the clear wells and the microplates were re-covered with parafilm and further incubated for 24 h at 37 °C. The MFC values of the extracts were recorded as the concentration in the last clear well of the microplates where there was no colour change. The experiment was performed three times with two replicates.

2.6. Phytochemical detection and quantification

2.6.1. Detection of phytochemicals

Detection of phytochemicals was performed on the various extracts and of the two biotypes and growth stages of the SAB plant using standard procedures for alkaloids (Sofowora, 1993; Makkar et al., 2007), saponins (Tadhani and Subhash, 2006), phenolics, flavonoids and tannins (Trease and Evans, 1983, 2002).

2.6.2. Quantitative determination of total phenolics, flavonoids and tannins

Standard methods were used to quantitatively determine total phenolics, flavonoids and condensed tannins on the crude extracts and of the two biotypes and growth stages of the SAB plant.

The method described by Makkar et al. (2007) was applied, using gallic acid as the standard to determine total phenolic content. The 50% MeOH plant extracts (50 μl) were transferred into test tubes in triplicate, 950 μl of distilled water were added followed by 500 μl of 1 N Folin-C reagent and 2.5 ml of 2% sodium carbonate ($NaCO_3$) added in the dark. Similarly, a blank containing 50% MeOH in place of the plant extracts at different concentrations of gallic acid were also prepared in triplicate. The test tubes containing the mixtures were incubated at room temperature for 40 min, and absorbance was measured at 725 nm using an UV–visible spectrophotometer (Varian Cary 50, Australia). Total phenolics were expressed as gallic acid equivalents (GAE) per gram dry weight.

As described by Zhishen et al. (1999) and modified by Marinova et al. (2005), the aluminium chloride ($AlCl_3$) method was used to determine flavonoid content using catechin as the standard. In triplicate, 250 μl of 50% MeOH extracts were measured into test tubes, 1000 μl of distilled water were added followed by 75 μl of 5% sodium nitrite ($NaNO_2$), 75 μl of 10% $AlCl_3$ and 500 μl of 1 M sodium hydroxide (NaOH) sequentially. Finally the mixtures were adjusted to 2.5 ml with 600 μl of distilled water. A blank containing 50% MeOH in place of the plant extracts and catechin at various concentrations were also prepared. Absorbance was measured at 510 nm using an UV–visible spectrophotometer. The flavonoid content was expressed as catechin equivalents (CAE) per dry weight.

To determine condensed tannins, the butanol-HCl assay using cyanidine chloride as the standard was employed. In triplicate, 250 μl of 50% MeOH plant extracts were measured into test tubes, 3000 μl of butanol-HCl reagent and 100 μl of ferric reagent were added; a blank containing 50% MeOH and cyanidine chloride of different concentrations were also prepared. All test tubes containing the mixture were vortexed, covered properly with a lid and incubated at 99 °C for 1 h. The mixtures were allowed to cool and absorbance was measured at 550 nm using an UV–visible spectrophotometer. Condensed tannins were expressed as cyanidine chloride equivalents (CCE) per dry weight.

2.7. Cytotoxicity assay

The tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) was used to determine the viable cell growth after incubation of African green monkey kidney (Vero) cells with the extracts. Vero cells were grown in Minimal Essential Medium (MEM) (Sigma) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). Cells of a subconfluent culture were harvested and centrifuged at 200 x g for 5 min, and re-suspended in MEM to 5×10^4 cells/ml. Cell suspensions (200 μl) were pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. MEM (200 μl) was added to wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37 °C in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. The MEM was aspirated from the cells and replaced with 200 μl of the extracts at differing concentrations prepared in MEM in quadruplicate. The microtitre plates were incubated at 37 °C in a 5% CO_2 incubator for 48 h with the extracts. Untreated cells and a positive control (doxorubicin chloride, Pfizer Laboratories) were included. After incubation, the MEM with plant extract was aspirated from the cells which were then washed with 150 μl phosphate buffered saline (PBS, Whitehead Scientific) and replaced with 200 μl of fresh MEM. Following the washing step, 30 μl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates incubated for a further 4 h at 37 °C. After incubation with MTT the medium in each well was carefully removed, without disturbing the MTT crystals in the wells, and the MTT formazan crystals were dissolved by adding 50 μl DMSO to each well.

The plates were shaken gently until the MTT solution was dissolved. The degree of MTT reduction was measured immediately by detecting absorbance in a microplate reader at a wavelength of 540 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT, but no cells, were used to blank the plate reader. The LC_{50} values were calculated as the concentration of extract resulting in a 50% reduction of absorbance compared to untreated cells.

