Phytochemical and pharmacological investigations of invasive *Chromolaena odorata* (L.) R.M. King & H. Rob. (Asteraceae)

By

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Abstract

Chromolaena odorata (L.) R.M. King & H. Rob. (Asteraceae) is an invasive weedy scrambling perennial shrub native to the Americas that has proven to be a significant threat to both natural and semi-natural ecosystems as well as to livelihoods in the tropics and sub-tropics (including sub-Saharan Africa). Two biotypes of *C. odorata* are invasive in sub-Saharan Africa. The Asian/West African biotype (AWAB) is the more widespread form on the continent (being present in West, Central and East Africa), while the southern African biotype (SAB) is restricted to south-eastern Africa. Although the negative impact of the plant has received considerable attention in Africa, its medicinal and pharmacological significance is only beginning to be explored. The AWAB plant is exploited as a source of medicine in West and Central Africa for the treatment of malaria, wounds, diarrhoea, skin infections, toothache, dysentery, stomach ache, sore throat, convulsions, piles, coughs and colds, possibly because of the presence of flavonoids, essential oils, phenolics, tannins and saponins. The plant is reported to have antibacterial, anti-inflammatory, antioxidant, anthelminthic, antifungal, cytotoxic, anti-convulsant, anti-protozoal, antispasmodic, anti-pyretic and analgesic properties. Though the above usefulness has been reported with reference to the AWAB plant, the SAB plant has not been investigated. Hence this thesis attempts to comparatively document the phytochemistry and ethnopharmacological importance of both *C. odorata* biotypes.

The antibacterial and antifungal activities of the leaf extract of the AWAB (mature non-flowering plants, AMNF) and the SAB (mature non-flowering plants, SMNF) were evaluated. Both biotypes exhibited good activity against *E. faecalis* (AWAB, 0.78; SAB, 0.78), but only the AWAB exhibited good activity against *K. pneumoniae* (AWAB, 0.78; SAB, 1.56) and *S. aureus* (AWAB, 0.39, 3.12), showing that the

AWAB extracts were more effective than those of the SAB. For the antifungal activity against *C. albicans* only the SAB exhibited good fungicidal (SAB, 0.78; AWAB, 1.56) and fungistatic (SAB, 0.78; AWAB, 1.56) activity. The results of a further investigation of the antimicrobial activities of the different growth stages of the SAB plant showed that all growth stages exhibited some level of activity against the tested bacterial and fungal strains, although young and mature non-flowering plants displayed the better activities.

Phytochemical analysis revealed the presence of saponins, phenolics, flavonoids and condensed tannins in varying amounts in the leaf extracts of the AWAB and SAB plants but alkaloids were only present in the AWAB plant. Quantitative determination showed that the AWAB contained higher amounts of phenolics and flavonoids than the SAB, but condensed tannins were higher in the SAB than the AWAB. In comparing the levels of phytochemicals between the three growth stages of the SAB, mature non-flowering plants contained the highest amount of phenolics, flavonoids and tannins compared to the young and flowering plants. This showed that the antimicrobial activity displayed by the extracts of the various growth stages of the SAB could not be correlated with the levels of various phytochemicals present.

The tetrazolium-based colorimetric (MTT) assay for cytotoxicity and Ames test for mutagenicity were used to evaluate the safety of the plant extracts prepared from the three growth stages of the SAB. The results from the cytotoxicity assay showed that the young plant extract which showed the best antimicrobial activity was more cytotoxic than the mature flowering and mature non-flowering plants. The Ames test using *Salmonella typhimurium* tester strains TA98 and TA102 without S9 metabolic

activation revealed that all plant extracts of the three growth stages of the SAB were non-mutagenic towards the *S. typhimurium* strains tested.

The screening of the plant extracts for pharmacological activity and phytochemical composition provided valuable preliminary information in that both the AWAB and SAB may be good sources of antimicrobial agents. This study further demonstrated that the leaf extract of the young and mature non-flowering plants of the SAB plants may be exploited for medicinal purposes. While the medicinal potential of the AWAB sub-type has been demonstrated in this thesis and by other workers, this is the first study that simultaneously examined the phytochemistry and pharmacological potential of the SAB plant. The results suggest that the SAB plant can be exploited in southern Africa as a source of traditional medicine. This serves the dual purpose of exploring a use for this burgeoning weed problem as well as finding a possible alternative to highly exploited plant species with the same medicinal potential

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Student Declaration

Phytochemical and pharmacological investigations of invasive *Chromolaena odorata* (L.) R.M. King & H. Rob. (Asteraceae).

I, Aitebiremen Gift Omokhua, student number: 214582485 declare that:

(i) The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development (RCPGD), School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa;

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We hereby declare that we acted as supervisors for this M.Sc. student:

Student's full name: Aitebiremen Gift Omokhua

Student number: 214582485

Thesis title: Phytochemical and pharmacological investigations of invasive *Chromolaena odorata* (L.) R.M. King & H. Rob. (Asteraceae).

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the School of Life Sciences Higher Degrees Office for examination by the University appointed examiners.

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Publications from this thesis

A.G. Omokhua, L.J. McGaw, J.F. Finnie and J. van Staden (2015). *Chromolaena odorata* (L.) R.M. King & H. Rob. (Asteraceae) in sub-Saharan Africa: a synthesis and review of its medicinal potential. Journal of Ethnopharmacology (in press).

A.G. Omokhua, L.J. McGaw, J. Chukwujekwu, J.F. Finnie and J. van Staden. Phytochemistry, pharmacological studies, cytotoxicity and mutagenicity of *Chromolaena odorata* (L.) R.M. King & H. Rob. (Asteraceae). (manuscript in preparation).

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College of Agriculture, Engineering and Science Declaration 2 – Publication

A.G. Omokhua, L.J. McGaw, J.F. Finnie and J. van Staden. *Chromolaena odorata* (L.) R.M. King & H. Rob. (Asteraceae) in sub-Saharan Africa: a synthesis and review of its medicinal potential. Journal of Ethnopharmacology (in press).

Contribution: Writing of the publication was done by the first author, under the supervision of the last three authors.

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Contribution: The experimental design, experimental work and the draft manuscript were done by me, with assistance from Professor L.J. McGaw and Dr. J.C. Chukwujekwu. Professors J.F. Finnie and J. van Staden and Dr. L.J. McGaw supervised the study.

Signed

CMC 2012

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Finally, I thank almighty God for strength and good health.

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List of Abbreviations

AWAB: Asian/West African biotype of *Chromolaena odorata*

AMNF: Asian/West African mature non-flowering *Chromolaena odorata*

$AlCl_3$: Aluminium chloride

CO_2: Carbon dioxide

DCM: Dichloromethane

DMSO: Dimethylsulphoxide

EtOH: Ethanol

H_2O: Water

His: Histidine

MeOH: Methanol

MEM: Minimum essential medium

MFC: Minimum fungicidal concentration

MIC: Minimum inhibitory concentration

NaCl: Sodium chloride

NaOH: Sodium hydroxide

$NaNO_3$: Sodium nitrite

4 – NQO: 4-nitroquinoline-N-oxide

PE: Petroleum ether

SAB: Southern African biotype of *Chromolaena odorata*

SMF: Southern African biotype of mature flowering *Chromolaena odorata*

SMNF: Southern African biotype of mature non-flowering *Chromolaena odorata*

SY: Southern African biotype of young *Chromolaena odorata*

Chapter 1: Introduction and literature review

1.1. Introduction

The widespread use of medicinal plants (both indigenous and alien) can be traced to the occurrence of natural products with medicinal properties in plants and their ability to synthesize a variety of chemical compounds **(LAI and ROY, 2004; AZEBAZE *et al.,* 2006; TAPSELL *et al.,* 2006; RIGIANO *et al.,* 2013)**. These natural products have become useful to humans as they remain a reservoir of natural medicines despite different approaches used for their application.

Chromolaena odorata (L.) King and Robinson (=*Eupatorium odoratum*) (Asteraceae) (Figure 1.1) is an alien invasive perennial shrub native to the Americas **(McFADYEN, 1989)**. It is considered to be a significant economic and ecological burden to many tropical and sub-tropical regions of the world where it impacts negatively on agriculture, biodiversity and livelihoods **(ZACHARIADES *et al*., 2009; UYI & IGBINOSA, 2013)**. Following its introduction into West Africa in the 1930's **(IVENS, 1974)** and South Africa in the 1940's **(ZACHARIADES *et al.,* 2011)**, the species has spread into many countries on the continent **(TIMBILLA *et al.,* 2003; ZACHARIADES *et al.,* 2013)**. The status of *C. odorata* as an agricultural and environmental weed has been a subject of major concern in the past four decades in West and southern Africa, probably because of its invasiveness in agro-ecosystems and conservation areas (**IVENS, 1974, LUCAS, 1989; GOODALL and ERASMUS, 1996; TIMBILLA *et al*., 2003; UYI *et al*., 2014)**. The invasive success of *C. odorata* is thought to depend upon a combination of several factors such as (i) high reproductive capacity; (ii) high growth and net assimilation rates; (iii) its capacity to

suppress native vegetation through competition for light and allelopathic properties; and (iv) its ability to grow in many soil types and in many climatic zones **(ZACHARIADES *et al*., 2009; UYI *et al.,* 2014)**.

Figure 1.1: *Chromolaena odorata* (drawn by A. Walters, first published in **HENDERSON and ANDERSON (1966)** South Africa National Biodiversity Institute, Pretoria).

While *C. odorata* has been declared a 'Category 1' weed under the Conservation of Agricultural Resources Act in South Africa because of its invasiveness in the north-

eastern parts of the country **(GOODALL AND ERASMUS, 1996; NEL *et al*., 2004; ZACHARIADES *et al.,* 2011)**. The situation in West Africa remains contentious despite much research and many discussions **(TIMBILLA *et al.,* 2003; UYI *et al.,* 2014)**, largely because of the perceived usefulness of the plant in the latter region. In view of its presence in large areas and its invasive capacity, the use of chemical, mechanical and other conventional methods of controlling the weed have proven unsustainable **(TIMBILLA *et al.,* 2003, ZACHARIADES *et al.,* 2009, UYI and IGBINOSA, 2013)**. Hence the use of biological control (using natural enemies to feed on the plant) has been advocated as an important long-term management strategy for control of *C. odorata* **(SEIBERT, 1989)**.

1.2. Descriptive biology and ecology of *Chromolaena odorata*

The biology of *C. odorata* and aspects of the plant's ecology have been documented by several authors. It is a weedy, scrambling, perennial plant belonging to the Asteraceae with straight, pithy, brittle stems which branch readily; it has three-veined, opposite, ovate triangular leaves and a shallow fibrous root system (**HOLM *et al*., 1977; HENDERSON, 2001)**. Capitula are borne in panicles at branch ends and are devoid of ray florets. The corollas of the florets vary between plants from white to pale blue or lilac and achenes are black with a pale pappus **(HOLM *et al*., 1977; McFADYEN, 1989)**. In open-land situations, *C. odorata* grows up to 3 m in height, but it can reach up to 5-10 m when supported by other vegetation. The plant grows vigorously and profusely throughout the wet season, forming a dense and impenetrable thicket. Growth ceases as flowering begins, normally together with a decrease in rainfall and length of day **(SAJISE *et al*., 1974)**. Flowering peaks in the southern hemisphere during the months of June and July and in the northern

hemisphere from December to January. The species can reproduce apomictically **(GAUTIER, 1992; RAMBUDA and JOHNSON, 2004)** and is a prolific producer of light, wind dispersed seeds. A single shrub can produce as many as 80 000 seeds in one season **(WITKOWSKI and WILSON, 2001)**. At the start of the wet season, established plants generate new shoots from the crown or from higher, undamaged axillary buds, while seeds in the soil, produced during the previous dry season, germinate **(McFADYEN, 1988, 1989)**. Stems branch freely and a large plant may have up to 15 or more branches of varying size from a single rootstock. The plant can grow on many soil types, but prefers well-drained soils **(ZACHARIADES *et al.,* 2009)**.

Chromolaena odorata has an altitudinal range of 1 000-1 500 m above sea level and is common in areas of rainfall above 1 500 mm per annum in its native range **(McFADYEN, 1988, 1991)**. In Africa, it is particularly present in areas with annual rainfall of about 600 to 2 000 mm and altitudinal regimes of <2 000 m above sea level **(GAUTIER, 1992; GOODALL and ERASMUS, 1996; TIMBILLA, 1998)**. It grows best in sunny, open areas such as roadsides, abandoned fields, pastures and disturbed forests but tolerates semi-shade conditions. It does not thrive under the shaded conditions of undisturbed forests or in closely planted, well-established orchards **(ZACHARIADES *et al.,* 2009)**. Two biotypes of *C. odorata* are known in its invasive range of distribution viz. the Asian/West African biotype (AWAB) which originated from Trinidad and Tobago and the southern African biotype (SAB), thought to have originated from Jamaica or Cuba **(PATERSON and ZACHARIADES, 2013; YU *et al.,* 2014)**. The two biotypes are known to differ in

morphology, genetics and aspects of their ecology **(PATERSON and ZACHARIADES, 2013; ARC-PPRI,** South Africa, unpublished data).

1.2.1. Genetic and morphological dissimilarities in *Chromolaena odorata*

The southern African biotype of *C. odorata* (hereafter referred to as SAB) is substantially different from the widespread invasive biotype found in Asia, West and Central Africa (hereafter referred to as AWAB) in the following ways:

(i) the leaves of the AWAB are usually large with fine hairs giving a soft texture particularly to younger leaves, with a grey-green to dark-green colour but often purple at the young stage, especially when growing in the sun, while the leaves of the SAB are distinctly small and smooth with a dark-green colour when growing in semi-shade but yellow-green in the sun and red when young (Figures 1.2 A and B);

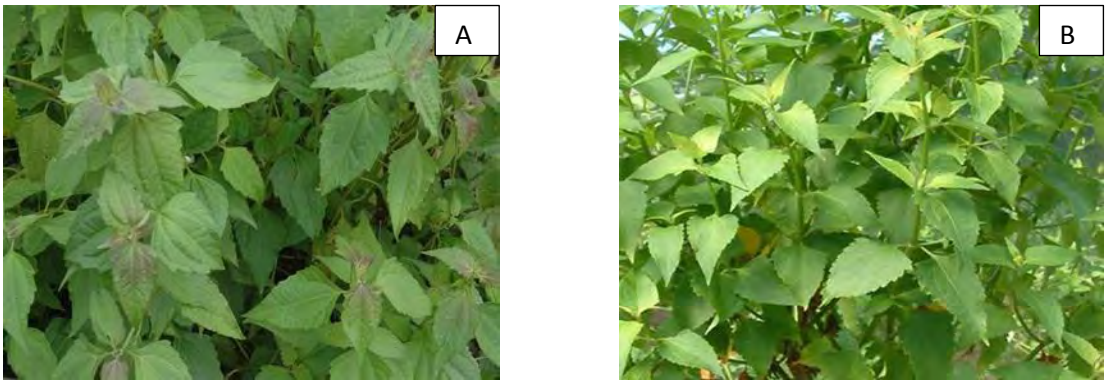

Figure 1.2: Leaves of the Asian/West African biotype (AWAB) of *Chromolaena odorata* (A) and of the southern African (SAB) *Chromolaena odorata* (B).

