THE CHEMOTAXONOMY AND BIOLOGICAL ACTIVITY OF *SALVIA STENOPHYLLA* (LAMIACEAE) AND RELATED TAXA

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DECLARATION

I, Angela Gono-Bwalya declare that this dissertation is my own work. It is being submitted for the degree of Master of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

.....Gono.....

.....13th.....day of .....October....., 2003.

To my late parents

Andrea Gono and Angeline Lisa Gono

ABSTRACT

Salvia stenophylla Burch. ex Benth. (Lamiaceae) is a perennial aromatic herb, which is widespread in the high altitude areas of the central and eastern parts of South Africa and also occurs in southwest Botswana and central Namibia. It is closely related to *Salvia runcinata* L. f. and *Salvia repens* Burch. ex Benth., with which it forms a species complex. The most recent revision of southern African *Salvia* species is that by Codd (1985). In this revision, the most important characters used in delimiting the three taxa were corolla size, calyx size and trichome density. As a result of intergrading morphological characters, the specific limits between the three taxa are not clear and positive identification of typical material is often difficult. Taxonomic delimitation through use of chemical characters was therefore the principle objective of this study. The taxa represented in this species complex are known in folk medicine and plant extracts have been used in the treatment of urticaria, body sores, and stomach ailments and as a disinfectant. *S. stenophylla* is reported to contain α-bisabolol, a compound that has anti-inflammatory properties. Based on the traditional uses of these plants and the international use of α-bisabolol to develop active cosmetic products, establishing a scientific rationale for the known uses was an important secondary objective.

Plant material was collected from a total of 30 natural populations (10 populations of *S. stenophylla*; six of *S. repens* and 14 of *S. runcinata*). Trichome studies were undertaken using scanning electron microscopy (SEM). The aerial parts of the plants were hydrodistilled to extract essential oil and analytical assessment of the oil was carried out using thin layer chromatography (TLC), gas chromatography (GC) and gas chromatography coupled to mass spectroscopy (GC/MS). Non-volatile compounds were extracted using methanol and analyzed by TLC and high performance liquid chromatography coupled to mass spectroscopy (HPLC/UV/MS). For the documentation of biological activity, antimicrobial activity was assessed by disc diffusion assay and the microplate dilution method. Antioxidant activity was assessed by TLC-DPPH and the anti-inflammatory activity was performed by COX-2 radiometric assay. To investigate whether observed chemical variations were genotypic or phenotypic, seeds from selected populations were cultivated, harvested and distilled upon maturity. A molecular study attempted to evaluate genetic differences amongst individual plants and populations using the amplified fragment length polymorphism (AFLPs) technique.

The SEM results did not reveal variation of the trichome types between the different taxa. TLC and GC/MS results showed variation in the qualitative and quantitative chemical composition within and between populations of the same and different taxa. The dominant compounds in *S. stenophylla* include; α-bisabolol (46.5%), limonene (38.1%), δ-3-carene (24.9%), γ-terpinene (20.3%), p-cymene (18.4%) and (*E*)-nerolidol (53.6%). *S. repens* accumulated major compounds like (*E*)-nerolidol (25.2%), ledol (25.4%), camphor (12.7%), β-caryophyllene (13.6%) and β-phellandrene (22.2%). *S. runcinata* has (*E*)-nerolidol (72%), α-bisabolol (41.1%), limonene (24.1%), α-pinene (45%) and β-pinene (15%) and 26% of guaiol in high percentages, and three chemotypes were identified. The HPLC/UV/MS results revealed the occurrence of rosmarinic acid, carnosic acid, carnosol, isocarnosol and oleanolic acid derivatives in the methanol extracts analysed. This was also portrayed in the TLC-DPPH assay. The chemical variation was also expressed in the biological activity assays that were performed. *S. stenophylla* extracts exhibited high antibacterial activity against Gram-positive bacteria and the essential oils exhibited good anti-inflammatory activity. The AFLP results showed congruency with the chemical composition data as it supported the erratic chemical variations within the species complex. In view of all these results, we concluded that the three taxa are closely related with the existence of intermediates (hybrids) within the species complex. Furthermore, the essential data suggest that *S. stenophylla* and *S. repens* are the closest allies within the complex.

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GENERAL INTRODUCTION

1.1 The genus *Salvia*

The Lamiaceae (mint family) is a widespread family that contains about 252 genera and upto 6700 species with a cosmopolitan distribution in warm and temperate regions of the world (Lawrence 1992; Wagstaff 1992; Pedersen 2000; Retief 2000). De Jussieu first named this family as Labiatae in 1789 and a major subfamilial classification was proposed in 1876 by Bentham, after which revisions were introduced by Briquet (1895–1897), Baker (1900), Brown *et al.* (1910), Launert and Schreiber (1969), Codd (1985), Zomlefer (1994) and Hedge *et al.* (1998) as cited by Retief (2000). The family is classified into six subfamilies, which include Viticoideae, Teucrioideae, Ajugoideae, Scutellarioideae, Lamioideae and Nepetoideae (Codd 1985; Retief 2000). In southern Africa, there are 35 indigenous genera and six naturalised genera that are represented solely by alien species. These genera comprise 266 species, 254 of which are indigenous and 12 of which are naturalised alien plants (Retief 2000). They occur mainly in the eastern parts of the region (Retief 2000).

The genus *Salvia* L., commonly known as sage, derives its name from the Latin name 'Salvere' which means 'be in good health or to be well' (Roberts 1986). According to the classification of Cantino *et al.* (1992), *Salvia* is one of the larger genera belonging to the subfamily Nepetoideae, which is a member of the tribe Mentheae *sensu* Bentham (1876). The genus *Salvia* constitutes nearly one quarter of the recognised genera of the Lamiaceae (Wagstaff 1992). The genus was first described by Linnaeus (1753 and 1754) followed by revisions of the genus by Bentham (1833; 1848 and 1876), Briquet (1896), Baker (1900), Skan (1910) and Launert and Schreiber (1969). The most recent revision of the genus in southern Africa was by Codd (1985). The genus contains about 900 species, which are widely distributed in the temperate, subtropical and tropical regions of the world, rarely in the arctic or alpine regions (Codd 1985; Harley and Heywood 1992; Hedge 1992; Rustaiyan *et al.* 1999, Tzakou *et al.* 2001). The major centers of diversity are the meditterranean, central Asia, the highlands of Mexico and the Andes Mountains of South America (Rodriguez-Hahn *et al.* 1992). In southern Africa there are 23 indigenous species of *Salvia*, and four additional species are naturalised. A number of other species have, however, been cultivated as ornamentals, for aromatic and aromatherapy purposes, confectionary and as

culinary herbs (Codd 1985; Retief 2000). It is the largest genus in the family and it is known throughout the world as important because of the useful essential oils (sage oils) contained in the foliage and other above ground parts (Ahmed *et al.* 1994). According to Bentham (1876) the genus is divided into four subgenera: *Leonia*, *Salvia*, *Sclarea* and *Calosphace*.

1.2 The species complex

Salvia stenophylla is very closely related to *S. runcinata* L. f. and *S. repens* Burch. ex. Benth and together forms a species complex. It is very difficult to distinguish the species from each other. In the present systems of classification, the delineation of *S. stenophylla*, *S. repens* and *S. runcinata* is based mainly on differences in the length of the fruiting calyces and corolla. *S. stenophylla* and *S. runcinata* are distinguished on the basis of trichomes, both secretory and non-secretory, distribution and density (Table 1)(Codd 1985). All three species belong to the subgenus *Leonia* of the section *Heterosphace* (Jequier 1980; Pedersen 2000).

1.2.1 *Salvia stenophylla* Burch. ex Benth.

Salvia stenophylla is commonly known as 'mountain sage'. It is an erect, bushy perennial odorous herb that usually branches from a woody taproot (Figure 1). The stem is sparsely hairy and the leaves are linear-oblong to oblong-lanceolate and narrow, from which it was named in Greek. The calyces are small and are depressed to the axis when in fruit (Hedge 1974). The corolla is pale blue to mauve with white patches at the center of the upper and lower lips, and navy blue nectar guides (Figure 2). It has a disjunct distribution and occurs in central Namibia and southwestern Botswana, in the north, and is widespread in the high altitude regions of the central and south-central parts of South Africa. It has also been recorded from Lesotho (Figure 7) (Codd 1985). It occurs mainly in grasslands on calcareous sandy or brackish soil, as a weed in seepage areas beside roadside bridges, waste places and occasionally pastures and croplands. *S. stenophylla* flowers from October to April.

1.2.2 *Salvia repens* Burch. ex Benth.

Salvia repens is a very variable species and is endemic to southern Africa (Codd 1985) Unlike *S. stenophylla* and *S. runcinata*, it arises from a creeping rhizome. The specific name '*repens*' is derived from the Latin word for creeping or prostrate in reference to habit (Wilson and Taylor, *in press*). Leaves are elliptic to obovate with entire to deeply incised or sometimes runcinate margins. The leaves are usually crowded at the base leaving the

Table 1. Summary of the diagnostic morphological characters used by Codd (1985) to delimit the *S. stenophylla* species complex

Characters	*S. stenophylla*	*S. repens*	*S. runcinata*
Habit	• 0.2 to 0.6m tall • arise from a taproot	• 0.25 to 0.8m tall • arise from decumbent creeping rhizome	• 0.15 to 0 7 tall • arise from taproot or occasional creeping rhizhome • hispid or crisped
Leaves	• petiolate or subsessile • narrower leaves with narrower segment • subglabrous with orange to red gland dots	• larger than *S. stenophylla and S. runcinata* leaves • petiolate at base, subsessile upper • concentrate at bottom of stem • pilose to tomentose	• shortly petiolate and sessile upper • pilose and gland-dotted
Inflorescence	• several to many spaced verticils • verticils 6 to 8-flowered.	• several to many verticals, widely spaced below and denser above • verticils 6 to 8-flowered.	• several to many verticils, widely spaced below and denser above • verticils 4 to 8-flowered.
Stem	• almost glabrous	• densely pubescent	• pubescent
Corolla	• pale blue to mauve	• pale blue or mauve to purple	• pale blue or sometimes white

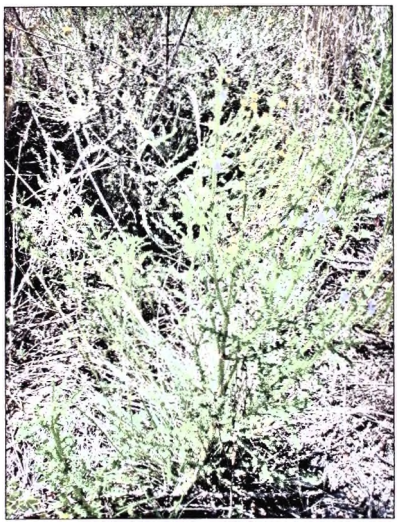


Figure 1. *Salvia stenophylla* habit.

Figure 2. *Salvia stenophylla* flower.

Figure 3. *Salvia repens* habit.

Figure 4. *Salvia repens* flower.

Figure 5. *Salvia runcinata* habit.

Figure 6. *Salvia runcinata* flower.

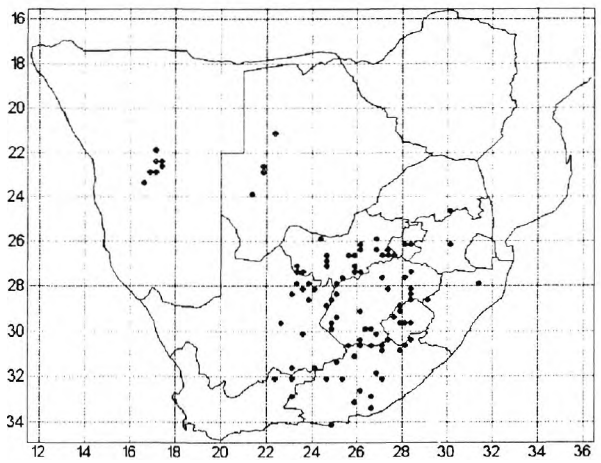


Figure 7. Distribution map of *S. stenophylla* (Codd, 1985).

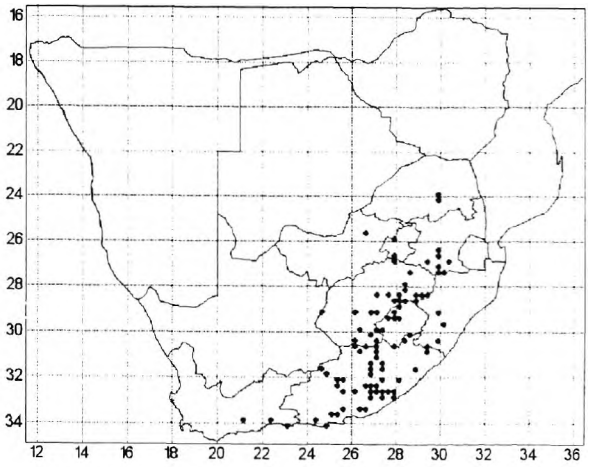


Figure 8. Distribution map of *S. repens* (Codd, 1985).

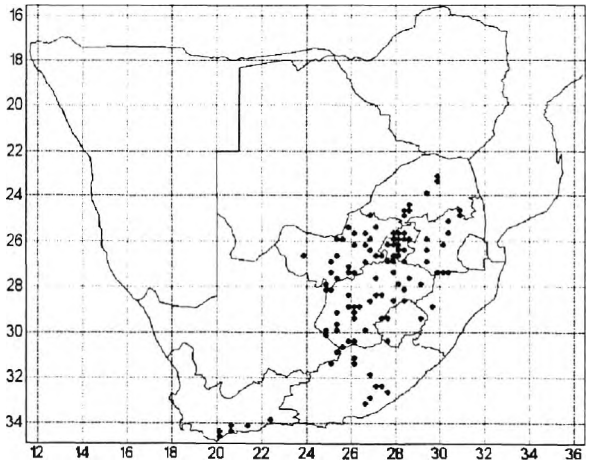


Figure 9. Distribution map of *S. runcinata* (Codd, 1985).