2.8. Mutagenicity assay

Samples were initially dissolved in 10% dimethylsulphoxide (DMSO) and were later diluted to the required concentrations using distilled water. The final concentration of DMSO was less than 1%. Samples were filter-sterilized and tested against *Salmonella typhimurium* strains TA98 and TA102 (100 μl/plate of a fresh overnight culture prepared by inoculating 100 μl stock bacteria in 10 ml Oxoid nutrient broth and incubating for 16 h at 37 °C) without an exogenous metabolic activation system (S9 mix), using a plate incorporation assay (Maron and Ames, 1983). The initial concentrations of the test samples used were 5, 0.5 and 0.05 mg/ml of which 100 ml of each were used in the assay (resulting in 500, 50 and 5 μg/plate). The plates for the negative control contained 100 μl of 1% DMSO without S9 mix. The positive control plates contained 0.2 μg/plate of 4-nitroquinoline-N-oxide (4-NQO). The colonies were counted manually after 48 h of incubation at 37 °C using a colony counter.

2.9. Statistical analysis

A Student t-test was used to compare the amount of total phenolics, flavonoids and tannins of leaf extracts between the AWAB and SAB plants, while General Linear Model ANOVA (GLM ANOVA) were used to analyse differences in total phenolics, flavonoids and tannins, among the growth stages of the SAB plant. When the results were significant, the differences among stages were compared using Tukey's Honesty Significant Difference (HSD) test because of the equality of sample sizes. Where the test statistics were not significant, no *post hoc* tests were done. With the exception of the Student t-tests that were performed using GENSTAT statistical software, version 14.0 (VSN International Ltd., UK), all other analyses were performed using SPSS statistical software, version 16.0 (SPSS INC., USA).

3. Results

3.1. Antimicrobial activity

3.1.1. Antibacterial activity of the two biotypes (AWAB and SAB) of Chromolaena odorata

The two biotypes exhibited inhibitory activity against all tested bacteria with MIC values ranging from 0.39 to 3.12 for the AWAB and 0.78 to 6.25 (mg/ml) for the SAB (Table 1). The AWAB showed the best activity overall when compared to the SAB.

Table 1
Antibacterial activity (MIC mg/ml) of the Asian/West African and southern African biotypes of *Chromolaena odorata*.

Extract	Biotype	*K.p*	*P.a*	*E.c*	*E.f*	*S.t*	*S.a*
70% EtOH	AMNF	**0.78**[a]	1.56	1.56	**0.78**[a]	3.12	**0.39**[a]
	SMNF	1.56	6.25	3.12	**0.78**[a]	3.12	3.12
Neomycin (μg/ml)		3.12	**0.78**[a]	3.12	**0.78**[a]	3.12	**0.39**[a]

AMNF = Asian/West African biotype mature non-flowering plant; SMNF = southern African biotype mature non-flowering plant; *K.p = Klebsiella pneumoniae; P.a = Pseudomonas aeruginosa; E.c = Escherichia coli; E.f = Enterococcus faecalis; S.a = Staphylococcus aureus; S.t = Salmonella typhimurium.*

[a] Values written in bold are considered active (<1 mg/ml). Values greater than 1 mg/ml but less than 6.25 mg/ml are less active in this study.

Table 2
Antibacterial activity of different growth stages of the southern African biotype of *Chromolaena odorata*.

Extract	Growth stage	MIC (mg/ml)					
		K.p	*P.a*	*E.c*	*E.f*	*S.t*	*S.a*
70% EtOH	SY	1.56	6.25	3.12	**0.39**[a]	6.25	**0.78**[a]
	SMNF	3.12	6.25	3.12	**0.78**[a]	3.12	3.12
	SMF	1.56	6.25	3.12	**0.78**[a]	3.12	3.12
PE	SY	1.56	6.25	3.12	**0.78**[a]	3.12	3.12
	SMNF	1.56	6.25	3.12	1.56	6.25	1.56
	SMF	6.25	6.25	>6.25	6.25	6.25	3.12
50% MeOH	SY	3.12	>6.25	6.25	**0.78**[a]	6.25	6.25
	SMNF	3.12	>6.25	3.12	3.12	3.12	6.25
	SMF	3.12	>6.25	3.12	6.25	6.25	6.25
H_2O	SY	6.25	>6.25	>6.25	6.25	6.25	6.25
	SMNF	6.25	>6.25	>6.25	**0.78**[a]	6.25	6.25
	SMF	6.25	>6.25	>6.25	3.12	6.25	6.25
	Neomycin μg/ml	3.12	**0.78**[a]	3.12	**0.78**[a]	3.12	**0.39**[a]

SY = the southern African biotype young plant; SMNF = the southern African biotype mature non-flowering plant; SMF = the southern African biotype mature flowering plant; *K.p = Klebsiella pneumoniae; P.a = Pseudomonas aeruginosa; E.c = Escherichia coli; E.f = Enterococcus faecalis; S.t = Staphylococcus aureus; S.a = Salmonella typhimurium.*

[a] Values written in bold are considered active (<1 mg/ml), while values less than 6.25 mg/ml are less active and values greater than 6.25 mg/ml are not active in this study.