(ii) the stems of the AWAB are hairy, with a grey-green to dark green colour, while those of the SAB are largely smooth and yellow-green in colour;

(iii) the AWAB has broad individual flowers with a pale lilac colour, bracts have sharp tips and are lax around the flower-head, while the SAB flowers are

often narrow with whitish colour; bracts have round tips and are tight around the flower-head (Figures 1.3 A and B);

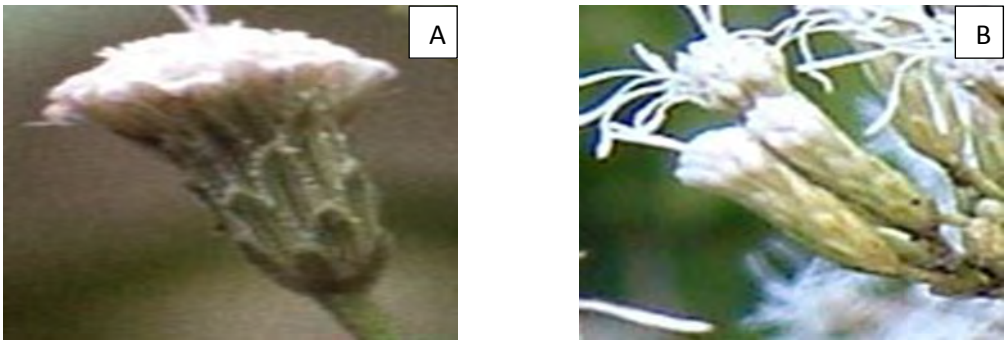

Figure 1.3: Flowers of the Asian/West African biotype (AWAB) of *Chromolaena odorata* (A) and of the southern African biotype (SAB) *Chromolaena odorata* (B).

(iv) the branches of the AWAB are not rigid, while the SAB has an upright posture, especially young growth in dense stands;

(v) the AWAB are more adapted to tropical conditions and may be more fire resistant, having a tendency to re-grow from the crown, while the SAB may be more cold-tolerant and more susceptible to fire; and

(vi) the AWAB has a very strong odour when compared to the SAB.

1.3. History, current distribution and impacts of *Chromolaena odorata* in Africa

Chromolaena odorata has a wide native range distribution from the southern U.S.A to northern Argentina, Central America and the Caribbean islands **(GAUTIER, 1992; KRITICOS *et al.,* 2005)**. A similar situation is increasingly becoming evident in its introduced range with the plant being present in Central, East, South and West Africa, Southeast Asia and parts of Oceania (Figure 1.4).

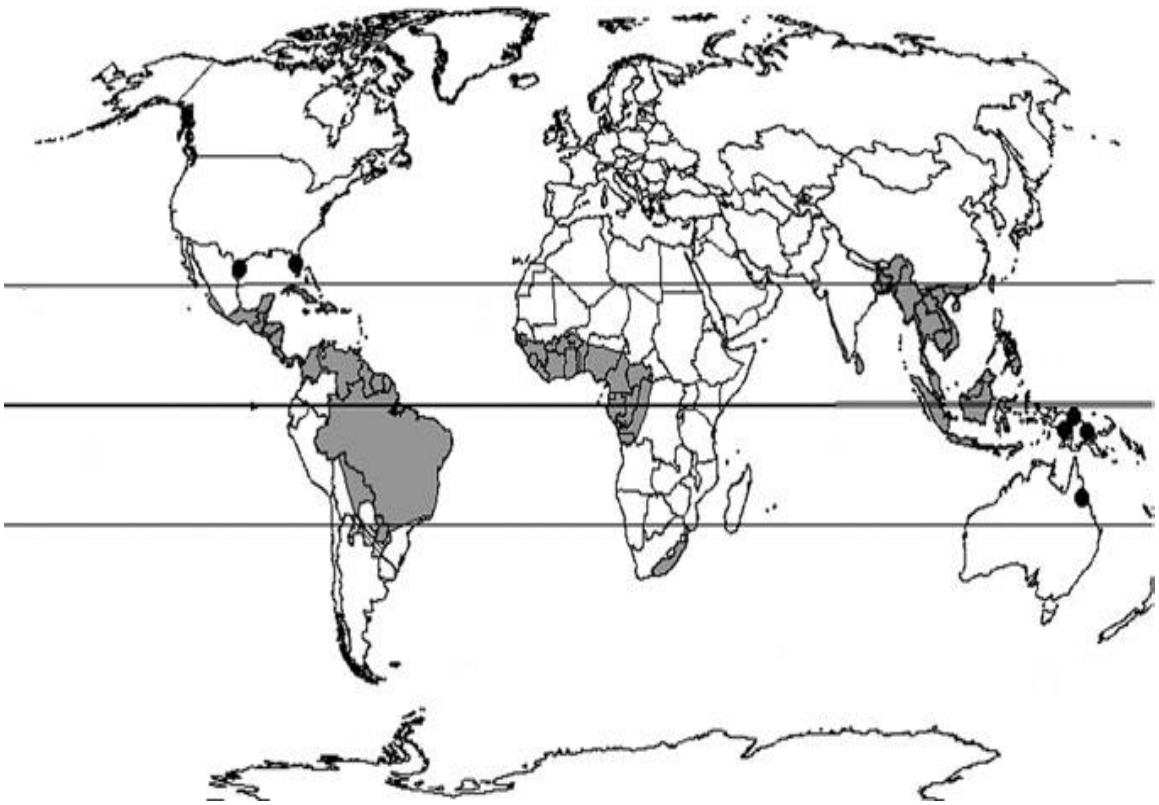

Figure 1.4: World distribution of *Chromolaena odorata* **(KRITICOS *et al*., 2005)**.

1.3.1. West Africa

The presence of *C. odorata* was first recorded in a forestry plantation near Enugu, in south-eastern Nigeria in 1942 and is thought to have resulted from contaminated seeds of the forest tree *Gmelina arborea* Roxb., imported from southeast Asia in 1937 **(IVENS, 1974; UYI *et al.,* 2014)**. The distribution of *C. odorata* in West Africa is shown in Figure 1.5. Following the introduction of the shrub, it quickly spread across many parts of Nigeria and to neighbouring countries, probably due to human and vehicular movement, road constructions and regional trades **(UYI *et al.,* 2014)**. Out of the 36 states in Nigeria, 23 states have already been colonised by *C. odorata*, especially the rainforest, mangrove forest, freshwater forest and the woodland savannah **(UYI *et al.,* 2014)**. *Chromolaena odorata* is thought to have been first introduced from Nigeria to Ghana **(TIMBILLA and BRAIMAH, 1996; HOEVERS and**

M'BOOB, 1996). The weed has occupied the high forest, semi-deciduous forest, coastal and forest savannah zones covering over two-thirds of the total land area in Ghana **(TIMBILLA *et al.,* 2003)**. As reported by **YEHOUENOU (1996)**, *C. odorata* spread into the southern Benin Republic and Togo around the 1970's and 1980's. The southern parts of Cote d'Ivoire have also been severely infested by *C. odorata* **(ZEBEYOU, 1991).** The spread of *C. odorata* to Liberia, The Gambia, Burkina Faso, Guinea and Sierra Leone have also been reported **(TIMBILLA *et al.,* 2003)**.

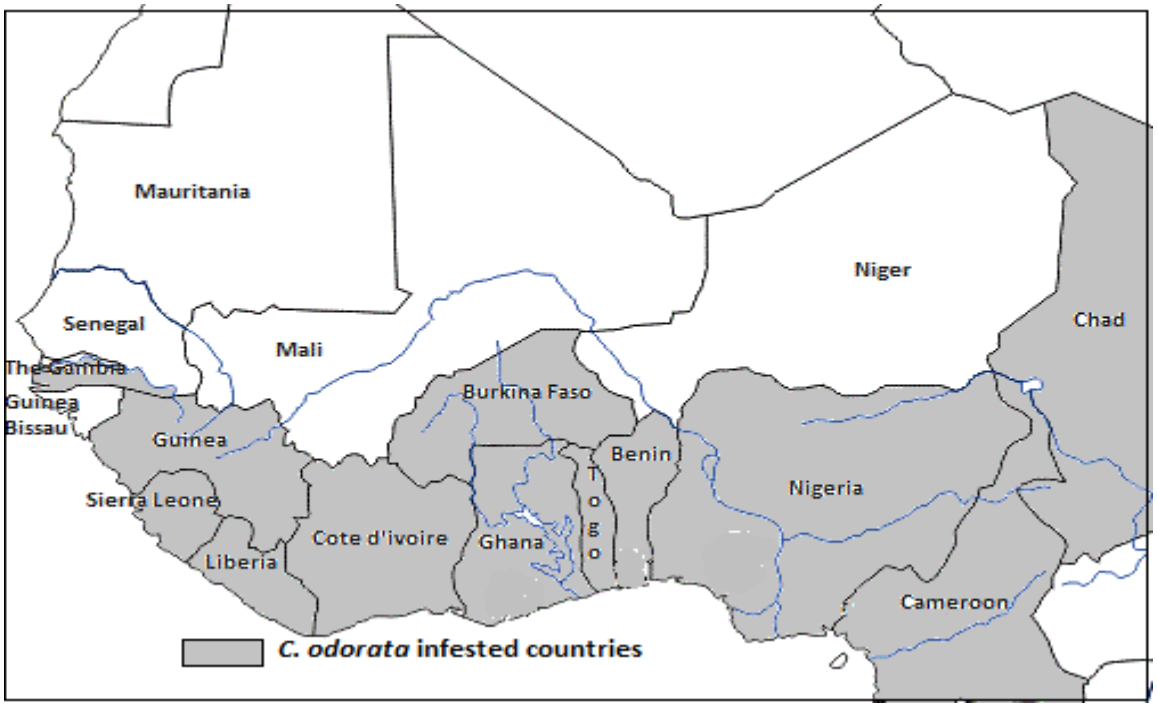

Figure 1.5: The distribution of *Chromolaena odorata* in West Africa and neighbouring countries (**UYI and IGBINOSA, 2013**).

Although the status of *C. odorata* in West Africa remains a subject of debate **(UYI and IGBINOSA, 2013; UYI *et al.,* 2014)**, its impacts on agriculture, livelihood and biodiversity have been documented **(LUCAS 1989; YEBOAH 1998; UYI and IGBINOSA, 2013)**. *Chromolaena odorata* competes with agricultural crops, causing decreases in crop yield by its suppressive abilities or allelopathic properties. It is a

major weed in crop plantations, such as cocoa, coffee, oil palm, cotton, rubber, cassava, banana, plantain and yam as well as vegetables in Nigeria **(UYI *et al.,* 2014)**. In Cote d' Ivoire, many farmers abandoned their coffee and cocoa plantations as the farm lands were invaded by *C. odorata* **(ZEBEYOU, 1991)**. Interest in establishing young cocoa, rubber and oil palm plantations by farmers in Nigeria is often lost due to problems created by *C. odorata* **(SHELDRICK, 1968; IKUENOBE and AYENI, 1998)**. Farmers in Ghana experience incomplete harvesting of crops as *C. odorata* drastically reduces their chances of returning to farmlands **(TIMBILLA and BRAIMAH, 1998)**. The weed harbours some crop pests such as *Zonocerus variegatus* (L.) (Orthoptera: Pyrgomorphidae) and *Aphis spiraecola* Patch (Homoptera: Aphididae) **(BOPPRÉ, 1991; UYI *et al.,* 2008)**.

Chromolaena odorata competes effectively with native plants and becomes dominant, especially because of its allelopathic properties. This may lead to the extinction of local plant species thereby reducing biodiversity of the ecosystem **(TIMBILLA *et al.,* 2003)**. Fields invaded by *C. odorata* are avoided by grazing animals possibly because of the presence of pyrrolizidine alkaloids (PAs) that may cause livestock death **(HOEVERS and M'BOOB, 1996; ZHAO *et al.,* 2001; KONE and KANDE, 2012)**.

1.3.2. Southern Africa

The *C. odorata* biotype in South Africa is believed to have originated from Jamaica or Cuba **(PATERSON and ZACHARIADES, 2013)** and was first recorded in 1947 **(ZACHARIADES *et al.,* 2011)**. It naturalised from a site east of Ndwedwe (290 30` S 300 56`E) near Durban **(HILLIARD, 1977)**. Its spread is restricted to the warmer sub-

tropical eastern and north-eastern parts of the country where it is present in KwaZulu-Natal, Mpumalanga, Limpopo and Eastern Cape Provinces **(GOODALL and ERASMUS, 1996; KRITICOS *et al.,* 2005)**. *Chromolaena odorata* spread has reached its southern ecological limit around the Port St Johns Region of the Eastern Cape Province **(ZACHARIADES *et al.,* 2011)**.

The plant was introduced probably from Asia into Mauritius before 1949 **(ZACHARIADES *et al.,* 2009)**, while it was discovered in Zimbabwe in the late 1960s and in northern Angola in the late 1970s **(GAUTIER 1992; HOEVERS and M'BOOB, 1996)**. Some parts of Mozambique, Swaziland and Malawi have also been infested by *C. odorata* **(ZACHARIADES *et al.,* 2013)**. The distribution of *C. odorata* in South Africa is shown in Figure 1.6.

Chromolaena odorata invades the forest and savannah biomes of South Africa, Lesotho and Swaziland **(ZACHARIADES *et al.,* 2011, 2013)**. It has become problematic in some southern African countries as it densely occupies roadsides and abandoned farmlands. The plant is thought to impact negatively on livestock grazing **(GOODALL and ERASMUS, 1996)**. Because of the scrambling nature of the plant, it develops dense free-standing shrubs forming canopies over other plants. These plants then become smothered **(ZACHARIADES *et al.,* 2009)**. The plant has the tenacity to invade both human-induced disturbed and undisturbed lands. The growth of *C. odorata* along riverbanks in South Africa has been reported by **LESLIE and SPOTILA (2001)** to interfere with the egg-laying of the Nile crocodiles and to alter the sex ratio in the progeny by shading the nests.

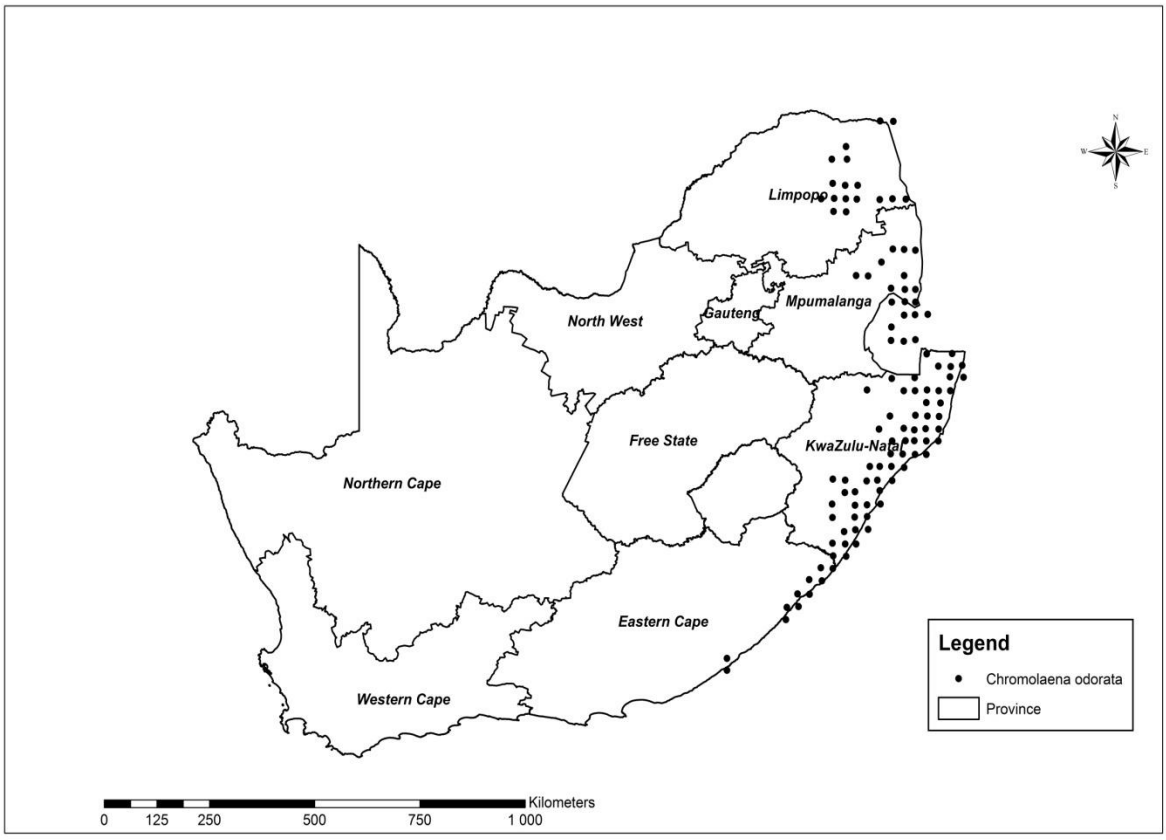

Figure 1.6: Distribution of *Chromolaena odorata* in South Africa and Swaziland. (From **UYI, 2014,** originally drawn by L. Henderson; data source: SAPIA database, ARC-Plant Protection Research Institute, Pretoria).