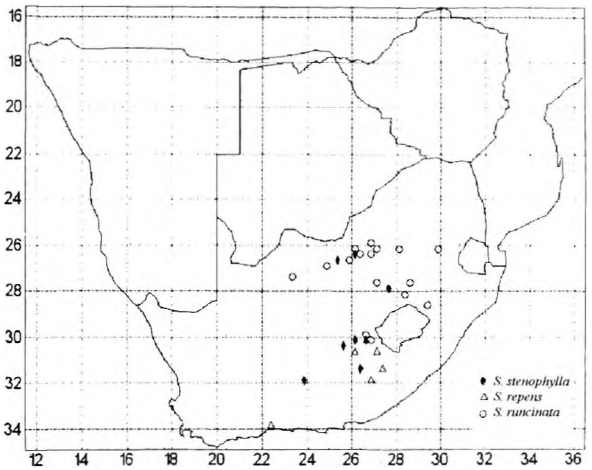


Figure 10. Distribution map of all localities from which material was collected for the study.

inflorescence clearly visible (Figure 3). The corolla is pale blue to purple, rarely white, and the mature calyx is usually maroon (Figure 4) (Goldblatt and Manning 2000). The flowering period is from October to February, and May. The distribution stretches from the Limpopo Province southwards through Gauteng, the Free State, Lesotho, Eastern Cape and eastern parts of the Western Cape. (Figure 8) (Codd 1985). It occurs in open woodland and grassland usually on clay soils. *S. repens* is used in folk medicine to treat sores, stomachache and diarrhoea, and is locally known as 'Kruipsalie' or 'Saliebossie' in Afrikaans and 'Mosisili' or 'Mosisili-oa-loti' in Southern Sotho (Goldblatt and Manning 2000). The species is divided into three varieties on the basis of corolla length and leaf shape. The recognised varieties are *S. repens* Burch ex. Benth var. *repens*, *S. repens* Burch ex. Benth var. *keiensis* Hedge and *S. repens* Burch ex. Benth var. *transvaalensis* Hedge.

Salvia repens var. *repens* occurs in mountain grasslands, riverbanks, open woodland or karroid veld, often on heavy clay or clay-loam soils. It has much or little branched ascending stems with leaves that are simple to runcinate and sparsely to freely gland-dotted. The corolla length is between 14 mm and 20 mm. *Salvia repens* var. *keiensis* variety is restricted to the Eastern Cape and North-West provinces of South Africa and occurs in open woodland and grasslands. It differs slightly from var. *repens* in that it has much branched ascending stems with elliptic to obovate leaves that are simple but sometimes lobed near the base. The corolla length is between 20 mm and 26 mm. Like var *keiensis*, *Salvia repens* var. *transvaalensis* has much branched erect stems with narrowly oblong or elliptic leaves, which are simple and occasionally lobed towards the base and freely gland-dotted. The corolla length ranges between 10 mm and 15 mm. It is restricted to the southern North-West Province and northern Free State and occurs in grasslands. Only two locations have been recorded for this variety. This study was, however, conducted at the species level and the varieties of *S. repens* were therefore not considered individually.

1.2.3 *Salvia runcinata* L. f.

Salvia runcinata is an erect, perennial and odorous herb endemic to southern Africa (Figure 9). It has a taproot or occasionally, a creeping root system (Figure 5) (Hedge 1974). Leaves are oblong-lanceolate to ovate and the margins are very dissected and toothed from which the specific name 'runcinate' was derived. The corolla is usually mauve with purple streaks (nectar guides), but can also be white. The mature calyx is green (Figure 6). It flowers from August and April.

Salvia runcinata is distributed mainly in the high lying parts of the eastern half of South Africa, and in Lesotho, but also extends westwards along the southern Cape coast. It has also been recorded from unspecified localities in Botswana and Zimbabwe (Codd 1985), which are not depicted on the distribution map provided in Figure 9. The plants are gregarious and often locally abundant. This species occurs mostly as a weed in disturbed areas such as roadsides, on dry heavy soils. It is used in folk medicine as a disinfectant and is known locally as 'hardesalie' or 'wildesalie' in Afrikaans, 'isicakathi' in Zulu and 'mosisili' in southern Sotho (Goldblatt and Manning 2000).

1.3 Commercial importance

The Lamiaceae (mint family) is best known for containing a wide range of compounds that are of economic importance (Richardson 1992). The family contains about 252 genera, 40 % of which have aromatic properties. The large number of aromatic taxa make this family commercially important because of their odours, infusions, tinctures, and flavours that have been and are still being used as components of herbal products (El-Gazzar and Watson 1970; Werker *et al.* 1985; Lawrence 1992; Ascensão *et al.* 1999). In the African culture and some third world countries, traditional medicine plays a major role in health care, especially in the rural areas, because of availability and cheaper cost (Shale *et al.* 1999). Plant extracts exhibit a range of biological activities, which include antimicrobial, antioxidant and anti-inflammatory properties (Biavati *et al.* 1996; Svoboda and Hampson 1999). The genus *Salvia* in particular is known throughout the world as being of commercial, medicinal and cultural importance because of the useful essential oils produced by the foliage (Ahmed *et al.* 1994). Many *Salvia* species have long been used in folk medicines, making this genus a popular choice for researchers.

1.3.1 Essential oils

Approximately 120 plant-derived chemical compounds are currently used as commercial drugs (Marston and Hostettmann 1999). Essential oils in particular, have been widely used in traditional folk medicines since ancient times (Cowan 1999; Nakatsu *et al.* 2000), and have gained commercial importance because of their application in pharmaceuticals (Janssen *et al.* 1988; Shapiro *et al.* 1994), food (Baricevic & Bartol 2000) and fragrance preparations (Nakatsu *et al.* 2000; Biavati *et al.* 1996), beverages, soaps, detergents and cosmetics. The best known and most widely used sage oils are extracted from *Salvia*

officinalis L. (Baricevic *et al.* 2001; Lawrence 1992; Tsankova *et al.* 1994; Tzakou *et al* 2001), *Salvia sclarea* L., *Salvia hispanica* L. (Ahmed *et al.* 1994), and *Salvia fruticosa* Mill (Skoula *et al.* 2000). The oil of *S. officinalis* is referred to as the oil of commerce because of the high levels of α-thujone, β-thujone and camphor. It is commercially cultivated along the Adriatic coast of Yugoslavia and Albania (Lawrence 1992). Basil oil (*Ocimum basilicum*) is also a renowned commercial oil globally and is currently produced in the following quantities per annum: India (15 tonnes), Bulgaria (7 tonnes), Egypt (5 tonnes), Pakistan (4.5 tonnes), Comores (4.5 tonnes), Israel (2 tonnes), Yugoslavia, USA, Madagascar (each 1 tonne) (Yayi *et al.* 2001; Lawrence 1992). Other commercially important oils are marjoram (*Majorana hortensis* Moench.) and rosemary (*Rosmarinus officinalis* L.) (Baratta *et al.* 1998).

The use of essential oils as an ingredient in pharmaceutical preparations is on the increase. Due to the lipohilic character of essential oils, they have easy passage through the blood-brain barrier and their affinity towards lipid-rich tissues like those of the central nervous system (CNS) facilitates an exchange of volatile molecules from blood into lipid tissues, making them responsible for a broad spectrum of biological activities (Buchbauer 1993). Some of the most popular widely used species belong to the genus *Mentha* L. Oil obtained from these species is reported to aid digestion, provide sinusites relief and aid breathing in people affected by colds. *Mentha longifolia* (L) in particular is used for headaches by the Zulu, Xhosa and Sotho in South Africa (Hutchings and van Staden 1994). John Parkinson's (1640) review, as reported by Richardson (1992) reveals the extensive use of mints as vermifuges, aphrodisiacs, fertility controllers, headache cures, rabies treatment, eye treatment, ear treatment, stomachache remedies, dandruff, leprosy, kidney stones and halitosis. Peppermint and spearmint are widely used as ingredients in toothpaste (Richardson 1992). The main active compounds in *Melissa officinalis* L. (lemon balm) essential oil have spasmolytic, sedative and moderate antibacterial properties (Schultze *et al.* 1992). In addition it contains tannins with antiviral activities. Dried roots of *Salvia miltiorrhiza* Bunge are reported to be widely used in China (Li and Chen 2001) for promoting blood circulation to remove blood stasis, relieving vexation, nourishing blood and tranquilizing the mind and cooling blood to relieve carbuncles. The plant is also reportedly used to treat heart diseases, particularly angina pectoris and myocardial infarction (rapid development of myocardial necrosis) (Rustaiyan *et al.* 1999). The plant

parts of *Salvia officinalis* L. are widely known for their anti-oxidative, antibacterial, fungistatic, virustatic, astringent, eupeptic and anti-hydrotic properties. Preparations of this plant also reduce plaque growth, inhibit gingival inflammation and have positive effects on caries prophylaxis, chronic bronchitis, tuberculosis, psoriasis and seborrhoeic eczemas (Rustaiyan *et al.* 1999; Baricevic *et al.* 2001). It is also reported to stimulate hair growth, tone up hair and remove dandruff (Roberts 1986). Thymol from the essential oil of *Thymus vulgaris* is used in pharmaceutical products because of its carminative and germicidal properties. The essential oil of *Thymus vulgaris* has been used as an antiseptic, antispasmodic, antitussive, expectorant, antifungal, antiphlogistic and rubefacient (agent that redden the skin due to mild irritation). It has also been used for digestive ailments, sedative, lotion, inhalation and enema, and nervous derangements (Roberts 1986; Echeverrigaray *et al.* 2001).

In southern Africa, *S. stenopylla*, *S. runcinata* and *S. repens* are used by the southern Sotho speaking people to disinfect a hut after sickness (Hutchings *et al.* 1996). A decoction of the roots, stems and leaves of *S. runcinata* have been used by Europeans for the relief and the treatment of urticaria (Hutchings *et al.* 1996). The Zulu use a paste of crushed leaves as a purgative for infants (Watt and Breyer-Brandwijk 1962; van Wyk *et al.* 1997). The Xhosa traditionally administer medicine made from leaves and mother's milk to newly born babies. A decoction of *S. repens* roots is taken in large doses before meals to treat stomachache and diarrhoea (Shale *et al.* 1999). Plant extracts may also be used to treat sores on the body (Watt and Breyer-Brandwijk 1962; Hutchings *et al.* 1996). The traditional uses of other *Salvia* species in southern Africa are summarized in the Table 2.

Table 2. Summary of the traditional medicinal uses of *Salvia* species in southern Africa as documented by Watt and Breyer-Brandwijk (1962), Hutchings *et al.* (1996), van Wyk *et al.* (1997).

Species	Traditional medicinal uses
Salvia africana-caerulea	Remedy for coughs, colds, chest problems, urine troubles, kidney infections, stomach ailments.
Salvia africana-lutea	Coughs, colds and female ailments.
Salvia chamelaeagnea	Coughs, colds, bronchitis, chest problems, flu and fever, convulsions, diarrhoea, female ailments, ear and headaches.
Salvia coccinea	Relief of lumbago, pulmonary tuberculosis.
Salvia disermas	Treatment of heart problems, high blood pressure, rheumatism, sores; and is used as a tea.
Salvia rugosa	Lotion for sores, and as a tea.
Salvia scabra	First medicine to Xhosa infants, purgative.
Salvia sclarea	Treat sores and swellings, stomachache and diarrhoea.
Salvia sisymbrifolia	Lotion on sores and swellings, treatment for sore throats.
Salvia triangularis	Mixed with *Helichrysum latifolium* and *Commelina africana* for barrenness in women.

Equally important has been the use of aromatic species to flavour food products (Lawrence 1992). These aromatics include culinary herbs such as mint (*Mentha*), sage (*Salvia*), thyme (*Thymus*), marjoram or oregano (*Origanum*), rosemary (*Rosmarinus*) and basil (*Ocimum*). It is the presence of terpenes in the essential oils that make them important, and Richardson (1992) states that the plants of greatest economic importance in the Lamiaceae are the two members of the genus *Mentha* L., from where spearmint (*Mentha spicata*) and peppermint (*Mentha* X *piperita* L.) are obtained. These species are widely used in flavours (La Croix 1984), for instance, the chewing gum industry in the United States. *Mellisa officinalis* is used as a strewing (diffusion) herb and the juice from the stems is rubbed on furniture to polish and perfume it. It has also been used to flavour ale and wine, and tea infused with this species is said to alleviate colds and influenza. *Hyssopus officinalis* is used to flavour liqueur Chartreuse (La Croix 1984; Roberts 1986).

Mints such as various *Lavandula* and *Thymus* species have pleasant smelling terpenes that are used in perfumes in the cosmetic industry. *Pogostemon* (patchouli) is used in scenting cosmetics (La Croìx 1984; Richardson 1992).

The existence of antimicrobial activity in the essential oils is of considerable benefit to the plant in that the plants protect themselves from plant pathogens (La Croìx 1984). Thus mints are also widely being used as insecticides to repel fleas and lice, leech killers and deterrants for moths that damage stored clothes (Richardson 1992) For example, *Rosmarinus officinalis, Salvia officinalis Origanum majorana, Melissa officinalis, Salvia elegans, Thymus* x *citriodorus* (lemon balm) and *Lavandula* species are used in pot-pourri, sachets and vinaigrettes (La Croìx 1984; Roberts 1986) The oil of basil has also been used to disinfect lettuce leaves (Cowan 1999). Terpenoids also play significant roles within the organisms producing them and they are known to function as mammalian sex hormones, insect pheromones, plant-growth substances, natural insecticides, defensive secretions of fish, ancillary pigments in photosynthesis, and as receptors in visual processes in animals (Banthorpe 1998). Some birds such as the Starling (*Sturnus vulgaris*) place aromatic plants in their nests to control fleas and other pests (Richardson 1992).

S*alvia,* although a large genus, is phytochemically poorly studied. This is especially the members of the subgenus *Leonia* to which the *S. stenophylla* species complex belongs (Jequier *et al.* 1980; Rodríguez-Hahn *et al.* 1992) However, the chemical composition of *S. stenophylla* has been investigated in the past years and it has been reported that *S. stenophylla* contains between 28.9% to 41% α-bisabolol, a commercially important compound (Jequier *et al.* 1980; Brunke and Hammerschmidt 1985; Lawrence 1992; Hoelscher *et al.* 2003). Four stereoisomers of α-bisabolol (Figure 11) have been isolated, these being; (-)-α-bisabolol, (-)-*epi*-α-bisabolol, (+)-α-bisabolol and (+)-*epi*-α-bisabolol (Brunke and Hammerschmidt 1985). (+)-e*pi*-α-bisabolol has been isolated from *S. stenophylla* and this compound together with manool (constituting about 2–4% of the oil) is responsible for the woody and persistent odour of *S. stenophylla* (Jequier *et al.* 1980; Brunke and Hammerschmidt 1985). Recently (+)-*epi*-α-bisabolol was also isolated from *Peperomia galioides* and was reported to have cicatrizant (wound-healing) activity (Villegas *et al.* 2001). The only source of naturally occurring (-)-α-bisabolol for commercial use has been *Matricaria chamomilla* essential oil, which contains about 17% of the

compound (Jager and van Staden 2000, Villegas *et al.* 2001). As such the oil of *S. stenophylla* could become a source of α-bisabolol since it contains up to 41% of this compound. (-)-α-bisabolol is reported to have anti-inflammatory activity and is therefore used as an additive to commercial skin care products for sensitive skins (Figures 12A & 12B).