3.1.2. Antibacterial activity of the extracts of the different growth stages of the SAB Chromolaena odorata

All bacterial strains were inhibited by the extracts from the three growth stages to various degrees. Except for *P. aeruginosa* which was not inhibited by any of the water extracts and 50% methanol, and *E. coli* which was not inhibited by any of the water extracts and SMF 50% methanol (Table 2). Petroleum ether and 50% methanolic extracts of the SY exhibited similarly good activities against *E. faecalis* while only the water extract of the SMNF showed good activity against *E. faecalis*. From the ethanolic extract of the different growth stages tested, only the SY plant extract showed good activity against *S. aureus*. The three growth stages exhibited good activity against *E. faecalis*, however, the best activity was noted from the SY plant extract. The PE extracts of the various growth stages exhibited some inhibitory activity against all tested bacteria except for the SMF which showed no inhibitory activity against *E. coli.* A good activity was only observed from the SY plant extract against *E. faecalis.* For the aqueous methanolic extracts of the three growth stages, some activity was observed against tested bacterial strains, except for *P. aeruginosa* which was not inhibited by any of the extracts. And only the SY plant extract showed good activity which was against *E. faecalis.* Apart from *P. aeruginosa* and *E. coli* (both Gram-negative) that were not inhibited by the aqueous extracts of various growth stages, some level of inhibition was observed against the other bacterial strains, but only the SMNF aqueous extract displayed good activity against *E. faecalis.* Overall, *E. faecalis* was the only bacterial species inhibited with good activity by the three growth stages, although activity varied with solvent extract used.

3.1.3. Antifungal activity of the Asian/West African and southern African biotypes

The ethanolic extract of both biotypes demonstrated antifungal activity by inhibiting the growth of *C. albicans* (Table 3). However, only the SAB showed good fungistatic and fungicidal activities, better than the control used.

3.1.4. Antifungal activity of the extracts of the different growth stages of the SAB Chromolaena odorata

The 70% EtOH, PE, 50% MeOH and aqueous extracts of the different growth stages showed some level of antifungal activity, but good

Table 3
Antifungal activity (MIC and MFC) of Asian/West African and southern African biotypes of *C. odorata* against *C. albicans*.[a]

Extract	Biotype	MIC (mg/ml)	MFC (mg/ml)
70% EtOH	AMNF	1.56	1.56
	SMNF	**0.78**[a]	**0.78**[a]
Amphotericin B (μg/m)l		1.56	6.25

AMNF = Asian/West African biotype mature non-flowering plant; SMNF = southern African biotype mature non-flowering plant; EtOH = Ethanol.
[a] Values written in bold are considered very active (<1 mg/ml), while values greater than 1 mg/ml but less than 6.25 mg/ml are less active.

activity was detected with the 70% EtOH extracts of the SY and SMNF plants with MIC's and MFC's of 0.78 mg/ml. (See Table 4).

3.2. Phytochemical determination

3.2.1. Determination of phytochemicals between the AWAB and SAB

Results from this study indicated the presence of phenolics, flavonoids, tannins and saponins in both biotypes, but alkaloids were only present in the AWAB (Table 5). Although quantitative determination showed that the amount of total phenolics appeared to be higher in the AWAB than SAB (Fig. 1a), this difference was not statistically significant ($t_2 = 2.10$; $P = 0.169$). The amount of flavonoids was significantly higher in AWAB compared to the SAB ($t_2 = 9.48$; $P < 0.001$) (Fig. 1b). The amount of condensed tannins significantly differed ($t_2 = -2.96$; $P = 0.042$) between the SAB and AWAB with the former having the highest concentration (Fig. 1c).

3.2.2. Determination of phytochemicals in the growth stages of SAB

Further detection of phytochemicals of the three growth stages showed that they all contain phytochemicals such as phenolics, flavonoids, tannins, saponins except alkaloids which were absent in all (Table 6). Although the amount of total phenolics in the leaves of the mature non-flowering (SMNF) plant seemed to be higher compared with that of the other growth stages, the difference was not statistically significant (GLM ANOVA: $F_{2,8} = 2.51$, $P = 0.161$; Fig. 2a). The amount of flavonoids differed significantly among the different growth stages of the SAB plants (ANOVA: $F_{2,8} = 125.4$, $P < 0.001$) with the SMNF having the highest amounts (Fig. 2b). Finally, the amount of condensed tannins was not significantly different among the different growth stages (ANOVA: $F_{2,8} = 4.07$, $P = 0.076$; Fig. 2c).