1.3.3. East and Central Africa

Chromolaena odorata appeared in East and Central Africa much later than in West and southern Africa. The spread and status of *C. odorata* in the region was recently reviewed by **ZACHARIADES *et al*. (2013)**. It was first confirmed present in Kenya in 2006, while it was recorded in the eastern part of Rwanda in 2003. The west of Busia where Uganda borders Kenya has also been infested by *C. odorata.* The presence of *C. odorata* in Tanzania was recorded between 2009 and 2010 near the eastern shores of Lake Victoria by these authors.

In the mid-1970s *C. odorata* was recorded in the central parts of the Democratic Republic of Congo **(GAUTIER, 1992; HOEVERS and M'BOOB, 1996)**. It is now present in the western parts of the country, and also the eastern parts close to the border with Burundi and Uganda **(ZACHARIADES *et al.,* 2013)**. *Chromolaena odorata* spread from the south-eastern states of Nigeria to Cameroon and has also been reported in Chad **(ZEBEYOU, 1991; HOEVERS and M'BOOB, 1996; TIMBILLA, 1998)**. The spread of the weed in this region has been possible through human and vehicular movements and dispersal of the seeds by wind and water **(ZACHARIADES *et al.,* 2013)**. Though not yet obvious in East and Central Africa, *C. odorat*a is expected to have a similar impact on agriculture, biodiversity and human livelihood, as has been reported in other regions where the species is currently invasive.

1.4. Beneficial attributes of *Chromolaena odorata*

Chromolaena odorata negatively impacts on agriculture, human livelihood, biodiversity and ecotourism in its introduced range where it has become invasive. Nevertheless, the usefulness of *C. odorata* as a fallow plant and with regard to its soil improvement properties and medical potential has been recognised.

1.4.1. Fallow and soil improvement properties

Many publications describe the importance of *C. odorata* as a fallow species in slash and burn fallow rotations practiced by farmers in West Africa **(TIAN *et al.,* 1998; AKOBUNDU *et al.,* 1999; TIAN *et al.,* 2005)**. Also, biogas can be produced from the plant by anaerobic digestion **(JAGADEESH *et al.,* 1990)**. It also has the ability to improve soil nutrients **(AMIOLEMEN *et al.,* 2012)** by increasing the essential

elements in the soil **(JIBRIL and YAHAYA, 2010)**. The plant is reported to be a good bioremediating and phytoremediating agent in heavy metal and crude oil-polluted soils **(ANOLIEFO *et al.,* 2003; AGUNBIADE and FAWALE, 2009; ALCANTARA *et al.,* 2013)**.

1.4.2. Nutritional value (human and livestock nutrition)

Chromolaena odorata leaves are a good source of protein, ash, carbohydrate, fibre and energy (**NWINUKA *et al.,* 2009)**. The leaf is also a rich source of calcium, sodium, magnesium, potassium, iron, zinc, copper, manganese and phosphorus **(NWINUKA *et al.,* 2009)**. **APORI *et al.* (2000)** suggested the use of *C. odorata* as a protein supplement in ruminant feeds because of the high crude protein content in the plant, although further investigations may be required to rule out toxicity effects on livestock. The plant is eaten by locals in southern parts of Nigeria as a vegetable **(personal observation)** probably because of its high nutritional content.

1.4.3. Medicinal potential

A medicinal plant is defined as any plant of which one or more of its organs contain substances that can be used for therapeutic purposes, or which can be used as precursors for the synthesis of useful drugs **(SOFOWORA, 1982)**. Though *C. odorata* has been known for its negative impact, the potential medicinal uses are enormous **(OWOYELE *et al.,* 2005; VAISAKH and PANDEY, 2012)**. Traditional healers in some parts of Africa explore the plant as a source of medicine in curing different ailments.

1.4.3.1. Traditional usage

Chromolaena odorata is used as a source of medicine in traditional medicinal practice in West Africa and countries in Asia. The plant is known for its medicinal properties especially in the treatment of wounds (**PHAN *et al*., 2001)**. The traditional medicinal usage of *C. odorata* in some West African countries is detailed in Table 1.1. Although several traditional uses of this plant have been recognised by locals in a number of West African countries and even in Cameroon (Central Africa), there is currently no known traditional usage for this plant in eastern and southern Africa.

A number of scenarios or hypotheses may partly explain the non-usage of the plant by traditional medicinal practitioners in these regions. Firstly, the recent arrival of the species into eastern Africa might suggest that traditional medicinal practitioners or locals are yet to fully understand the benefits of the plant, as recently introduced plant species may not be utilized by locals. Secondly, knowledge of the medicinal usefulness of the plant may elude practitioners or locals in South Africa because the plant is restricted to very limited parts of the country. Thirdly, ethnobotanical studies on indigenous knowledge of the medicinal usage of the plant are still scanty. Finally, the *C. odorata* biotype invasive in South Africa may not have (or may have fewer) medicinal properties compared with the AWAB which has been recorded to have medicinal usage by locals in West Africa and Southeast Asia. Due to the documented medicinal potential of this plant in a number of West African countries, studies on the invasive biotype of the species in South Africa are needed to either validate or invalidate the above conjectures.

Table 1.1: Ethnopharmacological usage of the Asian/West African *Chromolaena odorata* biotype in its distribution range.

Category of use	Description of traditional usage	References
Coughs and colds remedy	The plant is squeezed in water and the extract is taken to cure colds and coughs	**MORTON (1981), TIMBILLA *et al.* (2003)**
Skin diseases	The leaf is squeezed in water to bath	**MORTON (1981)**
Wounds and antiseptic	The leaves are squeezed and the juice is directly applied to the wound	**ADJANOHOUN *et al.* (1981), INYA-AGHA *et al.* (1987)**
Dysentery	The leaves are squeezed and taken as a tonic	**GILL (1992)**
Headache	The leaves are squeezed and taken as a tonic	**GILL (1992)**
Toothache	The leaves are squeezed and the juice is applied to the aching part	**GILL (1992)**
Malaria fever	A decoction of the leaves with *Azadiracta indica* is prepared and the water is taken	**AYENSU (1978), GILL (1992), IDU AND ONYIBE (2007)**
Antiseptic	The juice of the leaves, sometimes mixed with water, is used to stop bleeding	**GILL (1992)**
Stomach problems	Fresh leaves are squeezed in water and the juice is taken as a tonic	**HOEVERS and M'BOOB (1996), IDU and ONYIBE (2007).**
Antiseptic and haemostatic	Fresh juice from the leaves is used to arrest bleeding in fresh cuts and nose bleeds	**PHAN *et al.* (2001), IDU and ONYIBE (2007)**
Diarrhoea	The leaves are squeezed with water and the decoction is taken as a tonic	**AMATYA and TULADHAR (2011), BHARGAVA *et al.* (2011)**
Skin eruption	The fresh leaves are squeezed and the juice is applied to affected areas of the skin	**AMATYA and TULADHAR (2011), BHARGAVA *et al.* (2011)**

Fungal infections	The leaves are squeezed and taken as juice	**NGONO *et al.* (2006)**
Stomach ulcers	The leaves are squeezed and the juice is combined with honey and taken as a tonic	**NUR JANNAH *et al.* (2006)**
Skin infection	The juice is squeezed from the leaves and applied to affected areas	**OWOYELE *et al.* (2005)**

1.4.3.2. Secondary metabolites/bioactivities

Phytochemical studies on the extracts of the AWAB of *C. odorata* have indicated the presence of tannins, terpenoids, cardiac glycosides, saponins, anthraquinones, phenols and alkaloids **(AKINMOLADUN *et al*., 2007; PANDA *et al.,* 2010; ANYASOR *et al.,* 2011; VIJAYARAGHAVAN *et al.,* 2013)**. About 44 different compounds have been isolated from *C. odorata* extracts using GC-MS **(RAMAN *et al.,* 2012)**. Because of the presence of these phytochemicals, the plant is said to have anthelmintic **(PANDA *et al*., 2010),** antioxidant **(RAMAN *et al*., 2012; VIJAYARAGHAVAN *et al*., 2013)**, analgesic and anti-inflammatory (**OWOYELE and SOLADOYE, 2006)**, anti-pyretic, antispasmodic, anti-inflammatory **(TAIWO *et al*., 2000),** analgesic **(CHAKRABORTY *et al*., 2011),** antimicrobial (**CHOMNAWANG, 2005)**, antimalarial **(ONGKANA, 2003)**, antioxidant and wound healing **(ANYASOR *et al*., 2011)** properties. Eupolin, a product from *C. odorata* leaves for soft tissue burns and wounds has been licensed for use in Vietnam **(PHAN *et al.,* 1998; RAINA *et al.,* 2008)**.

1.5. Aims and objectives

The current study was aimed at elucidating the phytochemical and pharmacological potential of two invasive biotypes of *C. odorata* (viz. the Asian/West African and the southern African biotypes).

The main objectives of the project were to investigate:

1. The antibacterial and antifungal activities of the Asian/West African (AWAB) and the southern African (SAB) biotypes of *C. odorata* and to identify the growth stage with the most suitable antimicrobial activities in the SAB plants;

2. The phytochemical composition of the Asian/West African and southern African biotypes of *C. odorata* and to identify the growth stage (of the SAB plants) with the most suitable pharmacological potential; and

3. The cytotoxicity and mutagenicity of different growth stages of the southern African biotype of *C. odorata* to obtain a preliminary indication of its safety for use as a source of medicine.

1.6. Rationale and general overview of the thesis

Invasive alien plants are known to pose a serious threat to both natural and semi-natural ecosystems **(TIMBILLA *et al*., 2003; ZACHARAIDES *et al*., 2009)**. However, the medicinal potential of some of these species has also been recognised **(IDU and ONYIBE, 2007)**. **Chapter 1** highlights the genetic and morphological dissimilarities in

C. odorata, the spread and impact of the plant as well as the ethnopharmacological usage of the Asian/West African *C. odorata* biotype (AWAB) in its invasive range.

While some literature exists on the phytochemistry and medicinal properties of the AWAB plants, literature on the SAB are scant or non-existent **(Chapter 1; OMOKHUA *et al*., 2015)**. Studies on the phytochemical composition and aspects of pharmacological investigations (the AWAB) usually make use of leaves of *C. odorata* plants from the wild (with no ecological or site history). So, to eliminate this potential variation, both biotypes were planted in a shade house environment. Hence, **Chapter 2** investigated the antibacterial and antifungal activities of the Asian/West African (AWAB) and the southern African (SAB) biotypes of *C. odorata*. A further objective of this Chapter was to identify the growth stage of the SAB plants with the best antimicrobial activity.

Chapter 3 comparatively investigated the phytochemistry of the two biotypes of *C. odorata* (AWAB versus SAB). The phytochemistry of the different growth stages of *C. odorata* (young, mature non-flowering and flowering plants) is yet to be clearly elucidated. Hence, this Chapter comparatively documented the composition and concentrations of potential medicinal compounds in the different growth stages of SAB plants.

Chromolaena odorata has been reported to be cytotoxic against a hepatocellular carcinoma (HepG2) cell line in an *in vitro* assay **(PRABHU and RAVI, 2012)**. Whether its cytotoxic activity is apparent only on certain cell types is yet to be established. In **Chapter 4**, cytotoxicity studies were carried out using the different

growth stages of the SAB plant on a non-cancerous cell line (Vero monkey kidney) in order to establish whether the plant is cytotoxic to normal cells. A further objective of this Chapter was to conduct mutagenicity tests in order to detect possible mutagenic ability of the SAB plant.

Chapter 5 presents a summary of the main findings of the study.

The section **'References'** provides a list of all the literature and materials cited in the thesis.

Appendix 1 represents a list of chemicals and solutions prepared and the protocols used in this study.

Appendix 2 details the stock solutions used in this study.

Appendix 3 provides a list of equipment/ brand and apparatus used in this study.

Chapter 2: Studies on the antimicrobial activities of the Asian/West African and southern African biotypes of *Chromolaena odorata* and between the different growth stages of the southern African biotype

2.1. Introduction

Infectious diseases are health disorders caused following infection with bacteria, fungi, viruses or parasites. These organisms cause serious health problems and cause many deaths worldwide. Reports show that 25% of the 57 million annual deaths globally are related to infectious diseases **(MORENS *et al.,* 2004)**. The use of antibiotics in the twentieth century for the treatment of diseases such as pneumonia, typhoid fever, dysentery, diarrhoea, and malaria has been successful in the past. However, some bacteria have developed resistance to many antibiotics **(BANDOW *et al.,* 2003)** leading to the emergence of multidrug-resistance which has created a situation in which there are few or no treatment options for infections caused by certain pathogenic bacteria **(WENZEL and EDMOND, 2000).** The development of new drugs for such diseases has become very important.

2.1.1. Bacterial infections

Bacteria are the most abundant unicellular organisms found on earth. They easily adapt to different environments which include air, water and land. Some bacteria are beneficial to humans and animals, while some are harmful. The beneficial ones are helpful to humans in many ways such as aiding digestion, preventing the establishment of colonies of pathogenic bacteria and are also useful for enrichment of soil, fermentation of alcohol beverages and cheese, and decomposition of organic sewage and toxic waste. Bacterial cells are prokaryotic in nature with rigid walls that help protect the cells from osmotic damage. Two different kinds of bacteria are

known: the Gram-negative and the Gram-positive bacteria, which differ in their cell wall structure **(SLEIGH and TIMBURY, 1998)**. The Gram-negative bacteria differ from the Gram-positive bacteria by the presence of an outer membrane high in lipopolysaccharides. They have a single layer of peptidoglycan and a periplasmic space which separates this layer from the cytoplasmic membrane. The Gram-positive bacteria have multilayers of peptidoglycan outside the cell membrane which retains crystal violet stain when washed with alcohol **(SLEIGH and TIMBURY, 1998)**.