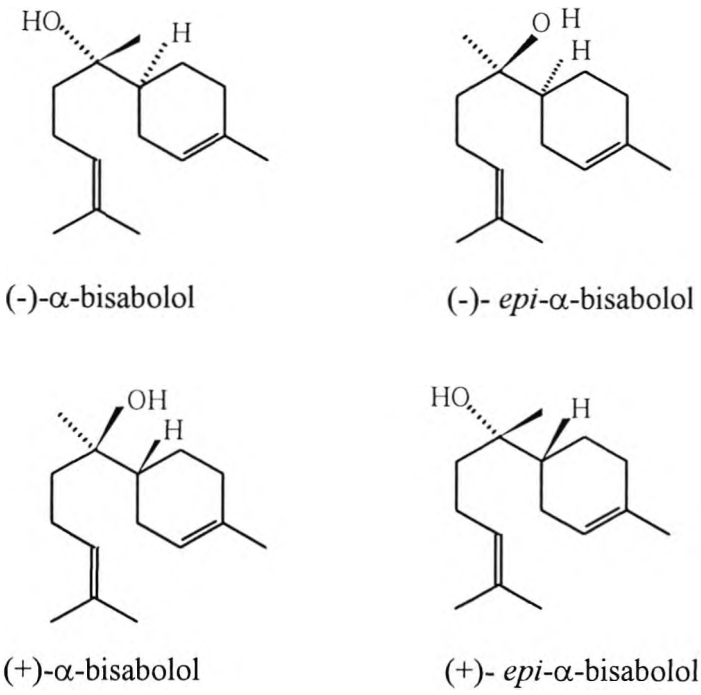


Figure 11. Chemical structures of four α-bisabolol stereoisomers.

Another compound of commercial importance contained in the members of the *S. stenophylla* species complex is *(E)*-nerolidol. An essential oil called cabreuva contains approximately 80% of (+)-*(S)*-*(E)*-nerolidol, which is especially used in perfumery (Joulain 1995). Apparently this essential oil was extracted from *Myrocarpus fastigiatus* Fr. Allem, a tree relatively abundant in Brazil, Paraguay and Argentina, but production has since declined to the point that it has nearly disappeared from the market. There are however, other sources such as a chemotype of *Eucalyptus nova-anglia* and *Zanthoxylum gardneri* Engl., which contain about 77% and 81% nerolidol respectively. Because these sources are trees, they can probably only be commercially harvested when they are mature, which takes many years. The advantages of a rapidly renewable source of natural nerolidol, e g annual plants are obvious. As the essential oil of *S. runcinata* contains approximately 71% of *(E)*-

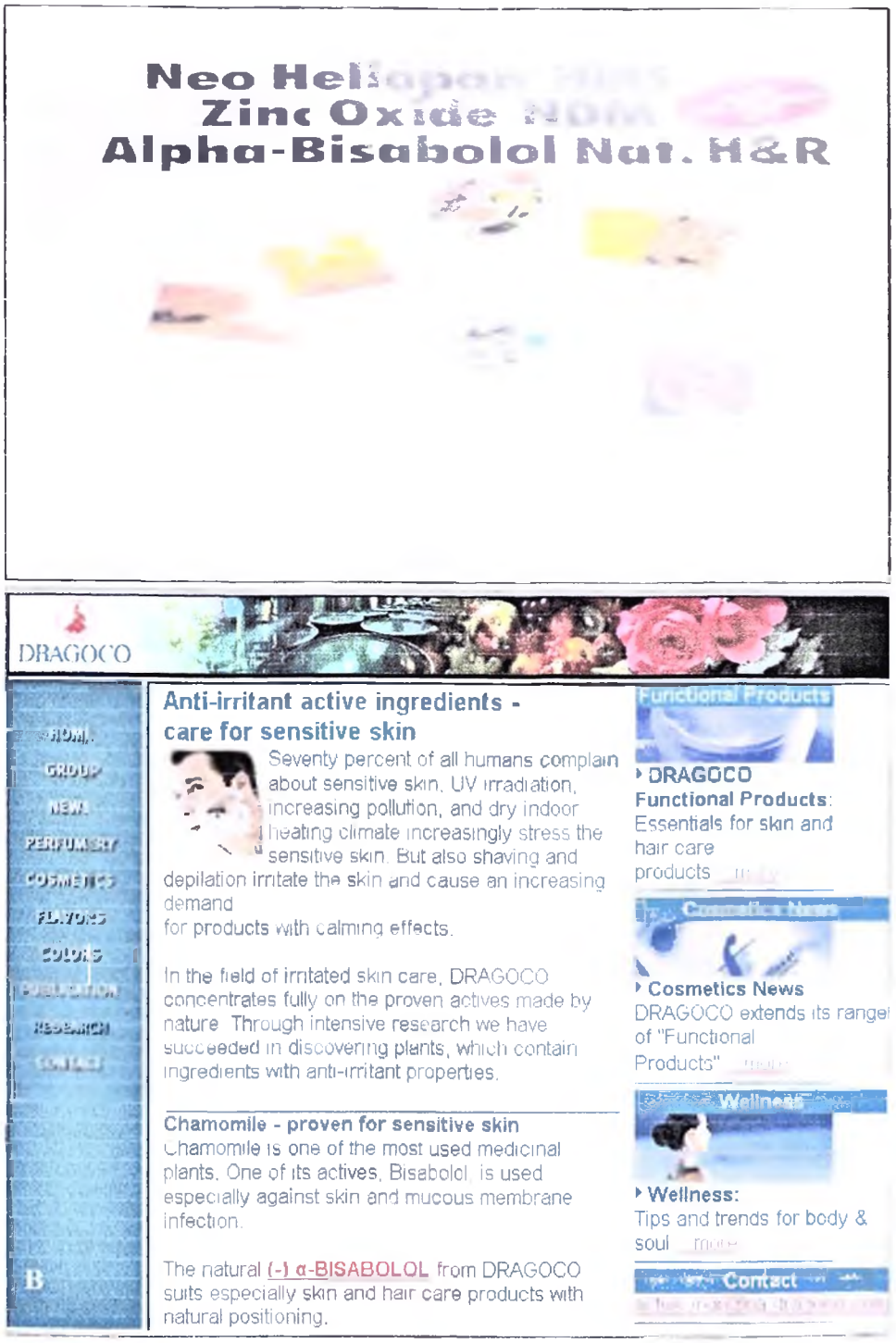
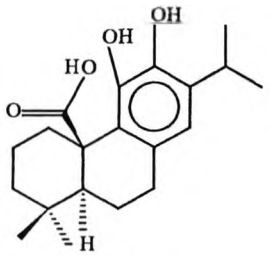


Figure 12 A & B are α-bisabolol advertisements by commercial companies: H&R and Dracogo (http://www.dragoco.com/eng/pages/kos_w_irri.htm) that produces the chemical compound for skin care products.

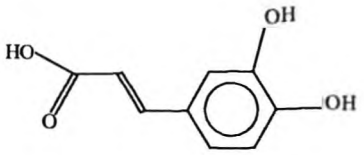
nerolidol, it may become an alternative source of the compound, since it grows and matures within one growing season.

1.3.2 Non-volatile compounds

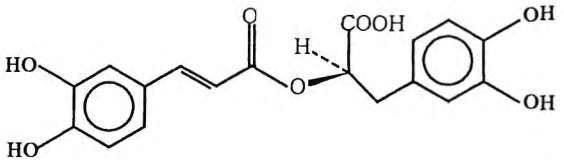
A number of non-volatile compounds that are of commercial importance have also been isolated and identified in the Lamiaceae (Lawrence 1992). The most common include caffeic acid, carnosic acid, oleanolic acid, rosmarinic acid and ursolic acid (Figure 13).



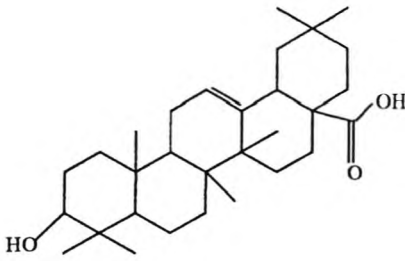
Carnosic acid



Caffeic acid



Rosmarinic acid



Oleanolic acid

Figure 13. Chemical structures of four common non-volatile compounds in the genus *Salvia*.

These compounds are reported to have significant biological activities. They are extracted from species such as *Salvia officinalis*, which exhibits antimutagen, antiviral and antioxidant activities (Culvelier *et al.* 1992 as cited by Perry *et al.* 2001). Oregano extracts have high contents of rosmarinic acid and other hydroxycinnamic acid, while *Thyme vulgaris* has high levels of thymol and carvacrol, which show high antioxidant and antimicrobial activity (Sotelo-Felix 2002). For instance caffeic acid has been implicated to be effective against viruses, bacteria and fungi (Cowan 1999). Carnosic acid and carnosol are reported to have stronger antioxidant properties than butylated hydroxytoluene and butylated hydroxyanisole in assays testing inhibition of lipoxygenase activity (Caruso *et al.*

2000; Sotelo-Felix 2002). Perhaps of greater significance, carnosic acid has shown to inhibit HIV protease activity *in vitro* (Paris *et al.* 1993 as cited by Caruso *et al.* 2000). Rosmarinic acid exhibits significant antioxidant and anti-inflammatory activity, as well as antimicrobial activity against certain bacteria and fungi. The antioxidant activity of rosmarinic acid is much higher than that of α–tocopherol and BHT (Chen and Ho 1997 as cited by Zheng and Wang 2001), making rosmarinic acid attractive to the pharmaceutical and cosmetics industries. Due to its antioxidant properties, rosmarinic acid shows promise as a preventative for atherosclerosis (the deposition of cholesterol-rich plaques in the artery walls). When combined with other polyphenols, it prevents the oxidation of LDL (low density lipoprotein), which is a primary instigator of plaque formation in oxidized form. Furthermore, combining rosmarinic acid with certain other available nutritional supplements, such as lycopene, synergistically enhances the inhibition of LDL oxidation. It is also used in treating bronchial asthma in Korea, where a high yielding rosmarinic acid herb called 'perilla' is used for allergic diseases, and also in HIV therapy where the HIV-1 integrase enzyme is inhibited and *Herpes simplex* infections (Petersen and Simmonds 2003). Rosmarinic acid has been applied for the treatment of diseases caused or propagated by complement activation, such as rheumatoid arthritis; toxic shock syndromes, capillary stabilization where microvascular injuries are inhibited, suppression of certain kinds of glomerulonephritis and kidney diseases. Other uses of rosmarinic acid have been in the treatment of inflammatory diseases such as gingivitis, peptic ulcers, arthritis, liver toxicity, ischaemic heart disease, cataracts, cancer and poor sperm motility (Petersen and Simmonds 2003). It is also widely used in the production of cosmetics. It is also reported that rosmarinic acid containing plants have been used to treat certain microbial diseases such as cholera.

In the food industry, to ensure nutritious and safe food, harmful effects of lipid oxidation have been prevented by addition of free radical scavenging antioxidants. This has become popular and has been effective in retarding fat oxidation of food, by increasing the shelf-life of food products, improving the stability of fats and oils rich in polyunsaturated fatty acids (Baratta *et al.* 1998; Peiwu *et al.* 1999). For instance extracts of *Rosmarinus officinalis* have been widely used as a preservative in the food industry due to the antioxidant activity of its constituents like carnosol and carnosic acid (Sotelo-Felix 2002). *R. officinalis* inhibits lipid peroxidation and its propagation in the liver. Rosmarinic acid is also applied to food

preservation and prevention of food poisoning, because of its antimicrobial and antioxidant properties. For example, to kill pathogens in sliced meat products, and in Japan 'perilla' a herb rich in rosmarinic acid is used to garnish raw seafood. These same properties, plus those of being anti-inflammatory and antiviral, also make this compound of interest as a nutritional supplement. It is absorbed well from the gastrointestinal tract and from the skin. Zheng and Wang (2001) also report that caffeic acid, ferulic acid and vanillic acid are natural antioxidants in fruits and vegetables. Rosmarinic acid is used as a defense compound, against pathogens and herbivores, by plants. It is a potent phagodeterrent against the tobacco hornworm (Petersen and Simmonds 2003).

1.4 Study objectives

Salvia stenophylla, *Salvia runcinata* and *Salvia repens* are very variable species linked by intergrading forms (Codd 1985). The specific limits between the three taxa are not clear. Because of the range of morphological variation that is displayed by the species complex, Hedge (1974) and Codd (1985) concluded that hybridization and introgression obscures the specific limits between the species and hence suggest that field observations should be carried out to help in the more precise delimitation of the taxa. This taxonomic uncertainty has also contributed to the undifferentiated use of the taxa in folk medicine. The chemical composition, and most importantly the chemogeographical variation, of *S. runcinata* and *S. repens* have not been investigated. A formal taxonomic revision of *S. stenophylla* and the related species has not been published since the account by Codd (1985).

Positive identification of species and infra-specific taxa in the *S. stenophylla* complex are extremely problematic, as is reflected by the large number of misidentified specimens in South African herbaria. Herbarium records further show that *S. repens* is often misindentified as *S. verbenaca*, which is a species with a sympatric distribution. *S. verbenaca* is, however, not closely related to *S. repens* and can be clearly differentiated by the densely pubescent throat of the calyx tube (Codd 1985).

Therefore, to attain a clear understanding of the specific limits between *S. stenophylla*, *S. runcinata* and *S. repens*, and to identify more diagnostic characters that may assist in the delimitation of the taxa at different taxonomic levels, this study sought;

(a) To resolve the taxonomic delimitation of the *Salvia stenophylla*, *Salvia repens* and *Salvia runcinata* species complex by chemical composition analysis.

(b) To investigate the essential oil chemistry of this species complex and identify various chemotypes to help the favourable selection of chemotypes for commercial uses.

(c) To document antimicrobial and anti-inflammatory properties of the species complex as they are used in traditional herbal preparations.

(d) To establish the scientific rationale for the traditional use of the *Salvia stenophylla*, *Salvia runcinata* and *Salvia repens* species complex.

MATERIALS AND METHODS

2.1.1 Sample collection

This study was based on fresh plant material that was collected from the wild during the growing season in 2002. The plant specimens were collected from various populations which were located by obtaining distribution data from herbarium specimens housed at the National Botanical Institute (NBI) and at the C. E Moss Herbarium (J), Oppenheimer Life Sciences building, University of the Witwatersrand. In an attempt to understand the variation patterns within and between these taxa and clarify the species limits in the species complex, specimens collected were assigned to three *a priori* groups, *Salvia stenophylla*, *Salvia runcinata* and *Salvia repens* in accordance with the taxonomic key provided by Codd (1985). To investigate whether variation occurs within a taxon at a single population, three individual plants of *S. stenophylla* from Biesiesvlei and three individuals of *S. runcinata* from Klerkskraal Dam were collected. Essential oils were obtained from *S. stenophylla* by microdistillation, and from *S. runcinata* by hydrodistillation. Thin layer chromatography and gas chromatography were performed on the extracts.