Table 4
Antifungal activity (MIC and MFC) of different growth stages of the southern African biotype of *Chromolaena odorata*.

Extract	Plant growth	MIC (mg/ml)	MFC (mg/ml)
70% EtOH	SY	**0.78**[a]	**0.78**[a]
	SMNF	**0.78**[a]	**0.78**[a]
	SMF	1.56	1.56
PE	SY	1.56	1.56
	SMNF	1.56	1.56
	SMF	1.56	>6.25
50% MeOH	SY	3.12	>6.25
	SMNF	1.56	6.25
	SMF	1.56	6.25
H_2O	SY	1.56	1.56
	SMNF	1.56	1.56
	SMF	1.56	1.56
	Amphotericin B μg/ml	1.56	6.25

SY = the southern African biotype young plant; SMNF = the southern African biotype mature non-flowering plant; SMF = the southern African biotype mature flowering plant; EtOH = Ethanol, MeOH = Methanol, PE = Petroleum ether, H_2O = Water.
[a] Values written in bold are considered very active (<1 mg/ml), while values less than 6.25 mg/ml are less active and values greater than 6.25 mg/ml are not active in this study.

Table 5
Qualitatively detected phytochemicals in AWAB and SAB *Chromolaena odorata* leaf extracts.

Phytochemicals	AWAB (AMNF)	SAB (SMNF)
Alkaloid	+	_
Saponins	++	++
Phenolics	+++	+++
Flavonoids	+++	+++
Tannins	+	++

- = Absent.
+ = Present.
++ = Moderate.
+++ = Abundant.

3.3. Cytotoxicity test

The result of the cytotoxicity testing of the extracts prepared from different growth stages of the southern African biotype (SAB) of *C. odorata* against Vero monkey kidney cells is presented in Table 7. In general, the dichloromethane extracts were more cytotoxic than the 70% methanol extracts, indicating that there may be more non-polar compounds with cytotoxic effects to mammalian cells in *C. odorata*. The young leaves were more cytotoxic than leaves harvested from the mature non-flowering and mature flowering specimens.

3.4. Mutagenicity test

From the results presented in Table 8, none of the extracts tested displayed any mutagenic property against the bacterial strains used, although the test was performed without any exogenous metabolic activation system. However, there was a marked reduction in the number of revertant colonies with regards to TA98 in the plates containing 70% methanolic extracts.

4. Discussion

The use of antibiotics has assisted globally in combating microbial infections, however its overuse has become a major factor in the emergence of multidrug resistant strains of several groups of microorganisms. The worldwide emergence of multidrug resistant *Escherichia coli* and many other ß-lactamase producers has become a major health challenge (Khan and Musharraf, 2004). In light of the evidence of the rapid global spread of resistant clinical isolates, the need to search for new antimicrobial agents is paramount. Plants have been reported to serve as potential alternatives as they contain novel biochemical properties that may be useful for the treatment of microbial infections. Though alien invasive plant species have been known to have adverse effects on the environment, including causing a decline in biodiversity possibly leading to extinction of indigenous species, ecological imbalances, a reduction in land value and depletion of water resources, some of these plants also possess novel medicinal properties which may serve as

Table 6
Phytochemicals detected in different growth stages of SAB *C. odorata* leaf extracts.

	Growth stage		
Phytochemicals	SY	SMNF	SMF
Saponins	+	++	++
Phenolics	++	+++	+++
Flavonoids	++	+++	+++
Tannins	+	++	+
Alkaloids	−	−	−

- = Absent.
+ = Present.
++ = Moderate.
+++ = Abundant.

Table 7
Cytotoxicity of extracts in different growth stages of the SAB biotype of *Chromolaena odorata* against Vero monkey kidney cells.

Plant part	Developmental stage	Solvent extract	LC_{50} (mg/ml)
Leaves	SY	DCM	0.031 ± 0.0007
		70% MeOH	0.217 ± 0.0321
	SMNF	DCM	0.151 ± 0.0139
		70% MeOH	0.618 ± 0.0318
	SMF	DCM	0.154 ± 0.0071
		70% MeOH	0.449 ± 0.0249
Doxorubicin (μM)			6.781 ± 0.3901

SY = southern African biotype young plant, SMNF = southern African biotype mature-non flowering plant, SMF = southern African biotype mature-flowering plant, DCM = Dichloromethane, MeOH = Methanol, SAB = southern African biotype.

alternatives to highly exploited indigenous plants with the same medicinal properties.