There are many bacteria that cause infections in humans, for example *Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhimurium, Enterococcus faecalis, Escherichia coli,* and *Pseudomonas aeruginosa. Staphylococcus aureus (S.a)* is the most common species of *Staphylococcus* which is known to cause serious health problems in humans. They are facultative Gram-positive bacteria which can be found in the skin, hair, scalp and armpit. Though the species is not always pathogenic, it is a common cause of skin infections such as boils, pimples, scalded skin syndrome (SSS), respiratory diseases such as sinusitis and also food poisoning, cellulitis, pneumonia, meningitis, osteomyelitis **(LOWY, 1998)**; endocarditis, sepsis and toxic shock syndrome (TSS). It causes post-surgical wound infection and can also occur as a commensal. It is estimated that 20% of the human population are carriers of *S. aureus* **(KLUYTMANS *et al.,* 1997)**, and the species can be found as part of the normal skin flora and in the anterior nares of the nasal openings **(KLUYTMANS *et al.,* 1997)**. *Staphylococcus aureus* produces DNAse (deoxyribonuclease) which is capable of breaking down DNA, lipase to digest lipids, staphylokinase to dissolve fibrin and beta-lactamase for drug resistance

(KORZENIOWSKI and SANDE, 1982). They also secrete exotoxins such as superantigen (PTSAgs) and exfoliative toxins (EF).The bacteria can produce potent protein toxins, producing cell surface proteins that bind and inactivate antibiotics **(KORZENIOWSKI and SANDE, 1982)**. Some drugs used for the treatment of *S. aureus* are penicillin and gentamicin **(KORZENIOWSKI and SANDE, 1982)**. Showing a clear resistance trend, *S. aureus* was reported to resist sulpha drugs in the 1940s, penicillin in the 1950s, methicillin in the 1980s and recently vancomycin in 2002 **(CDC, 2002)**. Penicillin resistance by *S. aureus* is mediated by penicillinase (a form of beta-lactamase) production which cleaves the beta-lactamase ring of the penicillin molecule, making the antibiotic ineffective.

Enterococcus faecalis (E.f) formerly known as *Streptococcus faecalis* **(SCHLEIFER and KILPPER-BALZ, 1984)** is a Gram-positive, non-motile, facultative anaerobic bacteria species which is usually found in the gastrointestinal tracts of humans and animals. They can also be found in the root canal-treated teeth of humans **(MOLANDER *et al.,* 1998)**. *Enterococcus faecalis* can cause bacteremia, endocarditis, urinary tract infections and meningitis **(MURRAY, 1990; HIDRON *et al.,* 2008).** The species has been reported to resist antibacterial drugs such as aztreonam, cephalosporin, clindamycin, oxacillin and trimethoprim. The bacterium may also be resistant to vancomycin **(AMYES, 2007; COURVALIN, 2006)**.

Escherichia coli (E.c) is a Gram-negative bacterium usually found in the intestinal tract of humans and animals. The bacteria can also be pathogenic, resulting in food poisoning, diarrhoea, wounds and urinary tract infections **(SLEIGH and TIMBURY, 1998)**. *E. coli* has been reported to resist sulphonamide and ampicillin-sulbactam in

patients administered these drugs in the United States of America and United Kingdom **(KAYE *et al.,* 2000; ENNE *et al.,* 2001)**.

Klebsiella pneumoniae (*K.p*), a member of the Enterobacteriaceae family, is a rod-shaped Gram-negative bacterium. It can be found in the natural environment such as soil and water and common sites of colonization in humans are usually the eyes, respiratory tract, genito-urinary and gastrointestinal tracts **(PODSCHUN and ULLMANN, 1998)**. Some of the infections caused by the species are urinary tract infections, community acquired pneumonia, bacteremia, chronic pulmonary disease, soft tissue infection, upper and lower respiratory tract infections, septicaemia and diarrhoea **(ROBERT *et al.,* 1990; EINSTEIN, 2000; RYAN and RAY, 2004; HARYANI *et al.,* 2007)**. *K. pneumoniae* is resistant to cephalosporin beta-lactam antibiotics **(BRADFORD *et al.,* 1997; RAHAL *et al.,* 1998; KEYNAN and RUBINSTEIN, 2007)**.

Pseudomonas aeruginosa (P.a) is a Gram-negative opportunistic bacterial pathogen usually present in diverse environments. It can be isolated from animals and humans. *Pseudomonas aeruginosa* can be found in swimming pools, hot tubs, whirl pools, contact lens solution, humidifiers, vegetables and soils. The species is able to tolerate a variety of physical conditions and survive with minimal nutritional requirements, and because of this it is able to persist for a long time in the environment. *Pseudomonas* infection is prevalent among patients with cystic fibrosis, acute leukemia, burn wounds and organ transplants. Patients suffering from these ailments can be colonized by this bacterium and are at risk of developing serious infections such as malignant external otitis, endocarditis, meningitis,

pneumonia, endophthalmitis and septicaemia **(PIER *et al.,* 1983; PIER, 2007)**. *Pseudomonas aeruginosa* can be resistant to multiple classes of antibacterial drugs even during the course of treatment. This makes it difficult to actually select the most appropriate antibiotics.

Salmonella typhimurium (S.t) is a Gram-negative pathogenic bacterium which can be found in the intestinal lumen. It causes gastroenteritis in humans and other mammals. Its outer membrane consists largely of lipopolysaccharides (LPS) which help protect the bacteria from the environment. They undergo acetylation of the O-antigen making them difficult for antibiotics to recognize **(SLAUCH *et al.,* 1995)**.

Most of these bacteria-causing infections are able to thrive in humans, especially in developing countries due to bad living conditions such as poor sanitation, an overcrowded environment and lack of awareness **(OTSHUDI *et al.,* 2000; HOTEZ *et al.,* 2007)**.

2.1.2. Fungal infections

Fungal infections have also been associated with life threatening diseases and death **(LEHRNBECHER *et al.,* 2010)**. Although several species of fungi are pathogenic in humans, *Candida* species, especially *Candida albicans*, are responsible for the most minor to severe fungal infections, particularly in immunocompromised patients. *Candida* is usually a harmless opportunistic pathogen found in the digestive tract, genitourinary tract, skin and mouth of humans **(TAMPAKAKIS *et al.,* 2009; KIM and SUDBERY, 2011)**. With frequent use of antibiotics and immunosuppressive drugs such as corticosteroids, the immune system becomes suppressed, creating an

enabling environment for fungal growth. Symptoms that can be noticed in a person with a *Candida* infection may be fatigue, depression, anxiety, or fibromyalgia. *Candida* infection can result in skin problems **(DAI *et al.,* 2011)**, oral candidiasis **(GIANNINI and SHETTY, 2011)**, intestinal candidiasis **(KUMAMOTO, 2011)** and fungal sinusitis **(IVKER, 2012)**.

The number of chemotherapies used against fungal infections is relatively small when compared to antibacterial infections. Drugs like clotrimazole, fluconazole, itraconazole, voriconazole, caspofungin, griseofulvin and amphotericin B are used for the treatment of fungal infections but these drugs are limited in number. Amphotericin B is mostly used because of its binding ability to sterols, thereby disrupting the cell membrane of the fungus and killing the organism **(DEACON, 2006)**, but its use can be hindered by considerable kidney toxicity. Though newer derivatives of the drug, e.g. liposomal amphotericin B, have been formulated, the high cost of formulation makes it not easily affordable **(BASSETTI *et al.,* 2011)**. *Candida* species are highly resistant to antibiotics because of their eukaryotic nature **(SAKLANI and KUTTY, 2008)**. There is a need for the development of new drugs, and novel compounds sourced from plant materials may help in the fight against fungal infections.

2.2. Antibacterial and antifungal activity

Microbial infection is one of the most common health problems experienced by humans worldwide. There are many synthesized antimicrobial drugs available but, because of numerous side effects and the development of antibiotic resistance, plant-derived medicines might provide valuable alternatives. The AWAB has been

reported to possess good antibacterial activity against *Vibrio cholerae* **(ATINDEHOU *et al.*, 2013)**, *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* **(IROBI, 1992; ANYASOR *et al.,* 2011; SUKANYA *et al.,* 2011)**. Other bacterial strains such as *Bacillus subtilis, Corynebacterium glutamicin, Streptococcus thermophilus* and *Vibrio parahaemolyticus* are also inhibited by *C. odorata* extracts **(RAMAN *et al.,* 2012)**.

The aqueous ethanol extract of *C. odorata* has been reported to exhibit very good antifungal activity against *Cryptococcus neoformans, Microsporum gypseum, Trichophyton rubrum* and *Trichophyton mentagrophytes* **(NGONO *et al.,* 2006)**. The above reports show that the AWAB is a promising source of antimicrobial drugs. There is the possibility that the SAB may also possess this same antibacterial and antifungal activity. Hence, it is essential to investigate and compare both biotypes for antimicrobial activities and also to further investigate the different growth stages (young, mature non-flowering and mature flowering plants) of the SAB in order to ascertain the best morphological growth stage at which the plant may have the best antimicrobial activity.

Table 2.1: Overview of reported antibacterial activities of Asian/West African biotype of *Chromolaena odorata* extracts

Extractant	Test model	Organism tested[1]	Result	MIC (mg/ml)	References
Ethanol	Agar diffusion	*Sa*	Sensitive	0.1	**ANYASOR *et al.* (2011)**
		Ec	Sensitive	0.8	**ANYASOR *et al.* (2011)**
		Pa	Resistant	Not stated	**ANYASOR *et al.* (2011)**
		St	Sensitive	Not stated	**ANYASOR *et al.* (2011)**
		Pv	Resistant	Not stated	**ANYASOR *et al.* (2011)**
		Kp	Resistant	Not stated	**ANYASOR *et al.* (2011)**
Aqueous	Agar diffusion	*Sa*	Sensitive	Not stated	**ANYASOR *et al.* (2011)**
		Ec	Resistant	Not stated	**ANYASOR *et al.* (2011)**
		Pa	Resistant	Not stated	**ANYASOR *et al.* (2011)**
		St	Sensitive	Not stated	**ANYASOR *et al.* (2011)**
		Pv	Resistant	Not stated	**ANYASOR *et al.* (2011)**
		Kp	Resistant	Not stated	**ANYASOR *et al.* (2011)**
Methanol	Agar diffusion	*Sa*	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Bs	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Cg	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		*St**	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Ec	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Kp	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Pv	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		St	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Vp	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
Aqueous	Agar diffusion	*Sa*	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Bs	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Cg	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		St	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Ec	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Kp	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		Pv	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
		St	Sensitive	0.01-1	**RAMAN *et al.* (2012)**

		Vp	Sensitive	0.01-1	**RAMAN *et al.* (2012)**
Crude extract	Agar diffusion	*St*	Sensitive	Not stated	**ZIGE *et al.* (2013)**
		Ec	Sensitive	Not stated	**ZIGE *et al.* (2013)**
Ethanol		*St*	Sensitive	Not stated	**ZIGE *et al.* (2013)**
		Ec	Sensitive	Not stated	**ZIGE *et al.* (2013)**
Aqueous		*St*	Sensitive	Not stated	**ZIGE *et al.* (2013)**
		Ec	Sensitive	Not stated	**ZIGE *et al.* (2013)**
Cyclohexane	Microdilution	*Ko*	Sensitive	1.25	**ATINDEHOU *et al.* (2013)**
		Se	Sensitive	1.25	**ATINDEHOU *et al.* (2013)**
		Ss	Sensitive	1.25	**ATINDEHOU *et al.* (2013)**
		Vc	Sensitive	1.25	**ATINDEHOU *et al.* (2013)**
Dichloromethane	Microdilution	*Ko*	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
		Se	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
		Ss	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
		Vc	Sensitive	0.156	**ATINDEHOU *et al.* (2013)**
Ethyl acetate	Microdilution	*Ko*	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
		Se	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
		Ss	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
		Vc	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
Butanol	Microdilution	*Ko*	Sensitive	1.25	**ATINDEHOU *et al.* (2013)**
		Se	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
		Ss	Sensitive	0.625	**ATINDEHOU *et al.* (2013)**
		Vc	Sensitive	0.312	**ATINDEHOU *et al.* (2013)**
Ethanol	Agar diffusion	*Pa*	Sensitive	8.0	**IROBI (1992)**
		Sf	Sensitive	6.0	**IROBI (1992)**
Ethyl acetate	Microdilution	*Bs*	Sensitive	7.0	**NAIDOO *et al. (*2011)**
		Bc	Sensitive	8.0	**NAIDOO *et al. (*2011)**
		Sa	Sensitive	8.0	**NAIDOO *et al. (*2011)**
		*Se**	Sensitive	7.0	**NAIDOO *et al. (*2011)**
		Ec	Resistant	No activity	**NAIDOO *et al. (*2011)**
		Pv	Resistant	No activity	**NAIDOO *et al. (*2011)**
		Ss	Resistant	No activity	**NAIDOO *et al. (*2011)**

		Ea	Resistant	No activity	**NAIDOO *et al.* (2011)**
Methanol	Microdilution	*Bs*	Sensitive	8.0	**NAIDOO *et al.* (2011)**
		Bc	Sensitive	7.5	**NAIDOO *et al.* (2011)**
		Sa	Sensitive	8.0	**NAIDOO *et al.* (2011)**
		Se	Sensitive	8.0	**NAIDOO *et al.* (2011)**
		Ec	Sensitive	8.5	**NAIDOO *et al.* (2011)**
		Pv	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Ss	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Ea	Resistant	No activity	**NAIDOO *et al.* (2011)**
Water	Microdilution	*Bs*	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Bc	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Sa	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Se	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Ec	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Pv	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Ss	Resistant	No activity	**NAIDOO *et al.* (2011)**
		Ea	Resistant	No activity	**NAIDOO *et al.* (2011)**

[1]*Bc, Bacillus cereus*; *Bs, Bacillus subtilis; Cg, Corynebacterium glutamicin*; *Ea, Enterobacter aerogenes*; *Ec, Escherichia coli*; *Ko, Klebsiella oxytoca*; *Kp, Klebsiella pneumoniae; Pa, Pseudomonas aeruginosa*; *Pv, Proteus vulgaris*; *Sa, Staphylococcus aureus; Se, Salmonella enterica*; *Se**, *Staphylococcus epidermidis*; *Ss*, *Shigella sonnei*; *Sf, Streptococcus faecalis; St, Salmonella typhi*; *St**, *Streptococcus thermophilus*; *Ss, Shigella sonnei*; *Vc*, *Vibrio cholerae*; *Vp, Vibrio parahaemolyticus*

2.3. Materials and Methods

2.3.1. 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) and Cedara (22° 38' 36.25" N, 120° 36' 12.36 E), near Pietermaritzburg on the 19th of 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 (29º 70' S, 31º 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 plant 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 or respective 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 (collection no. 1 and 2) 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 airtight containers at room temperature.

2.3.2. Preparation of plant extracts

Two g 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 g from SY, SMF and SMNF of the SAB were also weighed into 50 ml conical flasks and extracted using redistilled 70% EtOH, 50% methanol (MeOH), petroleum ether (PE) 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 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 experiment.

2.3.3. Antibacterial screening

2.3.3.1. Preparation of microbial stock cultures

Bacterial stock strains used for the assay were cultured in Mueller-Hinton (MH) agar 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 strains were streaked and sub-cultured on the plates. The inoculated bacterial plates were incubated for 24 h at 37°C to allow the bacterial colonies to develop. Bacterial growth was controlled by storing the plates at 4°C until required for bioassays.

2.3.3.2. *In vitro* antibacterial bioassay

All plant extracts, AMNF, SMNF of the two biotypes and SY, SMF and SMNF of the SAB *C. odorata* were tested for antibacterial activity as described by **ELOFF (1998)** through determination of the minimum inhibitory concentration (MIC) using the microdilution bioassay in 96-well microplates.

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 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 microplates, 50 µl of *p*-iodonitrotetrazolium (INT) was 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.

2.3.4. Antifungal Screening

2.3.4.1. Preparation of fungal stock culture

Candida albicans used for the antifungal bioassay was cultured in Yeast Malt (YM) agar. The autoclaved molten YM agar was poured into Petri dishes and allowed to gel following the procedure described in **Section 2.3.3.1.**

2.3.4.2. *In vitro* antifungal bioassay

The antifungal activity of extracts of both *C. odorata* biotypes was evaluated by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) using the microdilution bioassay in 96-well microplates as 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 *C. 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 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 x 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 was 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 minimum inhibitory concentration (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 and the concentration of the last clear well was recorded as the MIC values.