The results obtained from the thin layer chromatography and gas chromatography showed that there is no significant chemical variation between plants of the same population (refer to figure 32 and 33). It was therefore decided that 'pool' plant material be collected from different plants within a single population. Plant material was collected from a total of 30 different populations (ten populations of *S. stenophylla*, six of *S. repens* and 14 of *S. runcinata)*. The localities visited are listed in Table 3 and the voucher material was deposited at J. A population was described as a group of plants of one species occurring at least 20 km away from another population of the same species. GPS and quarter degree squares were recorded, and these were used to map the distribution with the Mappit program (Arnold *et al.* 1996). Quarter degree squares for plant samples that didn't have grid references, were obtained in the gazetteer of the Cape Provincial Museums of Leistner and Morris (1976).

Table 3. Specific localities and voucher material of *S. stenophylla, S. repens* and *S. runcinata.*

Taxon	Locality	GPS and quarter degree readings	Collecting numbers
S. stenophylla	Bethulie	30° 41'18" S; 26° 04"11" E	nil
S. stenophylla	Biesiesvlei	26° 14.624' S; 26° 51.689' E	AV/AG 61
S. stenophylla	Dordrecht	30° S; 26° E	ADC/AV 56
S. stenophylla	Lady Grey	30° 48'00" S; 27° 12'20" E	ADC/AV 70
S. stenophylla	Molteno	31° 21'11" S; 26° 16'37" E	nil
S. stenophylla	Murraysburg	31° S; 23° E	nil
S. stenophylla	Sannieshof	26° 37' 16" S; 25° 34'57" E	AV/AG 75
S. stenophylla	Smithfield	30° 10' S; 26° 40' E	ADC/AV 58
S. stenophylla	Springfontein	30° 15'07" S; 26° 41'42" E	ADC/AV 81
S. stenophylla	Steynsrus	27° 55.571' S; 27° 32.740' E	ADC/AV/AG 66
S. repens	Dordrecht	30° S; 26° E	ADC/AV 55
S. repens	George	33° S; 22° E	nil
S. repens	Indwe	31° 24'10" S; 27° 11'56" E	ADC/AV 74
S. repens	Knapdaar	30° 43'32" S; 26° 11'21" E	ADC/AC 78
S. repens	Lady Grey	30° 41'57" S; 27° 06'03" E	ADC/AC 71
S. repens	Queenstown	31° S; 26° E	ADC/AC 54
S. runcinata	Bergville	28° 40' S; 29° 17' E	nil
S. runcinata	Bethlehem	28° 01'30"S; 28° 22'45" E	ADC/AG 67
S. runcinata	Coligny	26° 19'47" S; 26° 27'32" E	nil
S. runcinata	Delareyville	26° S; 25° E	AV/AG 73
S. runcinata	Klerkskraal Dam	26° 15.173" S; 27° 09.920" E	ADC/AV 59
S. runcinata	Koster	25° 56.583' S; 26° 48.931' E	ADC/AG 64
S. runcinata	Kroonstad	27° 30.065' S; 27° 08.367' E	ADC/AG 65
S. runcinata	Kuruman	27° 27'31" S; 23° 27'82" E	AV/AG 70
S. runcinata	Lichtenburg	26° 13.758' S; 26° 08.524' E	AV/AG 62
S. runcinata	Melville Koppies	28° S; 28° E	nil
S. runcinata	Smithfield	29° 51'40" S; 26° 17'16" E	ADC/AV 58
S. runcinata	Tweeling	27° 26.800' S; 28° 30.950' E	ADC/AG 68
S. runcinata	Ventersdorp	26° 14.626' S; 26° 51.688' E	AV/AG 60
S. runcinata	Vryburg	26° 53'76" S; 24° 56'24" E	AV/AG 72
S. runcinata	Roossenekal	25° 15' S; 29° 45' E	ADC/AV 66

2.1.2 Greenhouse cultivation

To establish the source of chemical variation between plants within and between populations, *Salvia stenophylla* seeds were collected from Biesiesvlei, *S. runcinata* seeds were collected from Koster, Lichtenburg, Coligny, Kuruman, Delareyville and *S. repens* were collected from Smithfield.

The seeds were sown in seedling soil in seed trays at a depth of 3 to 5 mm and placed under a mist spray until they germinated. The seedlings were transplanted into potting soil in 15 cm pots when a size of 50 mm in height was reached. The pots were watered once a week and the plants were harvested when they started flowering. This study was done to investigate whether the chemical composition of the taxa is genetically or environmentally influenced.

2.2 Micromorphology

2.2.1 Trichomes

Trichomes are unicellular and/or multicellular appendages, which originate from epidermal cells only and develop outwards on the surface of various plant organs (Wekker 2000). Trichomes vary in size, morphology, surface microstructure, number of cells, origin and secretion (Werker 2000). The occurrence of trichomes and cellular structures are used extensively by taxonomists as an aid to identify taxa as they are constant in species or show constant range of form, implying that when a plant posseses hairs, they are usually of a type or types characteristic to that group (Cutler 1979). Trichomes can be studied from two different perspectives (Johnson 1975), firstly by the nature of individual trichomes and secondly by the characteristics that they collectively impart to the surface of occurrence. The nature of individual trichomes is used taxonomically more than the aspect of indumentum cover, since trichomes of different structure may form a similar type of indumentum (Oosthuizen 1983). Besides, the environment has a greater influence in modifying the nature of the indumentum (Johnson 1975) than it does in changing the type of trichomes. In addition, Judd *et al.* (1999) also considers the density and distribution (indumentum) of trichomes on the plant to be fundamentally important to the taxonomic delimitation of taxa in plant systematics.

Different methods of classification of trichomes have been reviewed (Theobald *et al.* 1979), although the final decision for classification is subjective (Werker 2000). Despite the variety

of systems for the classification of trichome types, they are mainly divided into two major categories as either eglandular (without a secretory function) or glandular (with a secretory function). The main characteristic for distinction within eglandular trichomes is their morphology while the characteristic distinction between types of glandular trichomes is morphology and the material they secrete, accumulate or absorb (Werker *et al.* 1985; Werker 2000). Eglandular trichomes are diverse in morphology and anatomy. They may be unicellular or multicellular, branched or unbranched (Hallanhan 2000). Unbranched multicellular eglandular trichomes have been found to be uniseriate, biseriate or multiseriate, distinctly articulated between cell walls or cross-walls on plant organs (Werker *et al.* 1985; Dudai *et al.* 1988; Werker 2000). Eglandular trichomes may show differences in length, size, and cell shape, and can be symmetrical or asymmetrical, having uniform width or varying width along the trichome and can be tapering or blunt at the apex (Werker *et al.* 1985; Werker 2000). Eglandular trichomes vary in wall thickness and in the material impregnated in their walls, which may render them soft, sinuous or stiff with bristles (Werker 2000).

Glandular trichomes, like eglandular hairs, may be unicellular or multicellular, uniseriate or multiseriate and are variously shaped. The outer surface of the trichome can be smooth or exhibit micro-ornamentation, which may show high diversity, causing it to appear micropapillate, warty, reticulate or striate (Werker 20000). These micromorphological structures may arise from the cell wall or from the cuticular or subcuticular inclusions (Werker 2000). There is variability in coverage, by both eglandular and glandular trichomes and different proportions of the types, between species and even between the two sides of organs such as leaves, calyces and corollas. Trichomes may appear on one side or both sides equally or unequally, sometimes along the margins and/or veins or restricted to intercostal regions in clefts (Werker 2000). In this study, the types of trichomes and indumentum cover were examined using the scanning electron microscope.

2.2.2 Scanning electron microscopy (SEM)

Plant material of *S. stenophylla* (Bethulie), *S. runcinata* (Klerkskraal Dam) and *S. repens* (Indwe) were collected from the wild and kept in a flower garden until the time they were processed for SEM observation. Small pieces of plant material (leaves and stems) were fixed in 2.5% glutaraldehyde in 0.075M phosphate buffer at pH 7.4–7.6 for one hour. The material was rinsed in 0.075M phosphate buffer three times for five minutes each, and then

postfixed in 1% aqueous osmium tetroxide for two hours after which they were rinsed three times in distilled water. The material was dehydrated with a series of different methanol concentrations, namely 30%, 50%, 70%, 90% and 100%, followed by critical point drying in liquid carbon dioxide. They were mounted on aluminium stubs using double-sided adhesive tape, and then sputter coated with gold for 40 seconds using a SEM autocoating unit E5200. The prepared stubs were viewed under a JEOL 840 SEM. The examined surfaces included the abaxial and adaxial surfaces of the leaves and stem surfaces of the plant material.

2.3 Chemical composition

Many different kinds of substances have been investigated as sources of taxonomic information. Plant secondary metabolites or natural products have in particular been used, for instance flavonoids (compounds that affect flower colour) and terpenoids (compounds that give plants their odour) are particularly useful for plant classification (Banthorpe 1998; Cowan 1999; Hallahan 2000; Spring 2000). Flavonoids have been used to delimit *Flaveria* species of the Asteraceae family (Agnese *et al.* 1999) and terpenoids have been the basis of taxonomic classification in the Lamiaceae (Lawrence 1992; Rodriguez-Hahn *et al.* 1992). Terpenoids have been used to define families, genera and species limits in plants (Lawrence 1992).

Terpenoids constitute the largest organic chemical group of natural compounds known, with over 2000 individual compounds described to date (Spring 2000). The terpenoid family is very diverse with structures ranging from simple linear hydrocarbons to highly complex derivatised polycyclic molecules. The unfying feature of this family of compounds is that all terpenoids can hypothetically be constructed from multiples of a fundamental five-carbon building block, isoprene unit $(C_5H_8)_n$ (Banthorpe 1998; Harbone 1998; Cowan 1999; Hallahan 2000). Thus they range from volatile mono- and sesquiterpenes (C_{10} and C_{15}) through the less volatile diterpenes (C_{20}) to the nonvolatile triterpenoids, sterols (C_{30}) and carotenoid (C_{40}) (Banthorpe 1998; Spring 2000). Each of these various classes are of significance in plant growth, metabolism and ecology. The mono- and sesquiterpenes are distillable fractions that provide plants with characteristic smells (Banthorpe 1998; Harbone 1998; Hallahan 2000). They are thus commercially important as the basis of natural perfumes, soaps, disinfectants, detergents, spices and flavourings in the food industry, medical preparations and vitamin supplements (Ahmed *et al.* 1994; Banthorpe 1998). They

have also been reported to have pharmacological or therapeutical activity (Sticher 1977 as cited by Nakatsu *et al* 2000).

Terpenoids are lipid-soluble and are located in the cytoplasm of the plant cell Terpenoids are extracted from plant tissues with petroleum, ether, chloroform, supercritical-CO_2 or steam distillation (for volatile terpenoids) and can be separated by chromatography on silica gel or alumina (Harbone 1998). For very small samples of about 0.5g, the microdistillation technique (Eppendorf) can be used where the oil from the sample is concentrated in little solvent. Non-volatile terpenoids can be extracted using solvents of different polarities. The methods of extraction of chemical compounds from plants significantly affect the chemical constituents and composition of the extracts. The most appropriate and convenient method to concentrate all the components of the extract should be selected (Nakatsu *et al* 2000). Terpenoids are a common constituent of essential oils, which may exist in various structural forms such as phenolics, aromatics, cyclic and acyclic compounds, acetonides and sulfur- and nitrogen- containing compounds (Nakatsu *et al.* 2000).

2.3.1 Extraction

The plant material was processed immediately or a day after harvesting to avoid possible temporal effects on the essential oil content. Extraction of the essential oils was done by hydrodistillation of the aerial parts of the plants in a clevenger-type apparatus for four hours (Figure 14). This technique is based on the evaporation of volatile compounds induced by steam. The oil was collected, dried through anhydrous sodium sulphate and stored in amber vials and refrigerated. Microdistillation was done on plant samples that were less than one gram. The leaves were crushed and placed in a sample vial with 10 ml of water. 2.5g NaCl and 0.5 ml water were placed in the collecting vial, and 300 µl of hexane was added into the collecting vial to trap the volatile compounds. The sample vials were then heated to 108 °C at a rate of 20 °C/minutes and kept at 108 °C for 90 minutes, and heated again to 112 °C at a rate of 20 °C/minutes and kept at this temperature for 30 minutes. Finally the samples were subjected to post-run for six minutes under the same conditions. The collecting vial was cooled to 1 °C during distillation and after distillation, the organic layer in the collecting vial was injected to the GC/MS (Figure 15).

Extraction of non-volatile compounds was done by solvent extraction method using methanol. Approximately 0.5 g of dried leaf material was weighed and ground to a fine

Figure 14. Hydrodistillation of plant material to obtain essential oil.

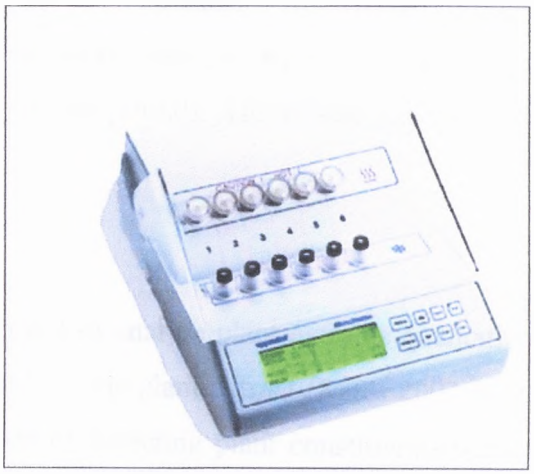


Figure 15. Microdistillation apparatus (Eppendorf) used to extract essential oils from small amount of plant material (< 1g)

Figure 16. Optimizing the mobile phase for the TLC separation of plant extracts.

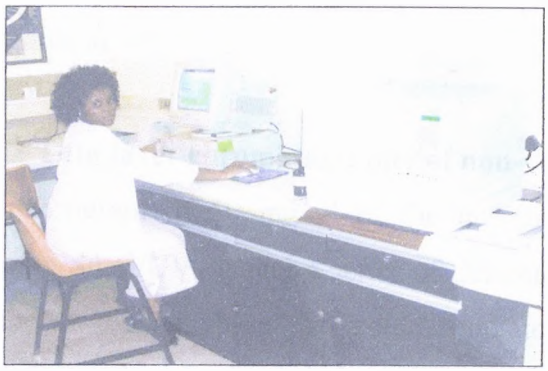


Figure 17. The analysis of essential oils by GC and GC-MS.

Figure 14. Hydrodistillation of plant material to obtain essential oil.

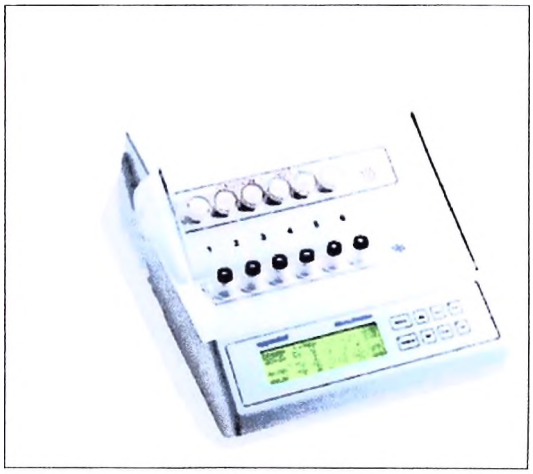


Figure 15. Microdistillation apparatus (Eppendorf) used to extract essential oils from small amount of plant material (< 1g)

Figure 16. Optimizing the mobile phase for the TLC separation of plant extracts.