Chromolaena odorata, an alien invasive plant which is widespread in the tropics and sub-tropics including South Africa, is exploited as a source of medicine in most of its introduced ranges except in South Africa. Hence this study was designed to investigate the antimicrobial activity, cytotoxicity and mutagenicity tests and phytochemicals screening of *C. odorata*. Results from the current study indicated that the AWAB and SAB of *C. odorata* leaf extracts inhibited the growth of some bacterial and fungal strains tested in the experiments. This shows that the SAB can also be exploited as a source of medicine in the treatment of microbial infections. With the three growth stages of the SAB tested for cytotoxicity, only the methanolic extract of the SY showed some level of cytotoxicity and none of the extracts showed any mutagenic effect on the TA98 and TA102 *Salmonella typhimurium* strains.

4.1. Phytochemical analysis

The commonly identified compounds in both biotypes and among the three growth stages of the SAB included phenolics, flavonoids, tannins and saponins except for alkaloids that were only present in the AWAB. This result is in accordance with those of Akinmoladun et al. (2007), who reported saponins, phenolics, flavonoids, alkaloids and tannins in the AWAB. The alkaloids in *C. odorata* have been identified as pyrrolizidine alkaloids (PAs) (Biller et al., 1994) and these authors reported only a low concentration of PAs in the leaves of AWAB plants. The roots of the AWAB contains five PAs *viz.* 7-angeloylretronecine, 9-angeloylretronecine, 3′-acetylrinderine, intermedine and rinderine (Biller et al., 1994). The absence of alkaloids in the leaves of SAB plants used for this study does not necessarily imply their absence, as quantifiable amounts of alkaloids are reported to be present only in the roots of *C. odorata*. The methods used here might not have been sensitive enough to detect alkaloids in the extracts. Therefore, further investigation on the SAB may be required to ascertain if these alkaloids reported in the roots of the AWAB are present in the roots of the SAB before we can rule out whether any part of the SAB does not contain alkaloids.

Results from the phytochemical quantification between the two biotypes (Fig. 1a-c) showed that both biotypes contained reasonable amounts of phenolics, flavonoids and tannins, with the AWAB containing a higher amount of phenolics and flavonoids. These results suggest that the AWAB may possess higher level of bioactivity compared to the SAB, but this can only be verified with further studies. The AWAB has been reported to contain phenolic compounds such as p-coumaric, protocatechuic, p-hydroxybenzoic, ferulic and vanillic acids, and these compounds have been reported to protect cultured skin cells and retard oxidative degradation of lipids (Phan et al., 2001). More than 40 flavonoid compounds have been isolated from the AWAB and flavonoids such as sinensetin and scutellareine tetramethyl ether isolated from this biotype have been reported to possess good antibacterial activity. However, none of these flavonoid compounds have been reported in the SAB, therefore isolation and identification of these novel compounds in the SAB is required to determine if these compounds are present and whether they also possess antibacterial properties. Tannins have been found to form irreversible complexes with proline-rich proteins (Akinpelu and Onakoya, 2006), resulting in the inhibition of the cell protein synthesis. This is important for the treatment of inflamed or ulcerated tissues (Akinpelu and Onakoya, 2006). The SAB which is reported to contain a higher amount of tannins in this experiment may be more effective with respect to the above properties than the AWAB, however an investigation of such a hypothesis is required.

Table 8
Mutagenic tests of extracts of different growth stages of SAB biotype of *Chromolaena odorata* using *Salmonella typhimurium* TA98 andTA102 assay systems in the absence of exogenous metabolic activation.

Plant part	Developmental stage	Extracting solvent	Dose (μg/plate)*	His + revertants/plate	
				TA98	TA102
Leaves	SY	DCM	5	19.33 ± 9.50	231.00 ± 32.97
			50	17.33 ± 6.80	218.33 ± 4.51
			500	22.33 ± 7.64	236.00 ± 29.46
		70% MeOH	5	9.67 ± 3.79	386.67 ± 54.31
			50	7.67 ± 1.15	374.67 ± 19.73
			500	5.33 ± 4.51	481.33 ± 34.02
	SMNF	DCM	5	24.67 ± 10.26	222.00 ± 34.83
			50	19.67 ± 4.16	213.33 ± 34.93
			500	20.33 ± 4.93	220.33 ± 26.27
		70% MeOH	5	6.67 ± 2.89	290.67 ± 89.47
			50	7.67 ± 3.51	417.33 ± 39.26
			500	8.67 ± 1.53	404.00 ± 22.27
	SMF	DCM	5	10.67 ± 2.08	324.00 ± 28.00
			50	9.67 ± 2.52	380.00 ± 18.33
			500	11.00 ± 4.00	378.67 ± 54.60
		70% MeOH	5	5.33 ± 0.58	392.00 ± 27.71
			50	6.33 ± 3.51	364.00 ± 18.33
			500	9.33 ± 4.93	372.00 ± 52.46
Positive (4-NQO)				116.00 ± 7.55	568.33 ± 114.34
Negative				18.67 ± 4.36	236.67 ± 14.42