To determine the 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.

2.4. Results and Discussion

The minimum inhibitory concentration (MIC) values of the extracts tested for antibacterial and antifungal activity, and the minimum fungicidal concentration (MFC) values in the bioassays are presented in Tables 2.2 to 2.5. The mature non-flowering plant leaves of the AWAB and SAB *C. odorata* extracted with 70% EtOH, as well as the young, mature flowering and mature non-flowering plant leaf extracts of SAB *C. odorata* extracted with 70% EtOH, 50% MeOH, PE and distilled water were screened for antibacterial activity *against K. pneumoniae, P. aeruginosa, E. coli, E. faecalis, S. typhimurium,* and *S. aureus,* and for antifungal activity against *C. albicans.* The various leaf extracts evaluated were quantified in terms of the MIC required for inhibition of the test microorganism growth as well as the lethal effect (MFC) of the extract against the test fungus in the *in vitro* bioassay. The MIC is referred to as the lowest concentration of the extract that produces an almost complete microbial growth inhibition in a liquid culture, while the MFC is the lowest concentration of extract that causes death of the test fungus in the *in vitro* assay.

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

Of the two biotypes investigated, both exhibited some level of inhibitory activity against all tested bacteria (Table 2.2). **FABRY *et al*. (1998)** suggested that for a plant extract to be considered to have good activity the MIC should be less than 8 mg/ml but clinically an extract or a compound is of little relevance if the MIC value is over 1 mg/ml **(GIBBONS, 2004)**. For this reason, extracts with MIC values between 1 and 6.25 mg/ml in this study were considered less active, while MIC values less than 1 mg/ml were considered very active.

Table 2.2: Antibacterial activity (MIC in 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**	1.56	1.56	**0.78**	3.12	**0.39**
	SMNF	1.56	6.25	3.12	**0.78**	3.12	3.12
Neomycin (µg/ml)		3.12	**0.78**	3.12	**0.78**	3.12	**0.39**

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.t* = *Staphylococcus aureus; S.a* = *Salmonella typhimurium.*
*Values boldly written are considered very active (< 1 mg/ml). Values greater than 1 mg/ml but less than 6.25 mg/ml are less active in this study.

Although the extract from the leaves of the AWAB plant had the lowest MIC and best activity against *K. pneumoniae* (0.78) and *S. aureus*, (0.39), *P. aeruginosa, E. coli and S*. *typhimurim* were less inhibited by the AWAB and SAB extracts. The extracts of both biotypes exhibited good activity against *E. faecalis* (0.78).

The Gram-negative bacteria were found to be less inhibited by both extracts than the Gram-positive bacteria. As indicated earlier 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 the bacterial growth without necessarily penetrating into the cells. The antibacterial activity exhibited by both biotypes may be associated with the presence of phytochemicals such as phenolics, flavonoids and tannins. Flavonoids such as sinensetin and scutellareine tetramethyl ether isolated from AWAB *C. odorata* have been reported to possess good antibacterial activity **(Table 2.1)**. The ethanolic extract of *C. odorata* has also been reported to increase the activity of tissue glutathione peroxidase and to decrease the concentration of malondialdehyde, thereby protecting the cells from oxidative damage **(NWANKPA *et al.,* 2012)**.

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

The antibacterial activity of the extracts of the different growth stages of SAB *C. odorata* leaves prepared using 70% ethanol, petroleum ether, 50% methanol and water were tested against *K. pneumoniae, P. aeruginosa, E. coli, E. faecalis, S. typhimurium* and *S. aureus* (Table 2.3).

Table 2.3: 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**	6.25	**0.78**
	SMNF	3.12	6.25	3.12	**0.78**	3.12	3.12
	SMF	1.56	6.25	3.12	**0.78**	3.12	3.12
PE	SY	1.56	6.25	3.12	**0.78**	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**	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**	6.25	6.25
	SMF	6.25	>6.25	>6.25	3.12	6.25	6.25
	Neomycin µg/ml	3.12	**0.78**	3.12	**0.78**	3.12	**0.39**

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.*

*Values boldly written 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.

Although all the aqueous ethanolic extracts showed antibacterial activity to various degrees against all tested bacteria, good activity was generally observed against the Gram-positive *E. faecalis* with the 70% ethanol extract with the young plant having the best activity. The young plant extract was the only one to have good inhibitory activity against *S. aureus*, another Gram-positive species.

The PE extracts of the various growth stages exhibited some inhibitory activity against all tested bacteria except for the mature flowering plant which showed no inhibitory activity against *E. coli.* A good activity was only observed from the young plant against *E. faecalis.* For the aqueous methanolic extracts of the young, mature non-flowering and mature flowering plants, some activity was also observed against tested bacterial strains, except for *P. aeruginosa* which was not inhibited by any of the extracts. The best activity was observed by the young plant 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 mature non-flowering aqueous extract displayed good activity against *E. faecalis.* **NAIDOO *et al.* (2011)** reported that the methanol leaf extract of the SAB (unspecified growth stage) 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,* but no activity was observed with the aqueous extract. 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, 2010)**.

Studies have shown that ethanolic extracts possess better antibacterial activity than water **(JÄGER, 2003)**, and this may be the reason why some traditional practitioners prepare selected plant remedies in alcohol **(SPARG *et al.,* 2000).** In this study, the antibacterial activity exhibited by various extracts may be due to the presence of phenolics, flavonoids, tannins and saponins, and other bioactive compounds **(COWAN, 1999)**.

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

The widespread AWAB and SAB *C. odorata* were investigated for antifungal activity against *Candida albicans* by determining the MIC and MFC (minimum fungicidal concentration) and the results are presented in Table 2.4. Determination of the fungistatic and fungicidal activity of a plant extract is important, as this will assess whether the extract only has the ability to reduce the multiplication rate or is also able to destroy the fungi completely.

Table 2.4: Antifungal activity (MIC and MFC) of Asian/West African and southern African biotypes of *C. odorata* against *C. albicans*.

Extract	Biotype	MIC (mg/ml)	MFC (mg/ml)
70% EtOH	AMNF	1.56	1.56
	SMNF	**0.78**	**0.78**
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.
*Values boldly written are considered very active (< 1 mg/ml), while values greater than 1 mg/ml but less than 6.25 mg/ml are less active.

Both biotypes inhibited the growth of the fungus but the SAB exhibited the best fungistatic (0.78) and fungicidal (0.78) activities. Aqueous and ethanolic leaf extracts of the SAB *C. odorata* have also been reported to exhibit some levels of antifungal activity against *Aspergillus flavus*, *Aspergillus glaucus*, *C. albicans*, *Candida tropicalis* and *Trichophyton rubrum* **(NAIDOO *et al.,* 2011)**. The authors found that the leaf ethanolic extract was more effective than the stem extract.

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

The young, mature non-flowering and mature flowering plant extracts of SAB *C. odorata* were investigated against *C. albicans* and MIC and MFC (minimum fungicidal concentration) values of different extracts are reported in Figure 2.5.

Table 2.5: 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**	**0.78**
	SMNF	**0.78**	**0.78**
	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.
*Values boldly written 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.

The 70% EtOH, PE, 50% MeOH and aqueous extracts of the different growth stages showed some level of antifungal activity, but a good activity was also detected with the 70% EtOH extracts of the young and mature non-flowering plants with both MIC

and MFC of 0.78 mg/ml. This shows that the leaves of the ethanolic extract of the young and mature non-flowering plants against *C. albicans* are fungicidal. A similar antifungal activity (though plant growth stage was not specified) was observed by **NAIDOO *et al.* (2011)** with the ethanolic extract of the leaves and stems of *C. odorata* against *C. albicans.*

2.5. Conclusions

The alien invasive plant *C. odorata* introduced into West Africa in the 1930s and southern Africa in the 1940s has become an economic and ecological burden to many countries in Africa. Two biotypes are recognised on the continent, viz the AWAB and SAB. Although the AWAB has been extensively exploited for its medicinal properties in West and Central Africa and in some countries in Asia, the SAB is yet to be recognised as a source of medicine in the region where it now occurs. The study showed that both biotypes possess antimicrobial activities against all tested strains, with the AWAB having the best antibacterial activity and the SAB the best fungicidal activity. This confirms that the SAB may be exploited for medicinal purposes relating to infectious diseases, as had been documented on the AWAB by various authors **(Chapter 1)** and also in this study. Further investigation of the different growth stages of the SAB showed that the leaves of the young, mature non-flowering and mature flowering plants exhibited antimicrobial activity, though the level of activity differed with the type of solvent used for extraction. However, to obtain a better result, the young and mature non-flowering plants are recommended for use. The isolation of bioactive compounds that may be responsible for antimicrobial activity as well as determination of the safety of the active plant extracts

will be of importance to reach a conclusion on their potential as sources of antimicrobial treatments.

Chapter 3: Studies on the phytochemical composition of the Asian/West African and southern African biotype of *Chromolaena odorata* and between the different growth stages of the southern African biotype

3.1. Introduction

Phytochemicals are naturally occurring chemicals found in plants **(BRIELMANN *et al.,* 2006)**. Plants are known to possess primary and secondary metabolites. While the primary metabolites such as proteins, amino acids, sugars, purines, pyrimidines, nucleic acids and chlorophylls play essential roles in the life of the plants, secondary metabolites, often referred to as phytochemicals, are generally used in defense mechanisms against enemies such as herbivorous animals, viruses, parasites and bacteria.

Many secondary metabolites are produced at various steps in metabolic pathways not directly related to photosynthesis or respiration **(COLEY *et al.,* 1985)**. Although these phytochemicals are abundant, not all plants have the capacity to produce them all. These phytochemicals are usually generated at specific developmental periods of plant life. Some of the phytochemicals produced by plants are alkaloids, saponins, phenols, flavonoids, tannins, essential oils, cardiac glycosides and steroids. With time, plants containing these phytochemicals have been shown to serve as sources of medicine for humans and animals **(see OMOKHUA *et al.,* 2015, and references therein)** because they may possess biological activities including antioxidant, antibacterial, antifungal, antipyretic, anthelmintic, antispasmodic, anti-inflammatory, and wound healing effects. Phytochemicals can also be used as antiseptics, fragrances, dyes, insecticides, stimulants and poisons **(CROZIER *et al.,* 2006)**.

3.2. Some phytochemicals in plants and their medicinal properties

3.2.1. Alkaloids

Alkaloids are a diverse group of low molecular weight organic bases containing secondary and tertiary or cyclic amines (**MAKKAR *et al.,* 2007)**. They form one of the largest single classes of plant phytochemicals **(ZULAK *et al.,* 2006)**. A large number of alkaloids isolated from plants are used in pharmaceutical industries, including morphine for pain relief, nicotine used in insecticides, cocaine which serves as a very good anaesthetic, reserpine for controlling high blood pressure, papaverine used as a muscle relaxant and quinine and artemisinin for the treatment of malaria **(WINK, 1998; BRIELMANN *et al.,* 2006)**.

The AWAB *C. odorata* has been reported to contain a number of pyrrolizidine alkaloids (PAs) which include 7-angeloylretronecine, 9-angeloylretronecine, 3'-acetylrinderine, intermedine and rinderine **(BILLER *et al.,* 1994)**. *Chromolaena odorata* employs these alkaloids as defensive compounds against generalist phytophagous insects because of their toxicity **(MACEL, 2011)**. Although PAs can be hazardous to the health of humans and animals because of their toxicity, PA-containing plants are still in use in many traditional medicines in Africa **(ROEDER and WIEDENFELD, 2011)**. Because the AWAB *C. odorata* contains alkaloids, it is possible that SAB plants may also contain these compounds.

3.2.2. Saponins

Saponins are naturally occurring glycosides which are found mainly in plants and in some marine organisms. They usually occur in plants as a mixture of structurally

related forms with similar properties. They are often characterized by surfactant properties, showing soap-like foam in aqueous solution. Some biological activities possessed by saponins can be attributed to their action on the cell membrane **(PLOCK *et al.,* 2001, FRANCIS *et al.,* 2002)**. Saponins have also been reported to possess haemolytic, antioxidant and antimicrobial properties **(FRANCIS *et al.,* 2002, MAKKAR *et al.,* 2007)**. Triterpenoid saponins isolated from *Platycodon grandiflorum* inhibit Hepatitis C virus replication **(KIM *et al.,* 2013)**.

3.2.3. Phenolics

Phenols are compounds having a benzene ring with a hydroxyl group directly bonded to it. Phenolic compounds are widely present in plants with about 8 000 phenolic structures known **(STRACK, 1997)**. Phenolics as a group include flavonoids and other related phenolic compounds such as tannins. **(BRIELMANN *et al.,* 2006)**. Phenolic compounds act as reducing agents, hydrogen donors, singlet oxygen quenchers, free radical scavengers, ion chelators, enzyme cofactors, catalysts of oxidative reactions, terminators of radical chain reactions, inhibitors of oxidases and free radical scavengers **(RICE-EVANS *et al.,* 1997; WEIQUN–WANG *et al.,* 1999; GAWLIK-DZIKI, 2008)**. Phenolic compounds possess biological activities such as anti-aging, anticarcinogenic, anti-apoptosis, anti-inflammatory, anti-atherosclerosis, endothelial function improvement and inhibition of angiogenesis and protection of the cardiovascular system **(HAN *et al.,* 2007)**.

Phenolic compounds such as protocatechuic, p-coumaric, ferulic, p-hydroxybenzoic and vanillic acids have been isolated from the AWAB *C. odorata* biotype and these compounds have been reported to help protect cultured skin cells and retard

oxidative degradation of lipids **(PHAN *et al.,* 2001)**. Whether phenolic compounds are also present in the southern African *C. odorata* biotype remains to be seen.

3.2.4. Flavonoids

Flavonoids are low molecular weight compounds which are comprised of a three-ring structure with various substitutions **(MIDDLETON *et al.,* 2000)**. Over 4 000 flavonoids with known structures have been isolated from plants **(HARBORNE, 1986)**. Flavonoids exert beneficial effects in a number of disease states including cancers, cardiovascular diseases and neurodegenerative disorders. They are active against diarrhoea, bronchitis and other microbial infections. Flavonoids can help prevent injury caused by free radicals through various mechanisms, such as direct scavenging of reactive oxygen species (ROS), activation of antioxidant enzymes, metal chelating activity, inhibition of oxidases, oxidative stress mitigation caused by nitric acid, reduction of α-tocopheryl radicals, a rise in uric acid levels and an increase in antioxidant properties of low molecular weight antioxidants**.** Flavonoids in plants possess antimutagenic and anti-carcinogenic activities as well as the ability to regulate cell proliferation **(CRAIG, 1999; KUNTZ *et al.,* 1999; MIDDLETON, *et al.,* 2000)**.

A number of flavonoid compounds have been isolated from the AWAB *C. odorata* **(BARUA *et al.,* 1978; IWU and CHIORI, 1984; WOLLENWEBER *et al*., 1995; LING *et al.,* 2007; JOHARI *et al.,* 2012)**. Because both biotypes of *C. odorata* are not genetically distinct enough to be classed as separate species, it is not impossible that similar flavonoids may also be present in SAB plants.