Figure 17. The analysis of essential oils by GC and GC-MS.

powder in a mortar. Methanol (10 ml) was added to the powder and left to extract for three hours in a water bath with temperature of about 40 °C The extract was passed through a sieve to remove all the debris. The methanol concentrate was passed through a sephadex LH-20 column to remove the abundant essential oil compounds. The eluent was then dried and the percentage yield calculated accordingly.

2.3.2 Thin layer chromatography (TLC).

Thin layer chromatography is a technique that is used to analyse plant extracts qualitatively by demonstrating the most characteristic constituents of the plant extract (Rates 2001; Grace *et al.* 2002). It is the simplest and cheapest method of detecting plant constituents because the method is easy to run, reproducible and requires little equipment (Marston and Hostettmann 1999). It can be used to screen plant extracts by using standard compounds for the identification of metabolites (Spring 2000). However, because plant extracts are complex mixtures of different compounds, other chromatographic techniques are necessary in characterizing chemical compounds in plant extracts. TLC plates were run for both the essential oils and non-volatile compounds in order to establish qualitative variation of different populations and of different taxa.

2.3.2.1 Thin layer chromatography of essential oils

One part of concentrated essential oil was diluted with seven parts of hexane and 2µl of the solution was applied on silica gel plates (Alugram Sil G/UV_{254}). Toluene/ethyl acetate (9.3:0.7) was used as the mobile phase. The developed plates were sprayed with vanillin and heated at 100 °C.

2.3.2.2 Thin layer chromatography of non-volatile compounds

Five microgram of 50 mg/ml of the methanol extract was applied on silica gel plates (Alugram Sil G/UV_{254}) and a series of different mobile phases were used to investigate the appropriate mobile phase that could separate the compounds (Figure 16). The solvents included; methanol/water/ethyl acetate (16.5:13.5:100), toluene/ethyl acetate (60:40), chloroform/methanol/water (64:50:10), n-hexane/ether (40:60), methanol/chloroform (30:60), glacial acetate/ethyl acetate/formic acid/water (11:100:11:26), chloroform/methanol (99.6:0.4) and toluene/dioxane/acetic acid (90:25:1). The plates were sprayed with 10 ml natural spray reagent composed of diphenylborinic acid, 2-aminoethyl

ether in 1% methanol (solvent A) and polyethylene glycol in 5% ethanol (solvent B) for colour development and then oven heated at 100 °C. The plates were viewed under UV light at 254 nm and 366 nm.

2.3.3 Gas chromatography (GC) and GC coupled with mass spectroscopy

Harborne (1998) reports that gas chromatography (GC) analysis is an indispensable tool for chemotaxonomic studies of essential oils. GC and high performance liquid chromatography (HPLC) are very useful for the separation of plant extracts (Spring 2000). For volatile compounds, GC coupled with the mass spectroscopy is the method of choice for the identification of terpenoids, fatty acids and many other volatile compounds in complex plant extracts (Spring 2000). For chemical compound screening, HPLC coupled with different detection methods like ultra violet (UV), nuclear magnetic resonance (NMR) or mass spectrometer (MS) provide very useful preliminary information about the content and nature of compounds found in the non-volatile extracts (Hostettmann 1999). Both GC and GC/MS techniques were performed on the *S. stenophylla* species complex essential oils. However, GC was done as a preliminary screening to observe the variation within and between populations, and further identification of the compounds was done by GC/MS.

A Shimadzu GC-17A gas chromatograph (Figure 17) fitted with J & W-DB1 column (30 m X 0.25 mm id., 0.25 mm film thickness) was used. The column temperature was programmed at 60 °C for one minute and then raised to 180 °C for the total analysis time of 80 minutes. The injection port and FID detector were at 250 °C. Helium was used as the carrier gas.

Gas chromatography coupled with mass spectroscopy (GC/MS) was completed by myself at the Medicinal and Aromatic Plant Research Centre (TBAM), Anadolu University in Turkey. Qualitative and quantitative analysis of the essential oil samples by distillation were carried out by mass spectra obtained with a computerized system consisting of a gas chromatograph coupled to a mass selective detector (Hewlett-Parkard GCD system), with electron impact ionisation of 70eV. The system was equipped with an Innowax FSC column (60 m x 0.25 mm) and helium as the carrier gas. Analysis was then carried out under the GC oven temperature being kept at 60 °C for 10 minutes and programmed to 220 °C at a rate of 4 °C/minute. Split flow was adjusted at 50mL/minute. The injector and detector temperatures were at 250 °C. Mass range was from m/z 35 to 425 Relative

percentage amounts of the separated compounds were calculated automatically from peak areas of the total ion chromatogram. Compound identification was done by the TBAM GC/MS library search of retention indices in comparison with literature values.

2.3.4 High performance liquid chromatography (HPLC)

The detection and identification of non-volatile compounds in methanol extracts was performed. Nine extracts of the *S. stenophylla* species complex (three from each *a priori* groups) together with the *S. stenophylla* isolate obtained from the bioassay guided column chromatography were subjected to HPLC/MS using a Waters 2690 HPLC system (Phenomenex Aqua C18 column, 250·2.1 mm) equipped with both a 996 photodiode array (PDA) detector and a Thermabeam mass selective detector (TMD) [electron impact (EI) mode], or alternatively a Z spray mass selective detector (ZMD) [electro-spray mode]. The TMD detector was operated in electron impact mode with the ionizer at 70 eV, with a gain of 10 and scanning a mass range of 50–550 amu. The flow rate of the HPLC was 0.2 ml/min and the gas flow through the nebulizer 30 L/h. The nebulizer temperature was 80 °C, the expansion region 90 °C and the source temperature 225 °C No flow splitter was used. The ZMD was operated in positive mode with no flow splitting and an HPLC flow rate of 0.2 ml/min.

To identify and confirm the presence of rosmarinic acid and carnosic acid in the extracts, rosmarinic acid and carnosic acid standards were co-injected with the samples and their retention times were compared with the retention times of some compounds detected in the test samples.

2.3.5 Multivariate analysis

The multivariate method of analysis can be performed on the phytochemical data to describe the full extent of chemical constitution variability within geographically separated populations and to delimit the taxa. This method of analysis is a numerical evaluation of the affinity or similarity between taxonomic units and the ordering of these units into taxa on the basis of their affinities (Sokal and Rohlf 1962). It is popular in studies on patterns of population variation and species delimitation (Verboom and Linder 1998). Sokal and Sneath (1963) believe that when taxa are established on the basis of an adequate representation of characters, the resulting classification will be natural. Classification in numerical taxonomy

is based on a matrix of resemblances, and it consists of various techniques designed to disclose and summarize the structure of the matrix. A rough, graphical representation of the structure can be obtained by differential shading of the elements of the matrix. The various computational methods for clustering processes the data equally efficiently whether they are ordered or not. The lowest taxonomic unit being classified in any study is called an Operational Taxonomic Unit (OTU). OTUs represent the tips of the dendrogram. The lowest ranked OTUs that can be clustered are individuals, which could represent infraspecific taxa. Higher taxonomic entities like genera, tribes or families can also be used as OTUs. Many kinds of analyses are contained in this package of multivariate analysis depending on the type of data set and the kind of information to retrieve from the data set.

2.3.5.1 The data matrices

The essential oil composition data was subjected to three analyses in the NTYSYS-pc package. These being the cluster analysis (CA), principal component analysis (PCA) and principal coordinates analysis (PCOORD). Data matrices were created based on the qualitative and quantitative characters obtained from the GC/MS results. From the 214 compounds identified, compounds (166) that occurred above 0.05% in at least one essential oil sample were selected for the quantitative data matrix. Character states for each specimen were listed in a matrix with the individual populations as Operational Taxonomic Units (OTUs), where n = 25 as columns of taxon localities (essential oils that were obtained by hydrodistillation only) and n = 166 as rows of compounds. Where the information was missing or character (compound) inapplicable a missing value code of zero (0) was assigned. Qualitative characters were scored as absent and present and a binary matrix was constructed with 197 characters. A third data matrix was constructed by combining the quantitative and qualitative matrices. This matrix was composed of 400 characters and 25 OTUs. A phenetic program NTYSYS-pc Version 2.0 (Rohlf 1998) was used to analyze the data matrices. The analyses were performed on standardized data for the quantitative and combined matrices. Standardization is done to reduce the effects of different scales of measurements in different characters.

2.3.5.2 Cluster analysis

Cluster analysis (CA) is more or less an automatic method for establishing and defining clusters of mutually high similarity coefficients among the entities in the resemblance matrix. The clusters are based on phenetic resemblances only and do not necessarily have

phylogenetic connotations (Sokal and Rohlf 1962; Sokal and Sneath 1963; McGarigal *et al.* 2000). The common aspect of cluster analysis is that it permits the delimitation of taxonomic groups, providing that the coefficients of relationships have been properly based on many characters chosen without bias and correctly computed. It is routinely used both in taxonomy and community ecology to classify individuals on the basis of multivariate attributes with a wide choice of clustering algorithms (Shaw 1998; McGarigal *et al.* 2000) The groups that are obtained can be defined using the concept of a phenon line, with an inclusion of an outgroup (Sokal and Sneath 1963; Sebola and Balkwill *in press*).

The patterns of similarity among OTUs were explored using the SIMINT module, which computes a variety of similarity and dissimilarity coefficients for interval measure on quantitative data using the Euclidean distance as a measure of similarity between samples. The SIMQUAL module was applied to compute similarity coefficient for qualitative data. The SAHN procedure followed for both data, which performs the various clustering algorithms (UPGMA cluster). This algorithm finds alternative trees when ties in the input matrix occur. A COPH module, was then applied to produce a cophenetic value matrix from a tree matrix produced by the SAHN algorithm. The cophenetic value matrix is used to test the goodness-of-fit or consistence of the cluster analysis to the data by using the MXCOMP to compare the original similarity or dissimilarity matrix that was clustered with the cophenetic value matrix. The dendrograms were lastly generated using the TREEG module.

2.3.5.3 Principal component and principal coordinates analyses

Principal component analysis (PCA) is designed for continuously varying or quantitative data or multistate data expressed on a scale of reasonable length (Parnell 1999). It thus condenses the information contained in a large number of original variables into a smaller set of new composite dimensions, with a minimum loss of information (McGarigal *et al.* 2000). Principal coordinates analysis (PCOORD) is very similar to PCA, except that the former incorporates binary data in the analysis. They are the most mathematically natural ordination techniques and the ordination diagrams can be used as an objective summary description of the results obtained (Shaw 1998). PCA finds the linear scaling of characters that maximizes the variation. However only the first few axes represent effective summaries of the data (Shaw 1998; Parnell 1999). An indication of the number of effective dimensions present in the character matrix is hereby achieved (Almeida and Bisby 1984; Shaw 1998; Mikkelsen and Selberg 2001).

To establish which characters contribute to the defining of phenetic clusters among the OTUs, principal component analysis (PCA) and principal coordinates analysis (PCOORD) can be performed on the quantitative and combined data matrices respectively. These were performed to establish the discriminate power for the selected constituents. From the PCA, evaluation of correlation matrix was calculated using the coefficient CORR of the SIMINT-programme. Eigenvectors of the correlation matrix were extracted using the EIGEN-module, which showed that the whole data set could be projected in the space defined by the first three principal components retaining a significant percentage of the total variation. The original standardized matrix was projected onto the first three of the principal axes to visualize the data structure with the PROJ-program. As for the PCOORD, evaluation of correlation matrix was calculated using the coefficient DIST of the SIMINT-program. The DIST output was the DCENTRED and eigen vectors were retrieved and projected in a three dimension space using the PROJ-program.

2.4 Amplified fragment length polymorphisms (AFLPs)

Genetic diversity studies can be used to address questions regarding genetic relatedness among individuals, population structure, phylogenetic relationships and mapping of quantitative trait loci (Mueller and Wolfenbarger 1999; Bottinni *et al.* 2002). For this reason a series of techniques and genetic markers have been developed to estimate genetic diversity that are useful for assessing genetic differences among individuals, populations and independently evolving lineages such as species (Mueller and Wolfenbarger 1999). Techniques used to generate genetic markers include allozymes, restriction fragment length polymorphisms (RFLPs), microsatellites, random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLPs).

DNA fingerprinting involves the production of a set of fragments reflecting the genetic constitution of an individual (Qamaruz-Zaman *et al.* 1998). DNA fingerprints can be obtained in two ways; (1) by classical hybridization-based fingerprinting which involves cutting the genomic DNA with a restriction enzyme, separating the resulting DNA fragments by electrophoresis according to size, and detecting polymorphic multilocus banding patterns by hybridization with a labeled, complementary DNA sequence; (2) PCR-based fingerprinting which involves amplifying a set of DNA fragments with the help of specifically or arbitrary chosen oligonucleotides (primers) and a thermostable DNA

polymerase, separating electrophoretically the amplified fragments and visualizing the polymorphic banding patterns. These fragments may be separated according to size by gel electrophoresis and visualized using autoradiography, silver staining or fluorescent dyes (Qamaruz-Zaman *et al.* 1998). Several molecular techniques have been developed to provide information on the diversity and genetic relationships in the family Lamiaceae. The existence of the complex systematics of *Salvia stenophylla*, *S. repens* and *S. runcinata* led us to choose AFLPs.

The amplified fragment length polymorphism (AFLP) technique is a multilocus technique used for broad applications including establishing or enriching maps, mainly in plants (Pérez-Enciso and Roussot 2002). It is a very cost-effective, easy and rapid technique that highly reproducibly generates hundreds of markers from small amounts of DNA (Fay *et al.* 2002; Pérez-Enciso and Roussot 2002; Qamaruz-Zaman *et al.* 2002). The technique is suitable for threatened or species-complex taxa and has been widely used in conservation genetics to determine levels of genetic variation within and between populations (Fay *et al.* 2002; Qamaruz-Zaman *et al. in press*). AFLP markers can be applied to evaluate gene flow and dispersal, outcrossing, introgression and cases of hybridization (Mueller and Wolfenbarger 1999). The usefulness of AFLP markers for systematics rests more on the rapid grouping of closely related lineages, which is crucial for biodiversity surveys or epidemiological research (Mueller and Wolfenbarger 1999). However, the main drawback of AFLPs is their biallelic and dominant nature, which causes the quantitative traits not to be categorized into discrete classes of heterozygosity and dominant homozygosity. This results in a significant loss of information, especially as regards mapping of quantitative trait loci (QTLs) (Pérez-Enciso and Roussot 2002). The genetic study was not part of the initial protocol, however, opportunity allowed us to do preliminary study of AFLPs.