Data presented are the mean ± standard deviation of six plates from two separate experiments each performed in triplicate.
4-NQO = 4-nitroquinoline-N-oxide, SY = southern African biotype young plant, SMNF = southern African biotype mature-non flowering plant, SMF = southern African biotype mature-flowering plant. DCM = dichloromethane, MeOH = Methanol, SAB = southern African biotype.
* Initial concentrations of the fractions were 0.05, 0.5 and 5 mg/ml (5, 50 and 500 μg/plate).

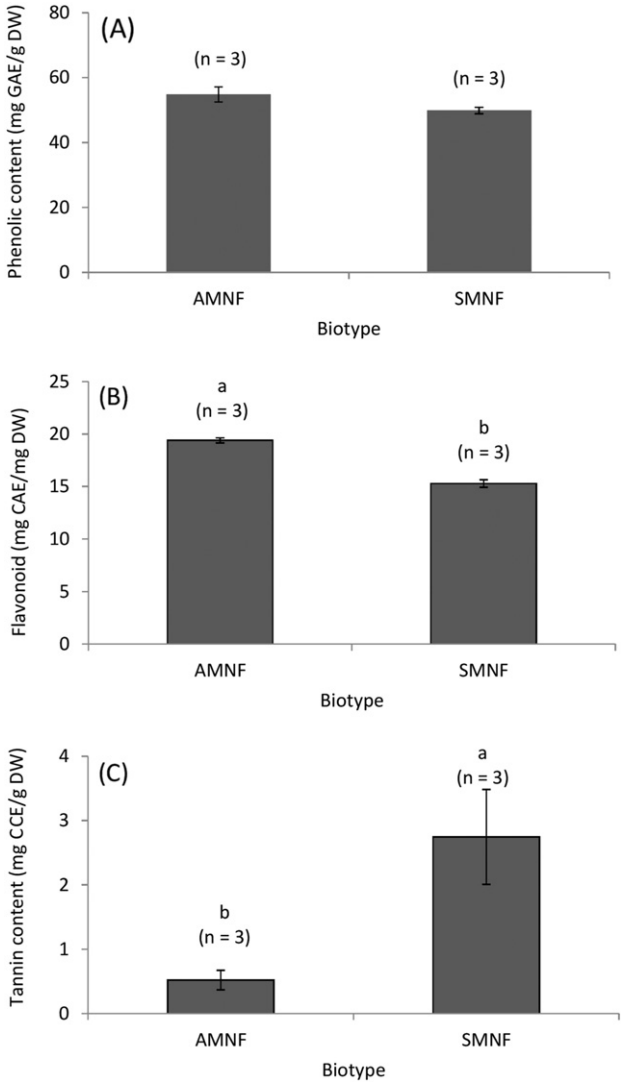

Fig. 1. (a) Total phenolic content, as gallic acid equivalents, detected in the leaves of AWAB and SAB *Chromoleana odorata* plants (b) flavonoid content as catechin equivalents detected in the leaves of AWAB and SAB *Chromoleana odorata* plants and (c) condensed tannins content as cyanidine chloride equivalents detected in the leaves of AWAB and SAB *Chromoleana odorata* plants. Values in each bar are means ± SEM. Sample sizes are given in parenthesis. DW = dry weight, GAE = gallic acid equivalents, CAE = catechin equivalents, CCE = cyanidine chloride equivalents, AMNF = Asian/West African biotype mature non-flowering plant, SMNF = southern African biotype mature-non flowering plant.