3.2.5. Tannins

Tannins are a group of phenolic compounds which are present in plants in condensed or hydrolysed forms **(BRIELMANN *et al.,* 2006)**. Medicinal properties of tannins such as antiparasitic activity against gastrointestinal nematodes, antimicrobial, antioxidant and anti-human-immunodeficiency-virus (HIV) activities have been documented **(ATHANASIADOU *et al.,* 2001; AKIYAMA *et al.,* 2001; FUNATOGAWA *et al.,* 2004; NOTKA *et al.,* 2004; HOSTE *et al.,* 2006)**. Although tannins have been found in the AWAB *C. odorata* **(AKINMOLADUN *et al.,* 2007)**, their presence in the SAB plant is yet to be elucidated.

3.3. Southern African biotype (SAB) *Chromolaena odorata*; a weed or medicinal plant?

Since the introduction of the SAB *C. odorata* into South Africa in the 1940s, the plant has been reported to be a threat to biodiversity because of its ability to suppress native vegetation through competition for light, high reproductive capacity, high growth and net assimilation rates and allelopathic properties, as well as its ability to grow in many soil types and in many climatic zones **(ZACHARIADES *et al.,* 2009; UYI *et al.,* 2014)**. The plant has been declared a 'Category 1' weed under the Conservation of Agricultural Resources Act (CARA) and the National Environmental Management: Biodiversity Act (NEMBA) on Alien and Invasive Species List in South Africa because of its invasiveness in the north-eastern parts of the country **(GOODALL and ERASMUS, 1996; NEL *et al.,* 2004; ZACHARIADES *et al.,* 2011)**. To control or eliminate the species, various strategies which include mechanical, chemical and biological methods are employed.

Although this species seems to be a threat to biodiversity and an economical burden, the current research shows that the plant may serve as a novel source of antibacterial and antifungal agents **(Chapter 2)**. Utilizing this plant as a source of medicine may serve as a form of control and also an alternative to highly exploited plants with similar medicinal properties. Since the AWAB biotype has been used as a medicinal plant, further investigation to compare the two biotypes to ascertain if phytochemicals reported in the AWAB are also present in the SAB, together with the quantity of these phytochemicals that may be present, is essential.

The age of a plant or its growth stage may influence the bioactivity displayed. The various growth stages of the SAB *C. odorata* were investigated for antibacterial and antifungal activity **(Chapter 2)**. In comparing the antimicrobial activity displayed by various growth stages, the young plant (SY) exhibited better activity against most of the bacterial strains tested, followed by the mature non-flowering plant (SMNF) and lastly the mature flowering plant (SMF). However, with the antifungal assay, the SY and SMNF both exhibited better activity than the SMF. If the phytochemicals that have been reported in the AWAB are also present in the SAB, there is the possibility that SY may be richer in phytochemicals than the SMNF and SMF. To verify this fact, it is important to further qualitatively and quantitatively investigate the phytochemicals present in the various growth stages.

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3.4. Materials and Methods

3.4.1. Plant collection

The leaves of the AWAB and that of the different growth stages of the SAB *C. odorata* plants were collected from the plants grown in the Botanical Garden of the

University of KwaZulu-Natal, Pietermaritzburg campus (see **Chapter 2** for details of the plant history).

3.4.2. Preparation of plant extracts

The leaves of the AWAB (AMNF) and that of the various growth stages (SY, SMNF and SMF) of SAB *C. odorata* were carefully collected and oven dried at 55°C for 72 h, after which the leaves were ground to powders using a blender. The powdered samples were stored in air tight plastic containers at room temperature until they were used for experiments. 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 (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.

3.4.3. Phytochemical detection between the AWAB and SAB and at different growth stages of the SAB

3.4.3.1. Alkaloid detection

As described by **MAKKAR *et al*. (2007)**, 0.5 g of various ground powdered leaves were measured into conical flasks and 20 ml of 50% MeOH were added, sonicated for 25 min and filtered through filter paper (No 1). From the filtrate, 5 ml were measured into test tubes in triplicate and a few drops of ammonium solution were added, shaken and left for 2 min. The formation of a precipitate indicated the presence of alkaloids. Another method described by **SOFOWORA (1993)** was also applied for the detection of alkaloids with little modification. To 0.1 g of various plant

samples, 5 ml of 1% hydrochloric acid were added and stirred in a water bath for 10 min. The mixtures were filtered under vacuum using filter paper. From each filtrate 1 ml was measured into test tubes and a few drops of Dragendorff's reagent were added. An orange-red colour indicated the presence of alkaloids.

3.4.3.2. Saponin detection

Using the froth test as described by **TADHANI and SUBHASH (2006)**, 2 g of plant samples were extracted with 20 ml of distilled water. From this, 2 ml were measured into test tubes and 10 drops of olive oil were added. The test tubes were corked and shaken vigorously for 5 min. The formation of an emulsion confirmed the presence of saponins. A further test was also carried out by adding 5 ml of 50% methanol to 0.1 g of various ground plant samples weighed into test tubes in triplicate. The test tubes were corked and shaken vigorously for 2 min. The appearance of a stable froth after allowing the mixture to stand for 45 min confirmed the presence of saponins.

3.4.3.3. Phenolic detection

The ferric chloride test was employed for phenolic detection. In triplicate, 0.1 g of various ground plant samples were extracted with 10 ml of 50% MeOH. Five ml of the various extracts were measured into test tubes and a few drops of 2% ferric chloride solution were added. A dark green colouration confirmed the presence of phenolic compounds.

3.4.3.4. Flavonoid detection

The sodium hydroxide (NaOH) test for flavonoids **(TREASE and EVANS, 2002)** with little modification was used to detect the presence of flavonoids. In triplicate, 0.1 g of

various ground plant samples were dissolved in 5 ml distilled water and the mixtures were filtered. Two ml of 10% NaOH were added. The appearance of a yellow colouration and disappearance after the addition of a few drops of HCl indicated the presence of flavonoids.

3.4.3.5. Tannin detection

In triplicate, 10 ml of distilled water were added to 0.5 g of various ground plant samples weighed into test tubes. The mixtures were filtered and a few drops of 1% ferric chloride were added to 2 ml of each filtrate. A blue-black colouration confirmed the presence of tannins.

3.4.4. Quantitative determination of phytochemicals between the AWAB and SAB and among the different growth stages of the SAB *C. odorata*

3.4.4.1. Folin-Ciocalteu (Folin-C) assay for total phenolics

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 was 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. A Student t-test was used to compare the amount of total phenolics of leaf extracts between the AWAB and SAB

and among the various growth stages of the SAB of *C. odorata* using Genstat version 14.0 (VSN International, Hemel Hempstead, UK).

3.4.4.2. Colorimetric assay for flavonoid determination

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 1M 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 was 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. A student t-test was used to compare the amount of flavonoids of leaf extracts between the AWAB and SAB and among the growth stages of the SAB of *C. odorata* using Genstat version 14.0 (VSN International, Hemel Hempstead, UK).

3.4.4.3. Butanol-HCl assay determination of condensed tannins

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 was also prepared. All test tubes containing the mixture were vortexed, covered properly with a lid and incubated at 99^{o}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. A Student t-test was used to compare the amount of tannins in leaf extracts between the two biotypes (AWAB and SAB) and among the three growth stages of the SAB of *C. odorata* using Genstat version 14.0 (VSN International, Hemel Hempstead, UK).

3.5. Results and Discussion

3.5.1. Determination of phytochemicals between the AWAB and SAB

3.5.1.1. Alkaloid detection between biotypes

The results of the qualitative detection of alkaloids in both biotypes (AWAB and SAB) are presented in Table 3.1. In the detection of alkaloids between the two biotypes, only the AWAB showed a positive result with few precipitates observed. The root of the AWAB has been reported to contain pyrrolizidine alkaloids (PAs) such as 7-angeloylretronecine, 9-angeloylretronecine, 3'-acetylrinderine, intermedine and rinderine **(BILLER *et al.,* 1994). BILLER *et al.* (1994)** reported only a low concentration of PAs in the leaves of AWAB plants. The absence of alkaloids in the leaves of SAB plants used for this study does not necessarily imply the absence of this type of compound in SAB plants because large 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 or quantify alkaloids in the extracts.

Table 3.1: Qualitatively detected phytochemicals in AWAB and SAB *Chromolaena odorata* leaf extracts.

Phytochemicals	AWAB (AMNF)	SAB (SMNF)
Alkaloids	+	-
Saponins	++	++
Phenolics	+++	+++
Flavonoids	+++	+++
Tannins	+	++

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

Further investigation is recommended to determine if alkaloids are also absent from the roots. Future studies should not only focus on the detection of alkaloids in the roots of the biotypes of *C. odorata* but also on the identification and quantification of alkaloids in this plant. Although alkaloids isolated from plants and plant parts containing alkaloids are known to play a role in ethnomedicine and pharmacology **(WINK, 1998; ROEDER and WIEDENFELD, 2011)**, certain of these compounds are also known to be poisonous to humans and livestock **(WINK, 1998; MACEL, 2011)**.

3.5.1.2. Saponin detection between two biotypes

The results observed regarding the qualitative detection of saponins are presented in Table 3.1. For both biotypes (AWAB and SAB) the presence of foam with a height of about 2 cm confirmed that both biotypes contain saponins. According to **FRANCIS *et al.* (2002)**, saponins dissolve in water to form colloidal solutions which foam after vigorous shaking. The result from this test agrees with that of **AKIMOLADUN *et al.* (2007)** for the AWAB, though no such experiment has been carried out previously on the SAB. The detection of this phytochemical on the SAB further confirms its

presence in the plant. This shows that both biotypes may serve as sources of antioxidant, anti-inflammatory, antimicrobial, antiviral, cytotoxic, anthelmintic, molluscicidal and anti-parasitic compounds (**AMOROS *et al.,* 1987; LACAILLE-DUBOI and RAGNER, 1996; SPARG *et al.,* 2004; ALI *et al.,* 2011; TAPONDJOU *et al.,* 2011; BI *et al.,* 2012; ZHANG and ZHOU, 2013)**.

3.5.1.3. Determination of total phenolics between two biotypes

The qualitative detection of phenolics in the biotypes (AWAB and SAB) were positive (Table 3.1) and this was confirmed by the presence of a dark-green colouration. The total phenolic contents of the leaves from the two biotypes (AWAB and SAB) investigated through quantitative determination based on the oxidation-reduction principle using Folin-C reagent are presented in Figure 3.1. The results suggest that both biotypes are rich in phenolics. Although the AWAB plants appeared to have a higher amount of total phenolics compared to the SAB plants, the difference was not statistically significant (t_2 = 2.10; P = 0.169). The AWAB has been reported to contain phenolic compounds such as p-coumaric, protocatechuic, p-hydroxybenzoic, ferulic and vanillic acids, and these phenolic compounds from AWAB have been reported to help protect cultured skin cells and retard oxidative degradation of lipids **(PHAN *et al.,* 2001)**. Isolation and identification of phenolic compounds present in the SAB is recommended to ascertain if the aforementioned and other phenolic compounds that can be useful in the pharmaceutical industries are also present.

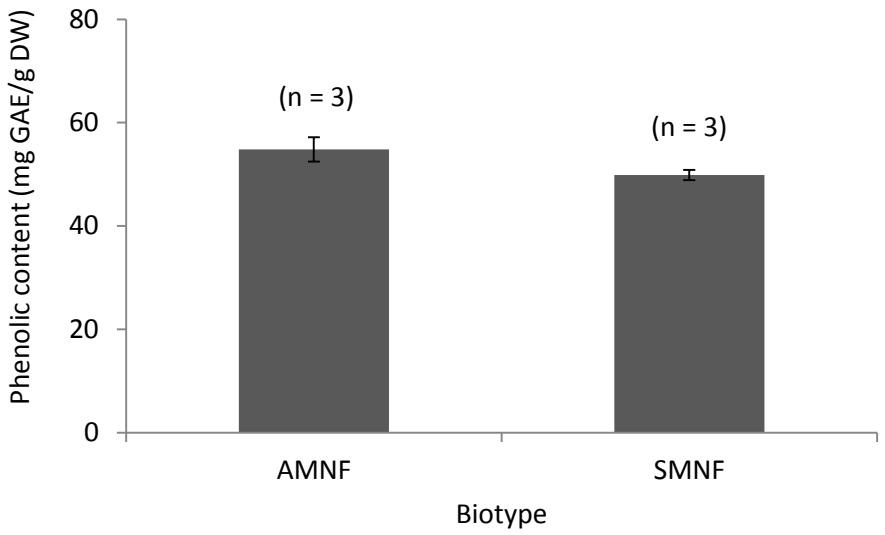

Figure 3.1: Total phenolic content as gallic acid equivalents detected in the leaves of AWAB and SAB *Chromolaena odorata* plants. Values in each bar are means ±SEM. Sample sizes are given in parentheses. DW= dry weight, GAE = gallic acid equivalents, AMNF= Asian/West African biotype mature non-flowering plant, SMNF = southern African biotype mature non-flowering plant.

3.5.1.4. Determination of flavonoid content between two biotypes

The qualitative detection of flavonoids in the AWAB and SAB using the sodium hydroxide test **(TREASE and EVANS, 2002)** confirmed the presence of flavonoids with a yellow colour change observed after the addition of diluted HCl as shown in Table 3.1.

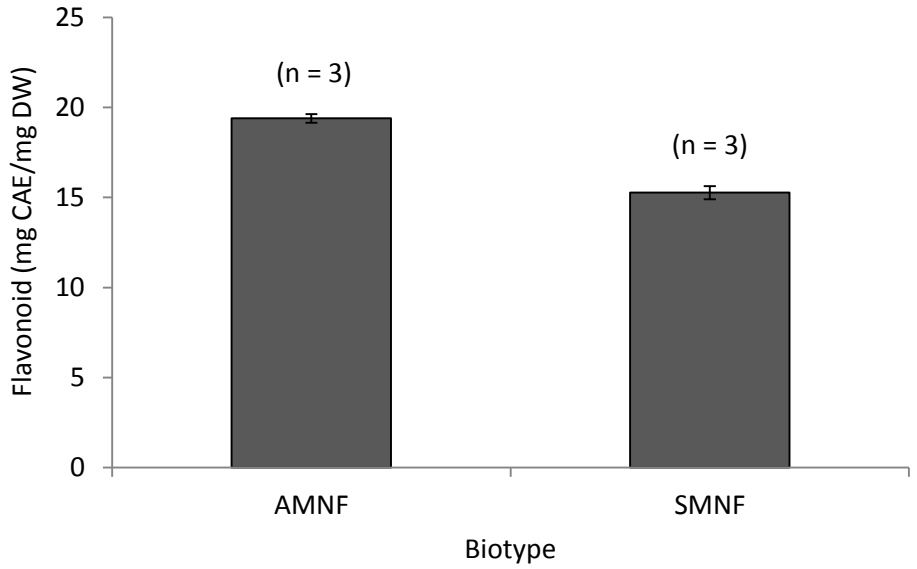

Figure 3.2: Flavonoid content as catechin equivalents detected in the leaves of AWAB and SAB *Chromolaena odorata* plant. Values in each bar are means ±SEM. Means followed by different letters are significantly different following student t test ($P < 0.05$) ($t_2 = 9.48$; $P < 0.001$). Sample sizes are given in parentheses. DW= dry weight, CAE = catechin equivalents, AMNF = Asian/West African biotype mature non-flowering plant, SMNF = southern African biotype mature non-flowering.