2.4.1 Sampling

Ten populations of *Salvia stenophylla*, *S. runcinata* and *S. repens* were sampled. Leaf material from each individual in ten populations was collected and stored in silica gel. Thus, three individual plants were collected from each population and so nine individuals of *S. stenophylla* were collected from Biesiesvlei, Sannieshof and Smithfield, nine individuals of *S. runcinata* from Bergville, Coligny and Klerkskraal Dam, and twelve individuals of *S. repens* were collected from Knapdaar, Indwe, Lady Grey and Queenstown, totalling thirty individual plants.

2.4.2 DNA extraction

DNA was extracted using the modified 2X CTAB (cetyltrimethyl-ammonium bromide) method of Doyle and Doyle, (1987). About 0.2 g of silica gel dried leaves were ground in 10 ml preheated 2X CTAB isolation buffer containing 40 µl of betamercaptoethanol. The slurry was then poured into 50 ml blue cap tubes and incubated at 65 °C for 10 minutes, after which 10 ml of SEVAG (chloroform:isoamylalcohol 24:1) was added while mixing the slurry gently but thoroughly. The tubes were horizontally placed on a shaker for 1 hour. The slurry was then spun at 8000 rpm for 10 minutes. The aqueous phase was decanted and 300 µl purified in a QiaQuick column (Qiagen) following the manufacturer's instructions for cleaning PCR products. The DNA was eluted in 100 µl EB buffer and 5 µl of the solution was run on a 1% agarose gel to verify the success of the extraction.

2.4.3 AFLPs fingerprinting

Fifteen microliters (and 20 µl for some specimens) of the DNA template was dried at 65 °C. For further use the DNA was reconstituted with 5.5 µl double distilled water (ddH_2O). AFLP procedure requires 500 ng of DNA per sample and DNA concentrations were estimated visually from an agarose gel using a pre-determined size-standard. The volume of DNA needed to obtain 500 ng was calculated. This volume was dried down to a pellet and resuspended in 5.5 µl of water (because this is the maximum volume of template DNA permitted by the AFLP procedure). The AFLP procedures were conducted according to the AFLP™ Plant Mapping protocol of Applied Biosystems Inc. (1996). There are four main steps that are involved in the procedure:

1. **Restriction ligation** involved digestion of the 5.5 µl aliquot of DNA template using 5.5 µl of two master mixes containing restriction enzymes EcoRI and Tru 9 (this is an isoschizomer for MseI used in the ABI protocol). This step generated template DNA fragments to which EcoRI and Tru 9 adapters were ligated in the same reaction, thereby generating primer binding sites for subsequent amplification. The restriction-ligation product was then diluted with Tris-EDTA (TE0.1) to 200 µl.

2. **Preselective PCR** involved the amplification of restriction fragments with a matching nucleotide at the 3' end. Thus 2 µl of the diluted restrion-ligation product,

0.5 µl AFLP preselective primer pairs and 7.5 µl core mix were placed together, microcentrifuged briefly, and then run in a GeneAmp 9700 thermal cycler. This resulted in a 16-fold reduction (4x4) in the number of amplified fragments. The success of this step was verified by running 4 µl of the DNA template on a 1.5% agarose gel.

3. **Selective PCR** involved the amplification of the preselective products using primer combinations that consist of fluorescently labeled EcoRI-based primers and Tru 9-based primer. At this stage there are eight EcoR1 and eight Tru9 primers to choose from, Therefore an initial primer trial was conducted using 24 different primer combinations to find a combination that yielded a suitable number of polymorphic fragments for the taxa under study. Thus 1.5 µl preselective product, 0.5 µl EcoRI primer, 0.5 µl Tru 9 primer and 7.5 µl core mix were added together and place in the thermal cycler. As a result of the primer trial. One primer combination (FAM EcoR1- ACT, Tru9-CAA) was selected to be used in the study.

4. **Fragment analysis** involved the separation of the fluorescently labeled DNA fragments on a 5% denaturing gel using an ABI 377 automated sequencer. 0.4 µl of the amplification products was loaded on the sequencer along with GSROX500 size standard in each lane (Figure 21). Gel analysis was carried out with Genescan Version 3.1.2 and and the subsequent AFLP profiles analysed using Genotyper Version 2.5. Bands were identified ranging from 50 to 500 base pairs and scored as present (1) or absent (0) resulting in a binary matrix.

5. **Analyses** of the binary matrix were performed using the UPGMA (unweighted pair group method using arithmetic averages) algorithm and NJ (neighbour joining; Saitou & Nei 1987) methods of the test version 4.0d80 of PAUP* (Phylogenetic analysis using parsimony and other methods; Swofford 2000). UPGMA belongs to the cluster analyses techniques for representing similarity or distance data in the form of an ultrametric tree. The tree was constructed by linking the least distant pairs of taxa, followed by successively more distant taxa, or groups of taxa. Ultrametric distances were defined as values that precisely fit a rooted tree with a constant molecular clock, thus the principle assumption for this method is that the

data are approximately ultrametric. Neighbour joining is conceptually related to cluster analysis but removes the assumption that the data are ultrametric. In contrast to cluster analysis, NJ keeps track of nodes on a tree rather than taxa or clusters of taxa. The raw data are provided as a distance matrix, and a modified distance matrix is constructed in which the separation between each pair of nodes is adjusted on the basis of their average divergence from all other nodes. Standard mean character differences were used to calculate distances for the construction of both UPGMA and neighbour joining (NJ) dendrograms.

2.5 Biological activity

2.5.1 Antibacterial activity

Many studies have been performed on the antimicrobial activities of plant extracts (Janssen *et al.* 1987). Antimicrobial activity involves the evaluation of the potency of antimicrobial agents by examining the dose that inhibits the growth of a suitable susceptible microorganism. This is not an easy task because the antimicrobial activity often does not depend on only one active compound (Buchbauer 1993, Nakatsu *et al.* 2000).

There are several common methods that are employed for antimicrobial assays. The method of extraction of essential oils and non-volatile compounds is important because chemical constituents and composition is affected (Nakatsu *et al.* 2000), which in turn can affect the antimicrobial activity. For instance, the most appropriate solvent for non-volatile extracts should be carefully selected to concentrate specific active compounds. This also applies to the selective extraction of essential oils (Nakatsu *et al.* 2000), and because of the volatility of essential oils, factors such as assay techniques, growth media, microorganisms and the type of essential oil should be considered when carrying out the antimicrobial screening (Janssen *et al.* 1987). The ability of the test compound to diffuse through the agar medium should also be considered (Nakatsu *et al.* 2000; Pauli and Kubeczka 1996). However, the most common method used is the disc diffusion method, which involves the application of the extract onto a filter paper disc, and the disc is placed onto solid medium seeded with the test microorganism. Another method is the incorporation of the plant extract into cooled agar or broth either in the presence or absence of a solvent (Nakatsu *et al.* 2000). Identification and isolation of the individual chemical compounds that are contained in the extracts is critical in understanding the origin of the biological activity.

2.5.1.1 Disc diffusion assay

Antimicrobial screening was done on both the essential oils (23 samples) and non-volatile extracts (22 samples) using six gram-positive bacteria; *Staphylococcus aureus* (ATCC 6538 and ATCC 25923), *Staphylococcus epidermidis* (ATCC 2223), *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6051), methicillin resistant *Staphylococcus aureus* (MRSA, clinical strain) and nine gram-negative; bacteria *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (NCTC 9633), *Salmonella typhimurium* (clinical strain), *Salmonella enteriditis* (clinical strain), *Yersinia enterocolitica* (ATCC 23715), *Serratia odorifera* (ATCC 33132), *Proteus vulgaris* (clinical strain), two yeasts; *Cryptococcus neoformans* (ATCC 90112) and *Candida albicans* (ATCC 10231), two moulds; *Aspergillus niger* (clinical strain) and *Alternaria alternata* (clinical strain)(Figure 18).

One milliliter of the bacterial spore suspension yielding an inoculum size of approximately 1 x 10^6 was placed in 100 ml of Tryptone Soya agar plate. The agar was prepared by mixing 30g of agar into 750 ml of distilled water and autoclaved at 121 °C for 15 minutes. This process is to sterilize the agar. The agar was poured into sterile bioassay plates in two phases. The base layer was half the unit of the total amount. It was left to solidify, and then the second layer seeded with the test organism was poured on top. After this preparation, paper discs impregnated with approximately 5 µl of the essential oil and 20 µl of 50 mg/ml methanol extract were placed on the seeded Tryptone Soya agar. Neomycin 30 µg and Nystatin 100 iµ discs were used for bacterial and fungal controls respectively. The plates were allowed to pre-diffuse for one hour in the fridge before incubation overnight at 37 °C for 18 hours. Pre-diffusion is done to enhance diffusion of the plant extract into the agar (Janssen *et al.* 1986). The sizes of the inhibition zones were measured from the edge of the disc. The inhibition zones are taken as a measure of efficiency of the test compound.

2.5.1.2 Minimum inhibition concentration assay

To evaluate the sensitivity of an organism towards the antimicrobial compound, minimum inhibitory concentration assays (MIC) were performed. The methanol extracts which showed good activity were selected for the well-dilution test to determine the antibacterial activity against *S. aureus* (ATCC 6538 and 25923), *S. epidermidis* (ATCC 2223), *B. cereus* (ATCC 11778) and *B. subtilis* (ATCC 6051). The extracts include *S. stenophylla* collected

from Sannieshof, Biesiesvlei, Springfontein and Molteno; *S. runcinata* collected from Roossenekal, Klerkskraal Dam, Bethlehem, Lichtenburg and Kroonstad, and samples collected from Indwe, Knapdaar and George for *S. repens*. To determine the minimum inhibition concentration (MIC), initial extract concentrations were prepared at 32 mg/ml in methanol. Serial dilutions of the initial concentration were then prepared in a microtiter plate (96 wells). A defined volume of the test plant extract was added to the first test well (100 µl sample + 100 µl sterile water), then 100 µl thereof was transferred to the second wells. This procedure was repeated till the last well. Control wells (just methanol) were used to observe the normal bacterial growth. Then 100 µl of the microbial suspension was added to each well. The plates were then incubated at 37 °C for 18 hours. Forty microlitres of 0.4 mg/ml *P*-iodonitrotetrazolium violet (INT) solution was added to the wells after incubation and the plates were left to stand for several hours. Readings were made after six hours and after 24 hours. MICs were determined as the lowest concentrations preventing visible microbial growth.

2.5.1.3 Thin layer chromatography bioautographic assay

To investigate the actual compounds responsible for the antimicrobial activity in the extracts, bioautography, a very convenient and simple method to test plant extracts and pure substances for their effects on both human pathogenic and plant pathogenic microorganisms can be performed (Marston and Hostettmann 1999). There are three methods that can be used to carry out this technique; (a) agar diffusion (in which the antimicrobial agent is transferred from the chromatogram to an inoculated agar plate through a diffusion process), (b) direct bioautographic detection of the TLC plate which is applicable to microorganisms that can grow directly on the TLC plate; and (c) agar-overlay, a hybrid of the two other methods. It involves the tranfer of active compounds from the stationary phase to the agar layer by diffusion process (Hostettmann 1999; Marston and Hostettmann 1999). Once the active compound is spotted on the TLC-bioautography plate, isolation can follow by column chromatography and thereafter analysis of the isolate using HPLC techniques.

The agar-overlay method was used and the assay was performed on methanol extracts of *S. stenophylla* plants collected from Biesiesvlei and Sannieshof. Approximately 5 µl aliquot of 50 mg/ml extract was applied on silica gel plates (Alugram Sil G/UV_{254}) and in order to establish the non-volatile compounds responsible for the antibacterial activity, two plates on

which carnosic acid (Sigma-Aldrich C-0609) and rosmarinic acid (Fluka Sigma-Aldrich 44699) standards were applied were developed in different mobile phases. One TLC plate was developed in toluene, dioxane and acetic acid (90:25:5) solvent and the other TLC plate was developed in ethylacetate; benzene; formic acid (2.4:2:1:0.6) solvent system. Different solvent systems were selected because rosmarinic acid and carnosic acid dissolve in solvent with different polarities. The developed TLC plates were sterilized under UV light for about one hour and then placed onto the base layer facing upwards. The seeded agar was evenly poured onto the TLC plate and pre-diffused for one hour. They were incubated at 37 °C for 18 hours after which they were sprayed with 0.2 mg/ml INT reagent. The inhibition zones were recorded as clear zones on a maroon background.

2.5.1.4 Column chromatography

Upon observing an inhibition zone at R_f value 0.54 on the TLC-bioautographic plate, a bioassay-guided fractionation was performed using the methanol extract of *S. stenophylla* collected from Sannieshof. Four gram of the extract was separated by column chromatography over silica gel 60 (0.063-0.2 mm/70-230, Mesh ASTM, Macherey-Nagel) using a chloroform/methanol (9.6:0.4) gradient solvent system. Fractions were collected in test tubes and TLC plates were developed in order to identify the particular fractions that exhibited an approximate R_f value of 0.5. Since bioautography established that the most active fraction against *Staphylococcus aureus* was at $R_f = 0.5$. The active fraction was further separated on a smaller column to yield 0.398 mg of the active isolate. The MIC value of the isolate was determined as earlier outlined.

2.5.2 Antioxidant properties

There are several methods that are used to determine antioxidant properties in plant extracts. Such methods include; ESR, chemiluminescence, oxygen radical absorbance capacity and enhanced chemiluminescence assays have been used for measuring radical scavenging activity and antioxidative potentials of non-volatile compounds. Blois' DPPH method and its variations, methyl linoleate and –caroten/linoleate models have often been adopted for antioxidant activity evaluation (Culvelier *et al.* 1992 as cited by Perry *et al.* 2001). Many *in vivo* methods such as thiobarbituric acid reactive species (TBARS) are used (Baratta *et al.* 1998).

The antioxidant activity of the methanol extracts in this study was assessed using the thin layer chromatography-2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Four microliters of 50 mg/ml methanol extract of the *Salvia stenophylla*, *Salvia runcinata* and *Salvia repens* were applied onto a silica gel plate (Alugram sil G/UV_{254}). The plate was developed in chloroform; ethylacetate; benzene; formic acid (2.4:2:1:0.6) mobile phase after which it was sprayed with DPPH radical. The presence of antoxidants was recorded as white spots against a purple background.