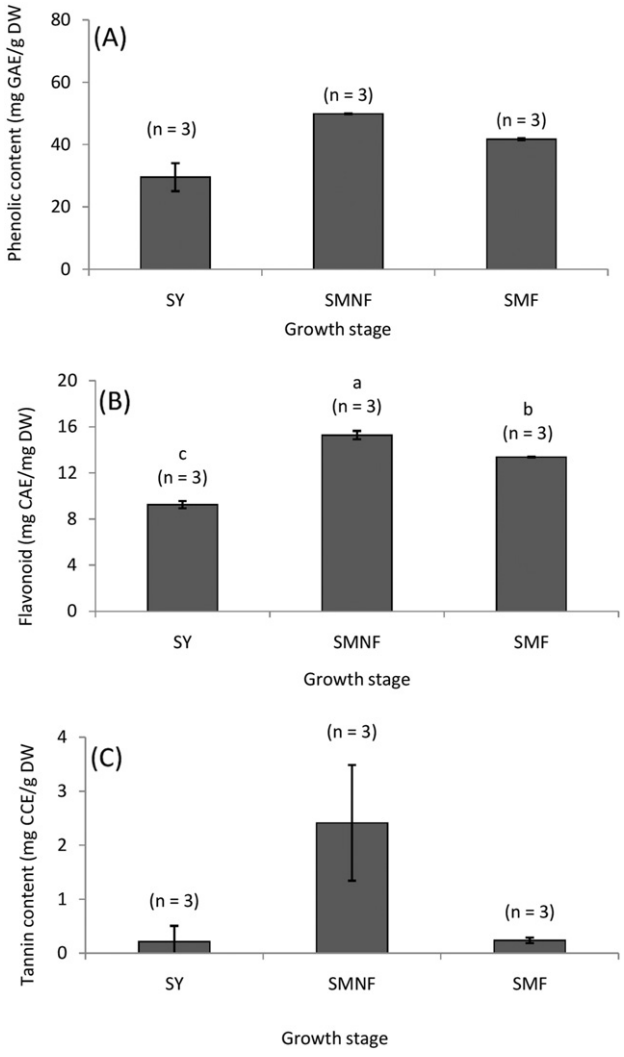

Fig. 2. (a) Total phenolic content, as gallic acid equivalents, detected in the three growth stages of southern African *Chromoleana odorata* biotype (b) flavonoid content as catechin equivalents detected in the three growth stages of southern African *Chromoleana odorata* biotype and (c) condensed tannins content as cyanidine chloride equivalents detected in the three growth stages of southern African *Chromoleana odorata* biotype. Values in each bar are means ±SEM. Sample sizes are given in parenthesis. DW = dry weight, GAE = gallic acid equivalent, CAE = catachin equivalents, CCE = cyanidine chloride equivalents, SY = southern African biotype young plant, SMNF = southern African biotype mature non-flowering plant, SMF = southern African biotype mature-flowering plant.

4.2. Antimicrobial activity

The antimicrobial activities displayed by both biotypes on the tested strains may be as a result of the presence of the above mentioned phytochemicals (Mendoza et al., 1997). Although the findings of this study demonstrated that both biotypes exhibited some level of antibacterial activity on tested strains, the AWAB had the best activity overall. Our results agree with those of Raman et al. (2012) who studied the ethanolic extract of the AWAB on some bacterial strains. These authors reported that all bacterial strains were inhibited by the extract. An MIC of 0.78 mg/ml similar to the value observed in this study was reported against *K. pneumoniae* and a lower MIC of 0.78 mg/ml against *E. coli* compared to this study. The results also agree with those of Nisit et al. (2005) who investigated the activity of the ethanolic extract against *S. aureus* and *E. coli*. Zige et al. (2013) also reported that the ethanolic extract inhibited the growth of *S. aureus* while the water extract inhibited *E. coli*. Irobi (1992, 1997) and Muskhazli et al. (2009) reported the antibacterial activity of the methanolic and ethanolic extracts of the AWAB on *S. aureus* and other bacteria. Sukanya et al. (2011), Bamba et al. (1993) and Caceres et al. (1995) also reported the antibacterial activity of the AWAB on some bacterial strains. In a study carried out by Vital et al. (2009), *S. typhimurium*, *B. subtilis* and *S. aureus* were inhibited by the leaf extract of AWAB *C. odorata*. With the exception of Naidoo et al. (2011) who reported the antibacterial activity against some bacterial strains of the SAB, no other studies have been conducted.

Antibacterial studies were further carried out on the SY, SMNF and SMF of the SAB to ascertain if a particular growth stage of this plant was more effective. The results showed that the SY was more active against the tested strains followed by the SMNF. Comparing the activities displayed by the various extracts, one can conclude that the quantity of phytochemicals present in a plant may not be proportional to the activity displayed by the plant - as there may be other bioactive components acting in synergy with these phytochemicals. However, isolation and quantification of these phytochemicals (*e.g.* phenolics, flavonoids and tannins) may be required in order to detect if there are some compounds of these phytochemicals analysed that are only present in the SY

which may have resulted in better activity. Naidoo et al. (2011) reported that the methanol leaf extract of the SAB (stage not specified) inhibited the growth of *Bacillus subtilis*, *Bacillus cereus*, *S. aureus* and *E. coli*, while the ethyl acetate extract inhibited *Streptococcus epidermidis*, *B. subtilis*, *B. cereus* and *S. aureus*. No activity was observed with the aqueous extract which is similar to the results observed in this study where different solvent extracts were used on the three growth stages of SAB. Good activity was observed using all extracts except for the aqueous extract of the SMNF. The authors suggested that the aqueous extract may be effective if the fresh leaves are boiled- as boiling may release the active compounds from the plant material (Coopoosamy et al., 2010). The suggestion made by Coopoosamy et al. (2010) may be worth exploring to ascertain if this conjecture is true - as boiling is one of the methods of preparation of some herbal medications used by traditional medicinal practitioners.