Employing the $AlCl_3$ colorimetric assay, the results expressed in mg CAE/g dry matter equivalents (Figure 3.2) showed that both biotypes contained a reasonable amount of flavonoids, although higher amounts were present in the AWAB plant ($t_2 = 9.48$; $P < 0.001$). This shows that both biotypes may be good sources of antifungal, antibacterial, antioxidant, anti-inflammatory, antioxidant, anticarcinogenic and antispasmodic agents **(HUSSAIN *et al.,* 2014)**. A flavonoid, 5-hydroxy-4,'7-dimethoxy flavone from AWAB *C. odorata* possesses good antimicrobial properties **(RAMAN *et al.,* 2012)**. **BARUA *et al.* (1978)** reported the presence of 2,'4-dihydroxy-4,'5,'6,'-trimethoxy chalcone, 2-hydroxyl-4,'5,'6,'4,'5-pentamethoxy chalcone, 4,-hydroxyl-5,6,7-trimethoxy flavone, scutellarein-tetramethyl ether and sinensetin (5,6,7,3,'4'- pentamethoxy flavones) in AWAB *C. odorata* collected in India. In a study carried out on antimicrobial activity of this biotype extract by **IWU**

and CHIORI, (1984) in Nigeria, kaempferol-4'-methyl ether, quercetagetin 3,5,7,3'-tetramethyl ether, naringenin-7-methyl ether and isosakuranetin were also present in the AWAB plants. **WOLLENWEBER *et al.* (1995)** isolated a large number of lipophilic flavonoid aglycones comprising 5 flavones, 10 flavonols, 5 flavanones, 2 dihydroflavonols and 3 chalcones from the AWAB plants. Another flavonoid that has been reported is laciniatin, (6, 4'- dimethyl ether) **(WOLLENWEBER *et al.,* 1995)**.

A new flavonoid, 3-hydroxyl-5,6,7,3,'4'-pentamethoxyl flavone, a novel pentamethyl ether of quercetagetin, was discovered by **WOLLENWEBER and ROITMAN (1996)** using column and thin layer chromatography. Quercetagetin-6,4'-dimethyl ether, a very rare flavonoid, was also discovered in the AWAB by **WOLLENWEBER *et al.* (1995)**. This flavone has previously only been found in *Brickellia laciniata* **(TIMMERMANN *et al.,* 1979)** and in two *Arnica* species **(MERFORT, 1985)**. Aromadendrin-7,4'-dimethyl ether which has also been detected in the AWAB **(WOLLENWEBER, *et al.,* 1995)** had previously only been reported in the bark of *Cephalanthus spathelliferus* **(LIMA and POLONSKY, 1973)**. Taxifolin-7-methyl ether has only been reported in *Prunus puddum*, *Artemisia glutinosa* and *Inula viscosa* (**WOLLENWEBER *et al.,* 1991)**.

There is a possibility that the AWAB can be effective against human small cell lung and breast cancer as studies carried out have demonstrated the presence of important compounds such as luteolin and acacetin **(SUKSAMRARN *et al.,* 2004)**. It is possible that SAB plants will also be effective against cancer cell lines. While this study detected and quantified flavonoids in the SAB plants, further studies should focus on the isolation and identification of the different types of flavonoids present in

this plant biotype. Such studies could help elucidate novel flavonoids that may be of pharmacological significance.

3.5.1.5. Determination of condensed tannins between two biotypes

In the qualitative detection of tannins the appearance of a blue-black coloration confirmed the presence of tannins in both biotypes as indicated in Table 3.1. The results obtained in the determination of condensed tannins using the butanol-HCl assay between the two biotypes (Figure 3.3) showed that both biotypes contain tannins, with leaves of the SAB plant having significantly higher amounts (t_2 = -2.96; P = 0.042). Both biotypes may not be a very rich source of tannins but the small amount present may act together with other phytochemicals present to influence the biological activity of the species.

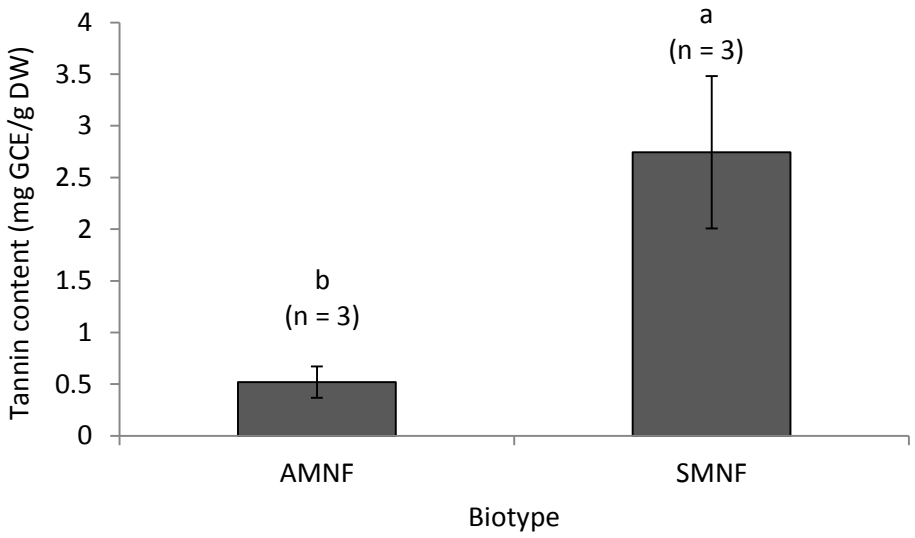

Figure 3.3: Condensed tannins content as cyanidine chloride equivalents detected in the leaves of AWAB and SAB *Chromolaena odorata* plants. Values in each bar are means ±SEM. Means followed by different letters are significantly different following Student t-test (P < 0.05). Sample sizes are given in parentheses. DW= dry weight, CCE = cyanidine chloride equivalents, AMNF = Asian/West African biotype mature non-flowering, SMNF = southern African biotype mature non-flowering plant.

3.5.2. Determination of phytochemicals in the growth stages of the SAB

3.5.2.1. Alkaloid and saponin detection

Investigation of the three growth stages of the SAB confirmed the absence of alkaloids in the SAB plants (Table 3.2), though it might be necessary to further investigate the root. Saponins were confirmed present in the young, mature non-flowering and mature flowering plant leaf extracts of the SAB by the appearance of foam of about 2 cm in height.

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Table 3.2: Results of the detection of phytochemicals in the different growth stages of SAB *C. odorata* leaf extracts

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

+ = present
++ = moderate
+++ = abundant
- = absent

3.5.2.2. Determination of total phenolics between the growth stages of SAB

The results of the quantitative determination of total phenolic content (mg GAE/g dry matter) on the different growth stages investigated showed that all the growth stages of the SAB are rich in phenolics (Figure 3.4). Although the amount of total phenolics in the leaves of the mature non-flowering (SMNF) plant seems to be higher (49.8 mg GAE/ g dry matter) compared with that of the other growth stages, the difference was not statistically significant (ANOVA: $F_{2,8} = 2.51$, $P = 0.161$; Figure 3.4).

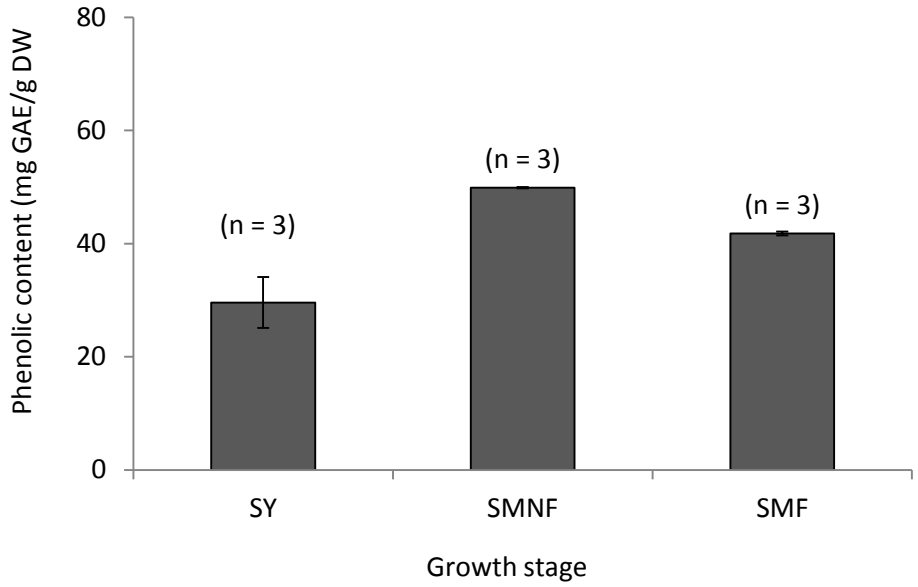

Figure 3.4: Total phenolic content as gallic acid equivalents detected in the three growth stages of southern African *Chromolaena odorata* biotype. Values in each bar are means ±SEM. Sample sizes are given in parentheses. DW= dry weight, GAE = gallic acid equivalent, SY= southern African biotype young plant, SMNF= southern African biotype mature non-flowering plant, SMF= southern African biotype mature flowering plant.

In the antibacterial assay **(Chapter 2)** the extracts from the leaves of the SY, SMNF and SMF plants exhibited moderate activity against all tested bacterial species, but good activity was observed by the three growth stages against *E. faecalis* and by the SY plant against *S. aureus.* Also, the same antifungal and fungicidal activity was exhibited by the SY and SMNF growth stages on *C. albicans* **(see Chapter 2)**. Considering the results in Figure 3.4, one can conclude that the amount of total phenolics present in a plant may not directly be responsible for the antimicrobial activity exhibited by the plant.

3.5.2.3. Quantitative determination of flavonoids in the AWAB and SAB *C. odorata*

Further quantitative determination of flavonoids among the different growth stages (Figure 3.5) of the SAB showed that the SY, SMNF and SMF are rich in flavonoids and that the amount of this compound differed significantly among the different growth stages of the SAB plants (ANOVA: $F_{2,8} = 125.4$, $P < 0.001$; Figure 3.5).

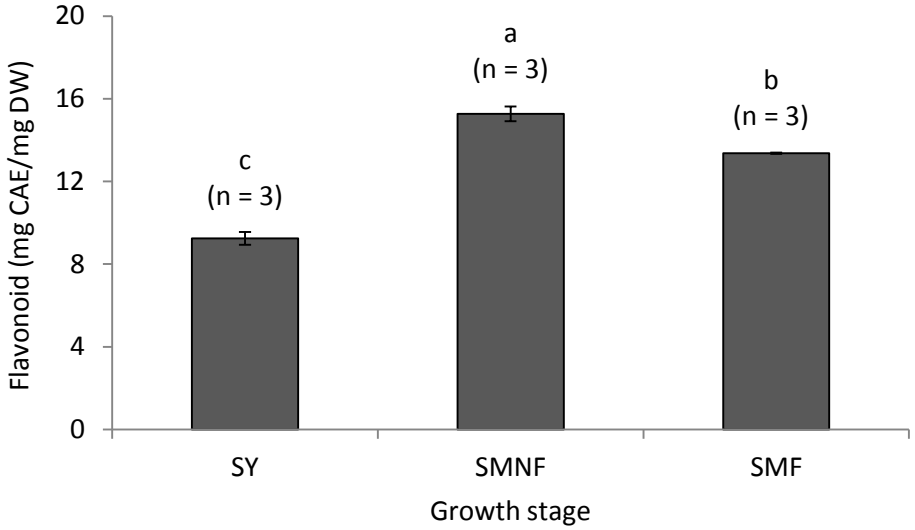

Figure 3.5: Flavonoid content as catechin equivalents detected in the three growth stages of southern African *Chromolaena odorata* biotype. Values in each bar are means ±SEM. Means followed by different letters are significantly different following Tukey's (HSD) test ($P < 0.05$). Sample sizes are given in parentheses. DW= dry weight, CAE = catechin equivalent, SY= southern African biotype young plant, SMNF= southern African biotype mature non-flowering, SMF= southern African biotype mature flowering plant.

The SMNF plants showed the highest flavonoid content while the SY plants showed the lowest amount. Considering the antimicrobial activity displayed by the three growth stages with the SY plant showing the best activity **(Chapter 2)**, it can be assumed that the amount of flavonoids present may not directly be responsible for the activity as they may be acting in synergy with other phytochemicals and unidentified bioactive compounds present in the different growth stages.

Investigation of other bioactivities, together with the isolation and identification of flavonoid compounds present in the different growth stages may be pertinent to further verify if the amount or type of flavonoids present may be a direct reason for such activity.

3.5.2.4. Tannin content in the three growth stages of the SAB

In the determination of condensed tannins among the three growth stages of the SAB, the amount of condensed tannins was not significantly different among the different growth stages (ANOVA: $F_{2,8} = 4.07$, $P = 0.076$; Figure 3.6).

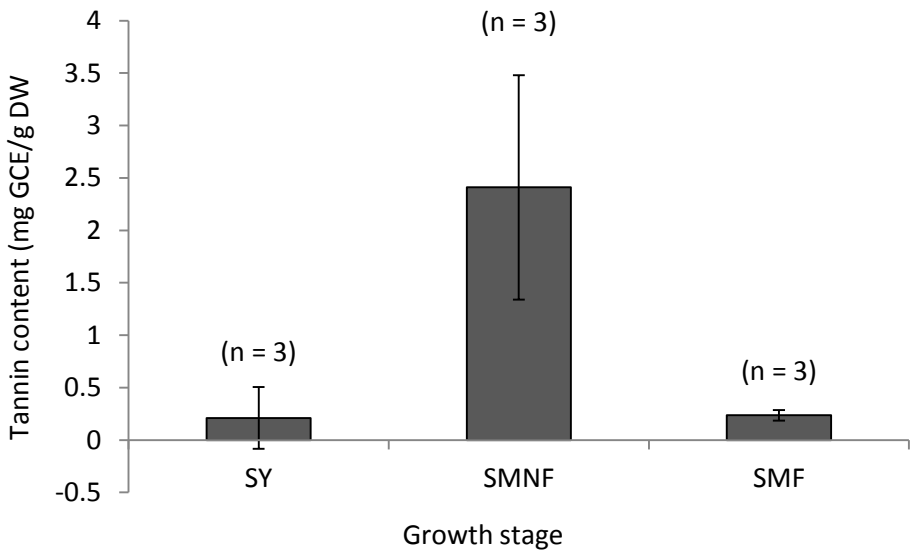

Figure 3.6: Condensed tannins content as cyanidine chloride equivalents detected in the three growth stages of southern African *Chromolaena odorata* biotype. Values in each bar are means ±SEM. Sample sizes are given in parentheses. DW= dry weight, CCE = cyanidine chloride equivalents, SY=southern African biotype young plant, SMNF= southern African biotype mature non-flowering plant, SMF= southern African biotype mature flowering plant.

Although the amount of tannins seems to be highest in SMNF plants, the antimicrobial activity displayed is not directly proportional but the low tannin content may be sufficient to contribute towards the anti-infective action of other phytochemicals present in the plant. Though pharmacological activities possessed by plants involve the interaction of various compounds, the presence of condensed tannins contributes mostly to the anthelmintic properties possessed by known medicinal plants **(JACKSON and MILLER, 2006)**. Proanthocyanidins, which include gallocatechin, epicatechin, catechin and epigallocatechin, help to inhibit the generation of chemiluminiscence by activating human polymorphonuclear neutrophils (PMN), which serve as a defence against infection such as inflammation **(POLYA, 2003)**. Tannins have also been reported to be cytotoxic with antitumor activity **(POLYA, 2003)**.

3.6. Conclusions

Phytochemical analysis of the AWAB (mature non-flowering) and SAB (mature non-flowering) of *C. odorata* and also on the different growth stages of the SAB, namely young, mature non-flowering and mature flowering was carried out. The results from the experiments showed that phytochemicals present in the AWAB such as phenolics, flavonoids, tannins and saponins, but not alkaloids, are also present in the SAB. However, phenolic and flavonoid contents were higher in the AWAB than the SAB while the tannin content was higher in the SAB than the AWAB. Further investigation among the growth stages of the SAB confirmed that the SAB is a good source of phytochemicals. Although the leaves of the mature non-flowering plant seem to contain the highest amount of phytochemicals such as phenolics, flavonoids and tannins compared to the young and mature flowering plants, the antimicrobial

activity exhibited does not appear to depend on or correlate with the quantity of these phytochemicals investigated.