2.5.3 Anti-inflammatory activity

All mammalian cells, except red blood cells, produce potent inflammatory mediators; among these are the prostaglandins (PGs), prostacyclins (PGI_2), thromboxanes (TXs), leukotrienes (LTs) (Mantri and Witiak 1994) and eicosanoids derived from the cyclooxygenase (COX) and lipoxygenase (LOX) pathways (Benrezzouk *et al.* 2001). Physiologically, the mentioned autocoids produce inflammation, pain, and fever, regulate blood pressure and induce blood clotting. Therefore, inhibition of the important biosynthetic enzymes, which are prostaglandin H synthase (PGH synthase or cyclooxygenase) and 5-lipoxygenase (5-LOX), that catalyse the formation of putative intermediates involved in the inflammatory process is the task of anti-inflammatory compounds. The mechanism by which these two enzymes operate is to convert by oxidation their common substrate arachidonic acid (AA) into prostagladins, thromboxanes and leukotrienes. Prostaglandins induce redness (erythema) and swelling (oedema) associated with heat and pain. These result in the development and inflammatory diseases like asthma (Benrezzouk *et al.* 2001), articular rheumatism and psoriasis (Zurier 1982 as cited by Shale *et al.* 1999).

However, cyclooxygenase (COX) exists in two isoforms, constituting COX-1, which is responsible for basal formation of prostaglandins (PGs) in many tissues, including the stomach, kidney and platelet and COX-2, which is induced by cytokines, growth factors and mutagens that catalyse the synthesis of high levels of PGs in the inflammatory response (Wallace and Chin 1997; Benrezzouk *et al.* 2001). Selective inhibitors of COX-2 inhibit prostaglandin synthesis at sites of inflammation but spares prostaglandin synthesis in the gastrointestinal tract and there not induce ulcers like standard NSAIDs (Wallace and Chin 1997). There has been research directed towards the development of highly selective inhibitors of COX-2, and as such essential oils can be assessed on COX-2. The enzyme is obtained from the sheep seminal vesicle microsomes. Cyclooxygenase (COX) converts

arachidonic acid (AA) into prostaglandin E_2 (PGE_2), hence as a mediator of inflammation. High yields of its production in the assay indicate no synthesis inhibition (Noreen *et al.* 1998).

The anti-inflammatory effect of seventeen essential oils of *S. stenophylla, S. repens* and *S. runcinata* was assessed on cyclooxygenase (COX-2) (Figure 19). The COX-2 (isoenzyme of COX-1) catalysed prostaglandin biosynthesis assay was done *in vitro* according to Noreen *et al* (1998). The enzyme (60 μl), which was activated with 1.25 ml co-factor, was added to the 20 μl prepared test samples after pre-incubating on ice for about 5 minutes. Prior to incubation at 37 °C, 20 μl (l-^{14}C) arachidonic acid was added, after which the test samples were incubated for 10 minutes. The reaction was terminated by adding 10 μl 2N HCl, and then 4 μl of carrier solution. The unmetabolized arachidonic acid was separated from the prostaglandin by column chromatography packed with silica gel 60 (particle size 0.063-0.200 mm) by eluting it with n-hexane/1, 4-dioxane/glacial acetate (70:30:1). The prostaglandin was eluted with ethyl acetate/methanol (85:15). The samples were counted using a liquid scintillation system (Figure 20), Beckman LS 6000 LL and the percentage inhibition was calculated using the following formula;

$$\% \text{ Inhibition} = 1 - \left[\frac{dpm(sample) - dpm(background)}{dpm(blank) - dpm(background)} \right] \times 100$$

Test samples were prepared by pipetting 17.5 μl water into eppendorf caps, after which 2.5 μl sample as well as 2.5 μl standard indomethicine (200 μM) was added. 2.5 μl DMSO was used for the blank and the background. The co-factor was prepared by weighing 6 mg adrenaline and 3 mg reduced glutathione in a 10 ml test tube. 10 ml TRIS buffer (pH 8.0) was added and the mixture was covered, shaken and stored on ice until the time it was needed. Prostaglandin carrier solution was a mixture of 0.2 μg/ml PGE_2 and PGF_{2a} each in ethanol.

The chemicals, buffers and solutions used for assay include; 1-^{14}C-arachidonic acid (Amersham), *l*-epinephrine (adrenaline-Sigma, E-4375), glutathione (Sigma, G-4251), hematin (Sigma, H-3281), indomethacin (Sigma, I-7378), prostaglandin PGE_2 (Sigma, P-4172), prostaglandin $PGF_{2\alpha}$ (Sigma, P-0424), silica gel (kieselgel 60, Korngroesse 0.063-0.2

mm, 70-230 mesh ASTM) (Merck, 7734), TRIS (Tris[hydroxymethyl] aminomethane) (Sigma, T-1378), phosphate buffer (0.1 M K-phosphate, 3 mM $MgCl_2$, pH 7.4).

Figure 18. Placing saturated discs onto agar.

Figure 19. ^{14}C arachidonic acid added to plant extracts with enzyme solution.

Figure 20. ^{14}C prostaglandin counted with scintillation counter.

Figure 21. Loading the DNA template onto the gene scanner.

RESULTS AND DISCUSSION

3.1 Distribution and habitat

The distributiom map provided in Figure 10, includes all localities from which material was collected and identified (using the key provided by Codd 1985) during this study. The following distribution patterns for the three species have emerged on the basis of the material collected and examined during this study:

- *S. stenophylla* is relatively sparsely distributed over a large geographic area, and occurs in the North-West, Free State, Eastern Cape and the Western Cape provinces.
- *S. repens* is restricted to the Eastern Cape and the Western Cape.
- *S. runcinata* is the most common and widespread species within South Africa, and was recorded from the North-West Province, Gauteng, Mpumalanga, the Free State and Kwa-Zulu Natal.

On the basis of the above distribution patterns (Figure 10), *S. stenophylla* is sympatric with both *S. repens* and *S. runcinata*. *S. repens* and *S. runcinata* are, however, seemingly allopatric, though there may be a slight overlap in distributions along the border between the Eastern Cape and the Free State provinces. The distribution patterns observed during this study therefore differ markedly from those depicted in the distribution maps provided by Codd (1985), which indicate that all three species are largely sympatric.

The distribution maps provided by the National Botanical Institute (Figures 7–9) contain many discrepancies that can, at least in part, be attributed to misidentifications resulting from the ambiguous nature of many of the points in Codd's (1985) key for the identification of southern African *Salvia* species. This confusion is furthermore not limited to the *S. stenophylla* species complex, as illustrated by the fact that three reported loacalities for *S. repens* (at Kuruman, De Rust and Prince Albert) obtained from the PRECIS Database of the NBI were all incorrect. Extensive searches at these localities revealed the presence of only *Salvia verbenaca,* and the specimens on which these locality records are based are likewise *S. verbenaca*.

The habitat of the three taxa, however, is fairly similar with all three showing a preference for disturbed areas within grassland and occasionally open woodland habitat. *S. repens*

material was collected from seasonally moist, disturbed places (e.g. roadside drainage areas) in grassland habitats (Figure 22). *S. runcinata* occurred mainly as a weed (often along roadsides) in mesophytic habitats as at Coligny and Koster, but occasionally also occurred in seasonally wet areas near dams and streams (e g. the Klerkskraal Dam and Delareyville localities (Figure 23)). *S. stenophylla* was also collected from occasionally or seasonally moist, disturbed areas in grassland (Figure 24). *S. stenophylla* specimens from Biesiesvlei and from Sannieshof may represent a new taxon, as they share a diagnostic (within the species complex) morphological character, namely two flowered verticils. These localities furthermore represent a highly specialised habitat, namely seasonally flooded marsh wetlands within the floodplain of the Harts River (Figure 25).

3.2 Greenhouse plant GC results

Of the seeds that were planted from seven populations, seeds from Coligny, Delareyville and Lichtenburg grew and where harvested. However, only the plant whose seeds were collected from Coligny had enough distillable material. Therefore, the essential oil was extracted by hydrodistillation and analysed by gas chromatography as outlined earlier. The profile is very similar to that obtained for the essential oil extracted from the natural population in the previous season (Figure 26). The essential oil from the wild has some additional minor compounds that cannot be detected in the essential oil of the cultivated plant material. This implies that environmental conditions influence the quantity of the compounds. The major compounds, (*E*)-nerolidol, β-caryophyllene, α-humulene and caryophyllene oxide are common in both essential oil samples (Table 4).

β-caryophyllene, α-humulene and caryophyllene oxide are higher in percentage in the oil sample extracted from the wild plants than from the cultivated essential oil sample. However, (*E*)-nerolidol (90%) is high in the cultivated essential oil sample. Thus enivironmental conditions can be manipulated to improve the quality and production of essential oils. In this case, the production of (*E*)-nerolidol can be maximized when need arises for commercial use.

Figure 22. The habitat of *Salvia repens* (Knapdaar).

Figure 23. Collecting of *Salvia runcinata* near Delareyville

Figure 24. The habitat of *Salvia stenophylla* (Sannieshof).

Figure 25. The habitat of *Salvia stenophylla* from Harts River floodplain (Biesiesvlei).

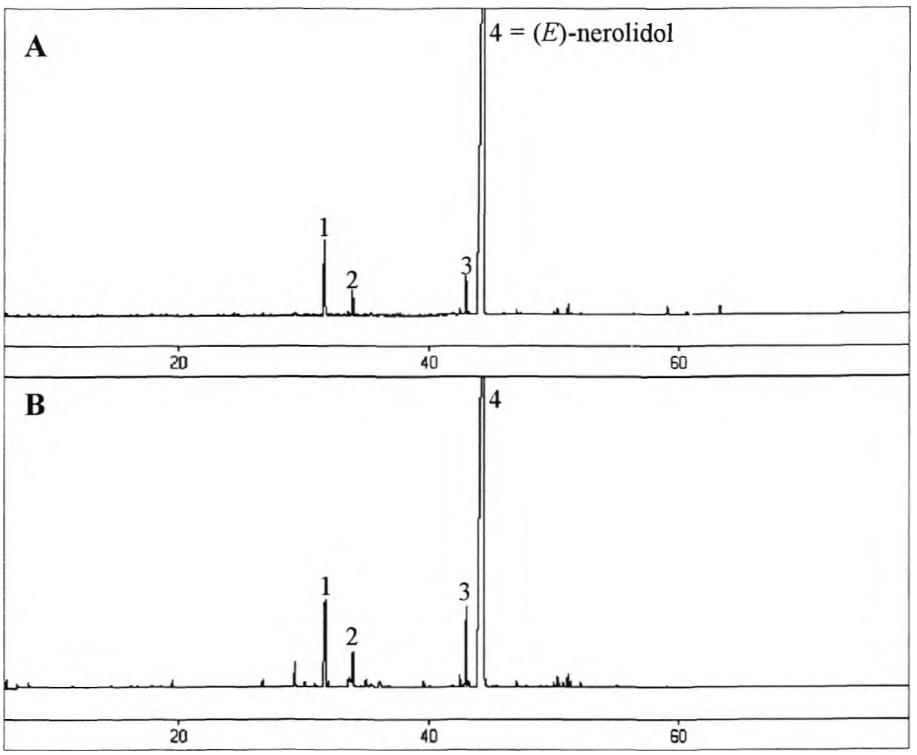


Figure 26. GC profiles for *S. runcinata* essential oils extracted from plants collected from Coligny, cultivated plant (A) and (B) wild.

The similarity of the GC profiles suggests that environmental factors did not significantly alter the chemical composition of the two essential oil samples. This implies that the genetic make-up of the plants influenced the biosynthesis of the major compounds, which in this case can be used as a taxonomic marker. Skoula *et al.* (2000) confirms this finding in that the observed variation in the oil yield and composition between naturally occurring *Salvia fruticosa* and cloned samples was solely due to genetic diversity and not environmental dynamics.

Table 4. Compounds comprising a high percentage of the essential oil samples from the wild and cultivated plant material from Coligny.

Peak number	GC/MS compound identification	Wild essential oil sample	Cultivated essential oil sample
1	β-caryophyllene	4.89%	4.50%
2	α-humulene	1.20%	1.06%
3	Caryophyllene oxide	2.92%	1.67%
4	(*E*)-nerolidol	87.22%	90.00%

TRICHOMES

3.3.1 Trichome type

In all taxa both eglandular and glandular trichomes are scattered on the abaxial and adaxial surfaces of the leaves and on the stem. Eglandular trichomes are more numerous over the veins and leaf margins. Glandular trichomes occur in two sizes and types, namely large peltate trichomes and smaller capitate trichomes. This pattern is a characteristic feature of the Lamiaceae (Ascensão *et al.* 1999). The glandular trichomes are defined according to the shape of the secretory head (Werker *et al.* 1985; Ascensão *et al.* 1995; Hallahan 2000). The following types of trichomes were observed on surfaces of the material of the three taxa;

Type A Peltate trichomes which are composed of one basal epidermal cell, one very short stalk cell and a large round head consisting of four to numerous secretory cells (Figures 27a and 28a). Usually the epidermal and neck cells are embedded into the epidermal tissue of the leaf. In the SEM micrographs, the heads show a smooth or wrinkled surface (Figure 28a) indicating the close attachment of the cuticle to the secretory upper cell walls. The surface is smooth when the accumulation of secretions in the subcuticular space distends the cuticle). In *S. stenophylla*, the head has four cells in the central circle, while the circumference has ten cells arranged in a circular pattern (Figure 28b). There was no raptured trichome that could be examined for the arrangement and pattern of cells in *S. runcinata* and *S. repens*. These trichomes occurred on all the leaf and stem surfaces.