The Gram-negative bacteria were less inhibited by both extracts than the Gram-positive bacteria. This may be due to the presence of the outer membrane in the Gram-negative bacteria acting as a barrier to antibiotics (Palombo and Semple, 2001). As a result, the Gram-negative bacteria are very resistant (Rabe and Van Staden, 1997). The observed activity by the AWAB against *K. pneumoniae* (a Gram-negative bacterium) could be as a result of active compounds inhibiting bacterial growth without necessarily penetrating into the cells.

Though both biotypes showed fungicidal activity against *C. albicans*, only the SAB had good fungistatic and fungicidal activity (Table 3). This may be due to the higher amount of tannins in the SAB than the AWAB. Aqueous and ethanolic leaf extracts of the SAB *C. odorata* have also been reported to exhibit some levels of antifungal activity against *Aspergillus flavus*, *A. glaucus*, *Candida albicans*, *C. tropicalis* and *Trichophyton rubrum* (Naidoo et al., 2011). A further investigation showed that the SY and the SMNF had better fungistatic and fungicidal activities than the SMF. Therefore, the best antifungal activity can be achieved with the SY and SMNF leaves of the SAB plant. This suggests that this plant may offer a new source of antifungal agents against the pathogenic *C. albicans*.

4.3. Cytotoxicity assay

Cytotoxicity is an adverse effect resulting from interference with the structures and/or processes necessary for cell survival, proliferation and function. These effects may involve membrane integrity, cellular metabolism, synthesis and degradation or release of cellular constituents, ion regulation and cell division (Seibert et al., 1996). The balance between therapeutic and toxic effects of compounds is an important parameter for evaluation of their usefulness as pharmacological drugs (Rodeiro et al., 2006). Vero cells which are a commonly used cell type for cytotoxicity tests were used in this experiment. Results from this experiment showed that only the SY had some level of toxicity compared to the SMNF and SMF respectively. This may contribute to the antibacterial activity exhibited by the SY plant (Table 7). Because of the good antimicrobial activities displayed by this plant in the *in vitro* assay, it may be necessary to further investigate this plant in *in vivo* studies to determine toxicity effects before the plant is used in therapeutics. Should the toxicity be high, the plant can possibly be used for topical applications to treat microbial infections, depending on the level of cytotoxicity.

4.4. Mutagenicity assay

Mutagenicity occurs as a result of substances that induce genetic mutations leading to alteration or loss of genes or chromosomes (Wink and Van Wyk, 2008). The Ames test which was used in this study to detect genetic damage induced directly or indirectly is based on the number of His + revertants in *S. typhimurium* strains that are produced by the crude plant extracts. Mutagenic potential of a test sample is assumed if (i) the number of revertant colonies of a test sample is at least double the number of revertant colonies of the negative control and/or (ii) there is any dose dependent increase in the number of colonies observed with the test sample (Verschaeve and Van Staden, 2008). None of the plant extracts showed a clear mutagenic effect against the bacterial strains used. This plant may therefore be considered as safe from this point of view, but further investigation involving metabolic activation will be necessary to confirm this.

5. Conclusion

Although the AWAB *C. odorata* biotype is used as a source of medicine in its invasive range and has long been a subject of ethnopharmacological investigations, the SAB *C. odorata* plant is yet to receive such attention and is not exploited as a source of medicine in southern Africa. In this study the AWAB and SAB were discovered to possess antimicrobial activities as they inhibited some of the bacterial and fungal strains investigated. Further investigation of different growth stages of the SAB showed that the young (SY) and mature non-flowering (SMNF) plants can be exploited as a source of antimicrobial drugs. Results from the cytotoxicity test showed that the SY plant extract which showed the best antimicrobial activity displayed some level of cytotoxicity, while none of the plant extracts were mutagenic. Based on the pharmacological activities, low toxicity and non-mutagenic effects observed in this study, the economical, ecological and environmental burdens posed by the SAB *C. odorata* in southern Africa can possibly be tackled through the use of the plant as a source of medicine in the treatment of infectious diseases. Our results suggest that SAB *C. odorata* may also serve as an alternative to highly exploited indigenous plants which may have the same medicinal potential, as it has been shown to contain important phytochemicals comparable with those found in the widely used AWAB.

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