Chapter 4: Cytotoxicity and mutagenicity evaluation of the different growth stages of the southern African *Chromolaena odorata* biotype

4.1. Introduction

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)**.

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)**. Mutagens are physical and chemical agents that are capable of changing the genetic material, usually DNA in organisms, and increase the frequency of mutations above the natural background level. These include ultraviolet (UV) and X-rays which are capable of deleting nucleotides. They can cause strand breaks, base damage and dimerization of bases in DNA. Mutagens are capable of initiating and promoting several diseases including infertility, growth mutation, arteriosclerosis and cancers. They can also cause disability and aging as well as genetic defects in offspring **(DE FLORA, 1998)**. Gene mutations can be assessed in bacteria as a change in their growth requirements, while in mammalian cells chromosome damage can be measured by observing the cell's chromosomes through magnification for breaks or rearrangements. The identification of compounds or chemicals capable of inducing

mutations is vital in safety assessments, as mutagenic compounds are capable of inducing cancer **(HECTH, 1999; SUGIMURA, 2000)**.

4.2. Plant cytotoxicity and mutagenicity

For many years plants have served as raw material for alternative medicines, and the use of plants as a source of food and medicine has continued to enjoy great patronage. According to the World Health Organization **(WHO, 2003)**, 60% of the world's population depends on medicinal plants and in some countries, traditional medicines are incorporated extensively into the public health system. A plethora of herbal drugs are available over the counter and at natural food stores. Self-medication with these substances has become a normal routine, but little is known about the safety of these herbal drugs.

Though there have been many findings on the advantages of the therapeutic use of medicinal plants, some of their constituents are potentially toxic, mutagenic and/or carcinogenic and can cause damage to DNA **(ALADE and IROBI, 1993; GADANO *et al.,* 2006)**. It is true that most plants used as sources of medicines are often not subjected to toxicological studies as required for modern pharmaceutical compounds. As they are based on the history of long term traditional use they are assumed to be safe **(EDZIRI *et al.,* 2011)**. Most herbal medicines escape toxicity testing before they are marketed, as most countries do not have laws that prohibit traditional medicines entering into the market **(SOBITA and BHAGIRATH, 2005)**. Many plants used as sources of food or medicine have been reported to have mutagenic effects detected by *in vitro* assays **(SCHIMMER *et al.,* 1994; KASSIE *et al.,* 1996; CARDOSO *et al.,* 2006; DEMMA *et al.,* 2009)**.

Chromolaena odorata possesses antibacterial and antifungal activity showing that it may be a source of antimicrobial agents **(Chapters 2)**. However, the safe use of this plant species is yet to be evaluated. A triterpene extracted from fresh leaves of *C. odorata* has been reported to be cytotoxic against a hepatocellular carcinoma (HepG2) cell line with an IC_{50} of 206.7 µg/ml in an *in vitro* cytotoxicity test using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay **(PRABHU and RAVI, 2012)**. Whether the plant may only be cytotoxic to cancer cell lines or also to normal human cells remains to be seen. Therefore it is important to investigate the cytotoxicity and mutagenicity of extracts and biologically active compounds from this plant to ascertain their safety and the existence of possible mutagens. This assessment can also be used for potential chemotherapeutic agents to minimize the adverse effect they may have on non-target tissues when they are used in clinical practice **(VERSCHAEVE *et al.,* 2004)**.

4.3. Materials and Methods

4.3.1. Preparation of plant extracts

Two g of ground plant material from the young, mature non-flowering and mature flowering plants were separately extracted with 20 ml of dichloromethane and 70% methanol following the procedure described in **Section 2.3.2.**

4.3.2. 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 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 resuspended in MEM to 5 x 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 amount 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.

4.3.3. 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.

4.4. Results and Discussion

4.4.1. Cytotoxicity assay

The cytotoxicity 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 4.1.

Table 4.1: Cytotoxicity of extracts of different growth stages of the SAB *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

Vero cells are a commonly used cell type for cytotoxicity tests as they are relatively easy to culture and are readily obtained. 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. This may contribute to the antimicrobial activities exhibited by the young plant **(Chapter 2)** as it is possible that the antimicrobial compounds present in the leaves may also be cytotoxic.

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4.4.2. Mutagenicity assay

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)**. From the results presented in Table 4.2, none of the fractions tested displayed any

mutagenic property (none of the results displayed any of the above listed trends: i and ii) 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.

Table 4.2: Mutagenic test of extracts of different growth stages of SAB *Chromolaena odorata* using *Salmonella typhimurium* TA98 and TA102 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.
* Initial concentrations of the fractions were 0.05, 0.5 and 5 mg/ml (5, 50 and 500 µg/plate).
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

4.5. Conclusions

Considering the results obtained in this study, the antimicrobial activities displayed by the young and mature non-flowering leaf extracts may be as a result of general toxicity. 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 in *in vivo*, the plant can possibly be used for topical applications to treat microbial infections, depending on the level of cytotoxicity. In the mutagenicity test without exogenous metabolic activation, none of the plant extracts showed a clear mutagenic effect. So 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.

Chapter 5: General Conclusions

5.1. Introduction

The challenge posed by the spread of the invasive alien plant, *C. odorata,* in southern Africa calls for concern. The species has been reported to smother native vegetation through competition for light as it forms shade over smaller plants due to its scrambling nature, allelopathic properties and high reproductive ability. For these reasons *C. odorata* has been declared a 'Category 1' weed under the Conservation of Agricultural Resources Act in South Africa because of its invasiveness in the north-eastern parts of the country **(GOODALL AND ERASMUS, 1996; NEL *et al*., 2004; ZACHARIADES *et al.,* 2011)**. Although different control methods which include mechanical, chemical and biological controls have been applied in attempting to control the plant, some of these methods seem not to be sustainable. Therefore, there is the need to find a way of utilizing the plant which may serve as a form of control.

Two biotypes of *C. odorata* are known, viz the Asian/ West African and the southern African biotype. The former has been exploited as a source of medicine where it is present while the latter is yet to be known for such a use although no reported studies have investigated this aspect. Hence, in this study the southern African biotype was compared to the Asian/West African biotype to ascertain if the southern African biotype contained some medicinal potential such as antimicrobial properties and phytochemicals that have been reported in the Asian/West African biotype. Further investigation was also carried out on the different growth stages of the SAB to confirm which stage of the plant is most active in antimicrobial studies, which of

the growth stages contain the highest amount of phytochemicals and how the quantity of the phytochemicals present may influence their bioactivities. To obtain indications of potential safe use of the plant species, the MTT test for cytotoxicity and the Ames test using *Salmonella* strains for mutagenicity were applied.

5.2. Pharmacological activities

The mature non-flowering leaf extracts of the Asian/West African and southern African biotypes were tested for antibacterial activity. Though both biotypes inhibited all tested bacterial strains to some degree, good activity was observed by extracts of both biotypes against *E. faecalis.* However, only the AWAB showed good activity against *K. pneumoniae* and *S. aureus.* Comparing their antifungal activity against *C. albicans,* both biotypes also exhibited some activity, but good fungicidal activity was only noticeable with the SAB extracts.

Further antibacterial activity studies were carried out on the young, mature non-flowering and mature flowering leaf extracts of the SAB using different solvents. All the 70% ethanolic extracts showed some level of activity with all tested bacterial strains but good activity by all the growth stages was only observed against *E. faecalis*. Only the young leaf plant extract showed good activity against *S. aureus*. The petroleum ether extracts of the young, mature non-flowering and mature flowering plants also showed some level of activity, except for the mature flowering leaf extract which did not inhibit *E. coli.* Good activity was only exhibited by the young plant against *S. aureus*. Extracts prepared using 50% methanol inhibited the bacterial strains but no activity was noticed against *P. aeruginosa.* Only the young plant exhibited good activity against *E. faecalis.* With regard to the water extracts,

although some level of activity was observed, none of the extracts inhibited *P. aeruginosa* and *E. coli.* Only the mature non-flowering plant leaf extract showed good activity against *E. faecalis.* Assessing their overall activity, the young plant showed the best activity against most of the bacterial strains tested, followed by the mature non-flowering and lastly the mature flowering plant. The above observations suggest that the young and mature non-flowering plants should be explored further.

In the antifungal activity tests, the leaves from all the growth stages extracted with 70% ethanol, petroleum ether, 50% methanol and water showed a broad spectrum of antifungal activity. Only the ethanolic extract of the young and mature non-flowering leaf extracts showed good fungistatic and fungicidal activity against *C. albicans*, suggesting that only the young and mature non-flowering plants may be used for product development.

5.3. Phytochemical analysis

Qualitative phytochemical analysis showed that phytochemicals such as phenolics, flavonoids, tannins, and saponins are present in both biotypes, but alkaloids were only present in the AWAB. For the quantitative determination for total phenolics, flavonoids and tannins, both biotypes were rich sources of total phenolics and flavonoids. Although higher amounts were present in the AWAB than the SAB, small amounts of tannins were present in both biotypes, with the highest amount recorded in the SAB.

Comparing the phytochemicals in the three growth stages of the SAB, total phenolics were higher in the SMNF plant, followed by the SMF plant and lastly the SY plant,

but the amount was not statistically significant. The flavonoid content was also higher in the SMNF plant, followed by the SMF plant and lastly the SY plant, and the difference was significant. The SMNF plant yielded a higher amount of tannins than the SY and SMF plants, but the difference was not significant. Considering the antimicrobial activities displayed by the three growth stages, where the SY plant exhibited good activities against some of the strains tested, followed by the SMNF plant, it can be concluded that the quantity of phytochemicals present in a plant may not be directly responsible for their antimicrobial activities, as they may be acting in synergy with other bioactive compounds. Results from this study demonstrated that the quantity of these phytochemicals in the plants of various growth stages were not related to their antimicrobial action displayed.

5.4. Cytotoxicity and mutagenicity test

To assess the safety of the extracts in the different growth stages of the SAB, the tetrazolium-based colorimentric (MTT) assay against Vero cells, and Ames test for *S. typhymurium* strains TA98 and TA102 without metabolic activation were applied. The results from the cytotoxicity test showed that the SY plant extract which showed the best antimicrobial activity **(Chapter 2)** was more cytotoxic than the SMF and SMNF plant extracts. None of the plant extracts of the various growth stages showed any mutagenic effects against TA98 and TA102, but plant extracts can only be considered reasonably safe upon further confirmation tests including *in vivo* studies.

5.5. Conclusion and recommendations

Although significant pharmacological activity identified in an *in vitro* assay does not confirm that a plant extract is a suitable candidate for the development of a new

drug, to some extent it does provide a basic understanding of the efficacy of a medicinal plant in traditional medicinal practice and its potential use as a source of novel chemotherapy. Based on the pharmacological activities observed in this study, the economical, ecological and environmental burdens posed by *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 related to the microbial strains that were inhibited by *C. odorata* extracts in this study. *Chromolaena odorata* may also serve as an alternative to highly exploited indigenous plants which have the same medicinal potential, as it has been shown to contain important phytochemicals such as phenolics, tannins, flavonoids and saponins.

The following recommendations on both *C. odorata* biotypes should be considered for future studies

- A further investigation on the seasonal variations of activity in the various growth stages is paramount, in order to determine the best season at which the plant material should be harvested to achieve the best results.
- It will be important to investigate other plant parts such as flowers, stems and roots of *C. odorata* for similar pharmacological activities to ensure proper use of the plant.
- The isolation and identification of bioactive compounds which may help in the development of antimicrobial drugs should be undertaken.
- Other biological activities should be tested, as this will help in the discovery of other possible bioactive compounds from the plant.

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APPENDIX

Appendix 1: List of chemicals used in this study and solutions prepared

Chemical	Company	Percentage prepared	Procedure
Ethanol	Merck Pharmaceuticals South Africa	70 %	70 ml of ethanol + 30 ml of dis. H_2O
Methanol	Merck Pharmaceuticals	50 %	50 ml of methanol + 50 ml of dis. H_2O
		70 %	70 ml methanol + 30 ml dis. H_2O
Petroleum ether	Merck Pharmaceuticals	Absolute	
Dichloromethane	Merck Pharmaceuticals	Absolute	
Ammonium solution	Merck Pharmaceuticals	Absolute	
Hydrochloric acid (HCl)	Merck Pharmaceuticals	1 %	1 ml + 99 ml dis. H_2O
Ferric chloride		1 % ferric chloride solution	1 g ferric chloride +100 ml of dis. H_2O
		2 % ferric chloride solution	2 g ferric chloride +100 ml dis. H_2O
Sodium hydroxide (NaOH)	Saarchem PTY Ltd	10 % NaOH solution, 1M NaOH	10 ml of NaOH + 90 ml dis. H_2O 2 g NaOH + 50 ml dis. H_2O
2 N Folin-Ciocalteu (Folin-C) reagent	Sigma-Aldrich	1 N Folin-C reagent	100 ml Folin-C reagent + 100 ml dis. H_2O in dark bottle
Sodium carbonate ($NaCO_3$)	BDH Chemicals Ltd Poole England	2 % $NaCO_3$	2 g of $NaCO_3$ + 100 ml of dist. H_2O
Gallic acid	Sigma Chemicals Ltd		0.01 g of gallic acid + 100 ml of 50 % MeOH
Sodium nitrite ($NaNO_2$)	BDH Chemicals Ltd Poole England	5%	0.25 g of $NaNO_2$) + 100 ml dis. H_2O
Aluminium chloride $AlCl_3$ from	Merck Pharmaceuticals	10% $AlCl_3$	0.5 g of $AlCl_3$ 5 ml of dis. H_2O

Butanol-HCl reagent *butanol	Merck Pharmaceuticals		5ml of HCl + 95 ml butanol
Ferric reagent	Saarchem PTY Ltd		0.83 ml HCl + 4.1 ml of dis. H_2O + 0.1 g ferric chloride
Cyanidine chloride	Roth Karlsruhe		0.1 g cyanidine chloride + 100 ml of 50 % methanol)

Appendix 2: Stock solutions used for experiments

Stock	Company	Solution
Mueller-Hinton (MH) broth (Oxoid)	England	10.5 g MH broth + 500 ml dis. H_2O
Mueller-Hinton (MH) agar (Oxoid)	Merck, Germany	12.5 g MH agar + 500 ml of dis. H_2O
Yeast malt (YM) broth	Merck, Germany	
Yeast malt (YM) agar	Becton Dickson, USA	
Plant extract (25 mg/ml)		0.025 mg + 1 ml dis. H_20
Neomycin (10 mg/ml)	Sigma-Aldrich Germany	20 µl neomycin to 480 µl sterile dis. H_2O
p-iodonitrotetrazolium chloride (INT)	Sigma-Aldrich, Germany	0.001 g INT + 5 ml sterile dis. H_2O to make 0.2 mg/ml
Sodium chloride (NaCl)		0.85 g NaCl + sterile dis. H_2O to make 0.85 % solution
Amphotericin B	Sigma Aldrich, Germany	(a) 0.005 g Amphoterecin B + 200 µl DMSO. (b). 100 µl of above solution + 900 µl of dis. H_2O

Appendix 3: List of equipment/ brand and apparatus used

1. UV-visible spectrophotometer (Varian Cary 50, Australia)
2. Sonication bath (Julabo GMBH, Germany)
3. Filter paper (Whatman No 1. GE Healthcare UK Ltd)
4. Vortex (Heidolph REAX 2000),
5. Microplate reader (Chromate 4300)
6. Microplates (Greiner Bio-one GmbH, Germany)