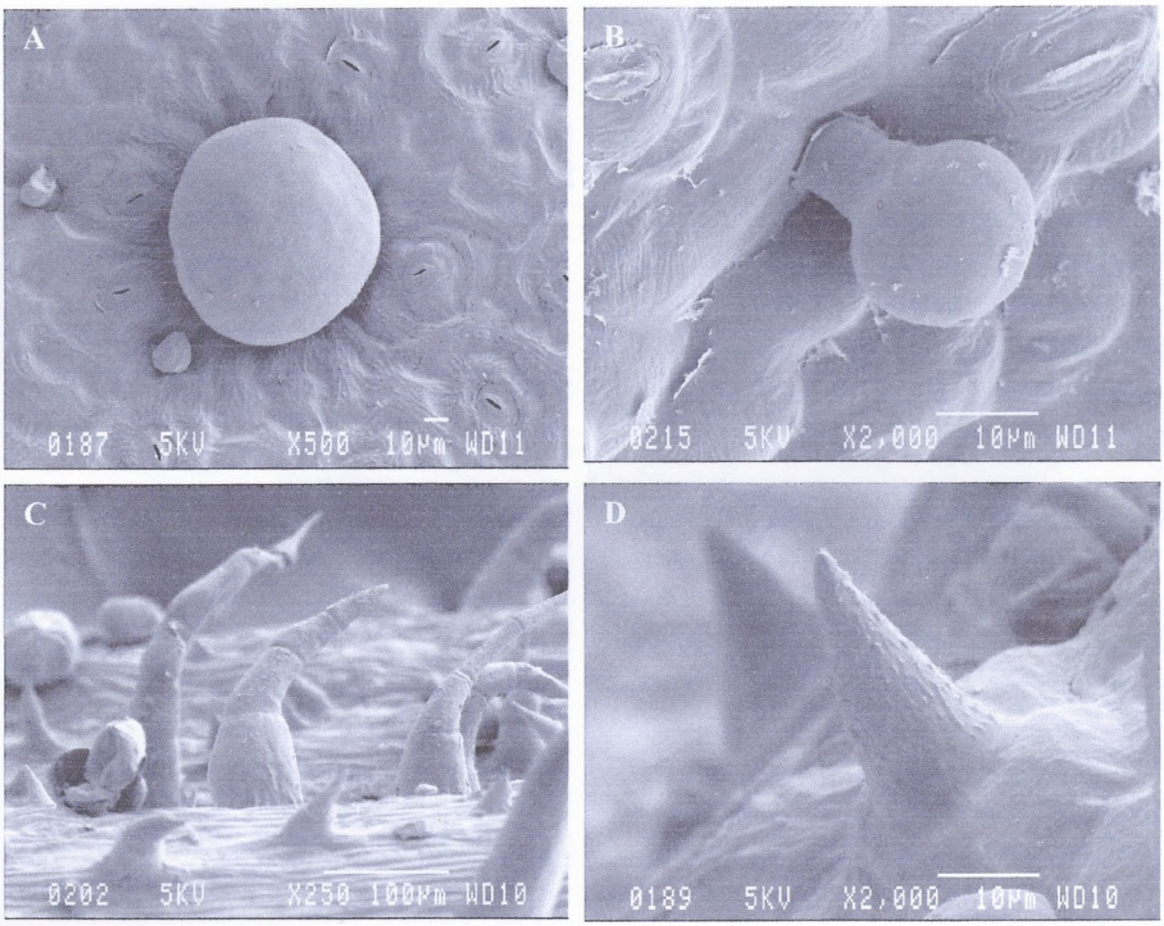


Figure 27. Scanning electron micrographs of the types of trichomes found on the leaf surfaces and stem of *S. stenophylla, S. runcinata* and *S. repens*. **A**. peltate trichome on abaxial surface of *S. runcinata* leaf (x 500); **B**. capitate trichome on the abaxial surface of *S. repens* leaf (x 2000); **C**. mature eglandular trichomes on stem of *S. runcinata* (x 250); **D**. papillae on the adaxial surface of *S. runcinata* leaf (x 2000).

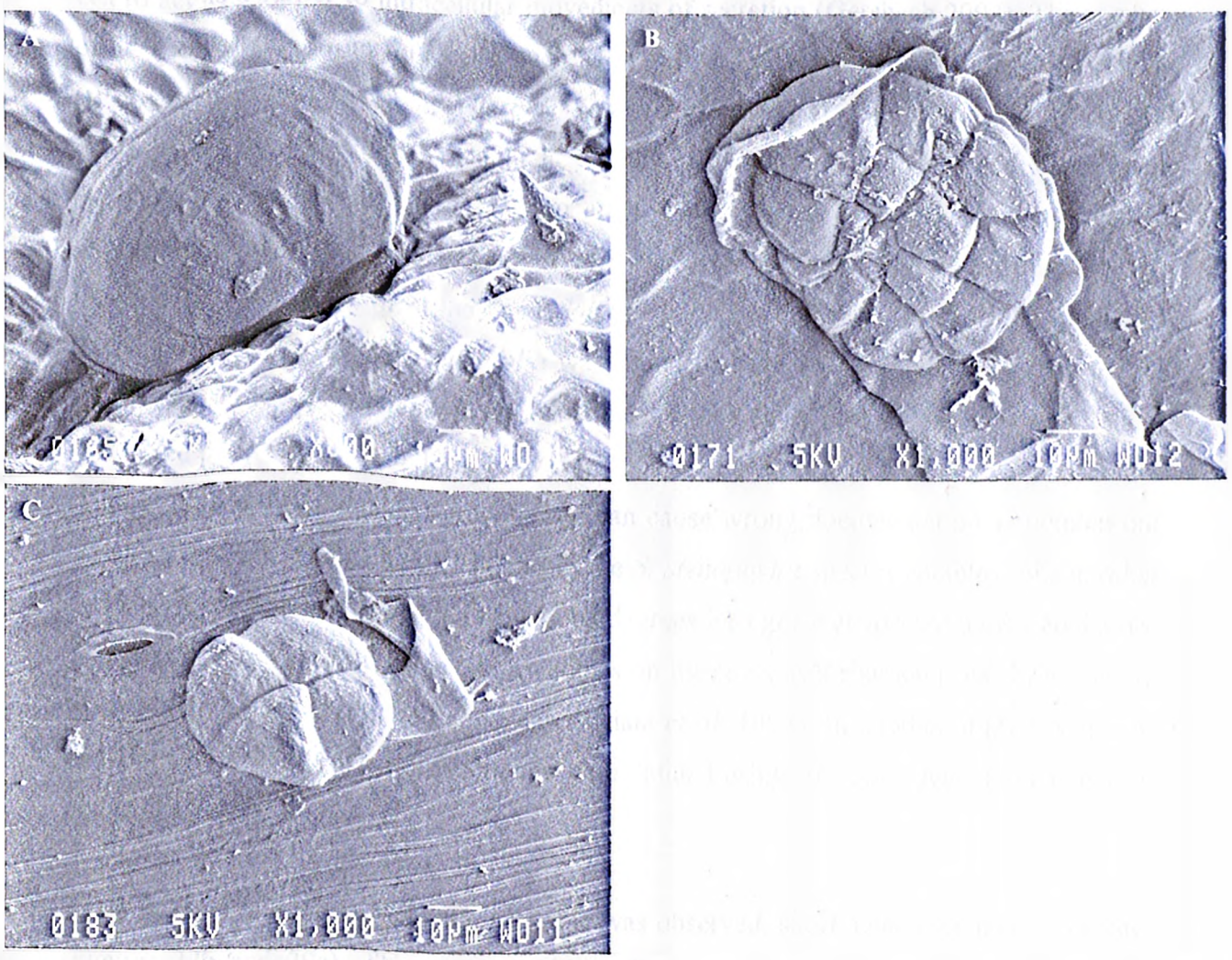


Figure 28. Scanning electron micrographs. **A.** peltate trichome embedded into the epidermal tissue of the adaxial surface of *S. stenophylla* leaf (x 800); **B.** mature peltate trichome with raptured cuticle revealing four central cells and ten peripheral secretory head cells (x 1000); **C.** immature peltate trichome on the stem of *S. runcinata* showing four secretory cells on the stem of *S. stenophylla* (x 1000).

Stalk cells in the Lamiaceae are reported to possess heavily cutinised cell walls, a feature that has a dual function of adding structural support to the trichome and enables the stalk cell to act as a barrier to intracellular movements of secretion (Gersbach 2002). The number of head cells range from four cells, such as in *Salvia blepharophylla* Brandegee ex Epling (Bisio *et al.* 1999) to 18 in *Micromeria fruiticosus* (L.) Druce (Werker *et al.* 1985). *Plectranthus ornatus* Codd has eight head cells (Ascensão *et al.* 1999), and *Calamintha menthifolia* Host has 12 cells (Hanlidou *et al.* 1991), which have also been observed in *Majorana syriaca* (L.) Rafn., *Melissa officinalis* (L.), *Salvia fruticosa* Mill, *Satureja thymbra* L. (Werker *et al.* 1985) and *Salvia officinalis* (Werker *et al.* 1985; Corsi and Bottega 1999). *Prostanthera ovalifolia* was found to have 16 head cells (Gersbach 2002) and in this study, *Salvia stenophylla* exhibited fourteen cells, four in the central circle and ten on the periphery (Figure 28b). However, four head cells can be seen in young trichomes (Figure 28c), a developmental process that can cause wrong documentation as pointed out earlier in the text. The peltate trichomes in the *S. stenophylla* species complex, like in other Lamiaceae species, are embedded to various degrees into grooves formed by the epidermis. The presence and absence of peltate trichomes on the calyx as a character, has been used to classify the *Stachys germanica* complex (Falciani *et al.* 1995). In another study (Demissew and Harley 1992), 13 species of *Stachys* were defined using trichome, seed surface and/ or pollen characters.

Type B. Only one type of capitate trichome was observed, short stalked capitate trichomes (Figures 27b and 29a). This corresponds to the capitate type (I) trichome according to Werker *et al.* (1985), and is the commonest capitate trichomes in the Lamiaceae. They consist of one or two secretory cells supported by one or two stalk cells and a basal cell (Figures 29a and 29b). The size of the whole trichome is much smaller than that of type A. This kind of trichome was observed on all the surfaces examined, and it has also been observed in *Salvia sclarea, S. fruticosa* (Werker *et al.* 1985) and *S. officinalis* (Werker *et al.* 1985; Bisio *et al.* 1998).

Type C. Two types of eglandular trichomes where observed (Figures 27c and 27d):

(1) Single, uniseriate, multicellular and erect simple trichomes. They vary in length consisting of 3 to 5 cells and are supported by a cellular pedestal formed by a group of about 4–6 epidermal cells arranged in a circle around the base (Figures 29c and 29d).

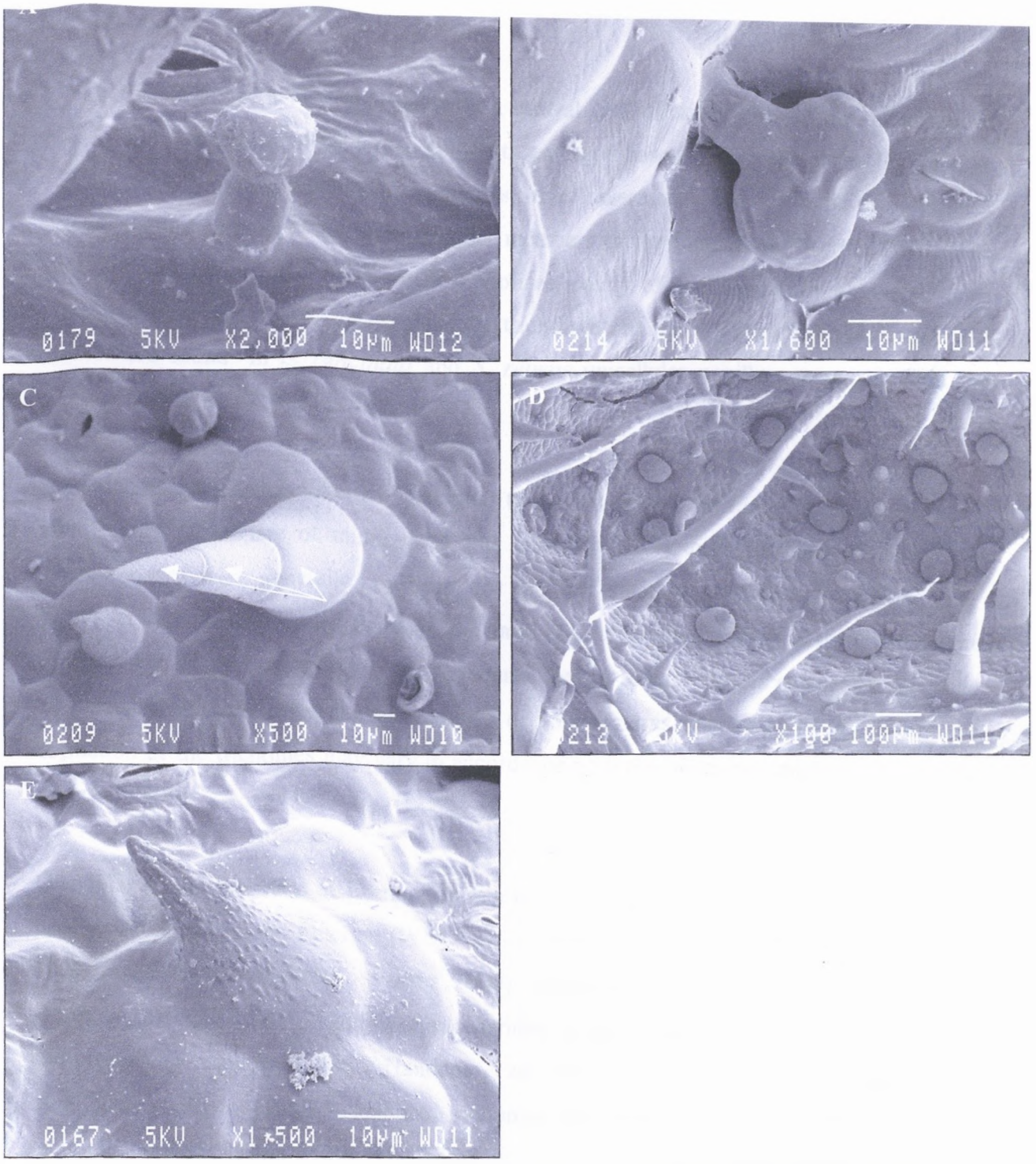


Figure 29. Scanning electron micrographs showing; **A.** type I capitate trichome on abaxial surface of the leaf of *S. stenophylla* (x 1500); **B.** capitate trichome with bicellular secretory head (x 1600); **C.** 3-cell eglandular trichome supported by a pedestal of 4 epidermal cells. The arrows point to the three cells (x 500); **D.** multicellular eglandular trichome composed of 5 cells (x 100); **E.** unicellular papillae surrounded by swollen basal epidermal cells on adaxial surface of *S. stenophylla* (x1500).

(2) Unicellar papillae associated with a central circumscribed area of the outer wall of the swollen basal epidermal cell, with enlarged bases and acute apices (Figures 27d and 29e). In eglandular trichomes, the lowermost stalk cells have very thick cutinised walls as reported in other members of the Lamiaceae (Werker 2000). The indumentum of *S. stenophylla* comprises mainly of papillae except along the veins and margin where longer eglandular trichomes composed of not more than four cells are found. In *S. runcinata* and *S. repens* unicellular papillae occurs only on the adaxial side of the leaves.

The glandular trichomes, like the eglandular trichomes, have been found to be unicellular or multicellular, uniseriate or multiseriate and are variously shaped. In this study, trichome characteristics were generally consistent for leaf and stem surfaces across the three taxa. However, the density varies between the taxa. The diversity of trichomes found on the epidermal surface of plant organs such as stems, leaves, bracts, calyces and seeds have been of considerable value to systematic studies of Angiosperms (Theobald *et al.* 1979). The formation of the trichomes may vary in accordance with the organ on which they are borne (Werker 2000).

Glandular trichomes vary in chemical composition of substances they secrete, mode of production, location and function (Werker *et al.* 1985; Maffei *et al.* 1989; Hanlidou *et al* 1991; Ascensão *et al.* 1995; Werker 2000). Glandular trichomes secrete a variety of secretory material, which include polysaccharides, sugars, salts, lipids, essential oils, resin and proteins (Werker *et al.* 1985; Hanlidou *et al.* 1991; Ascensão *et al.* 1995). Suggestions have been made to classify glandular trichomes according to the composition of their secretion products (Fahn 2000). The difficulty, however, with this type of classification is that some glandular hairs secrete more than one type of substances (Werker 2000). For instance, the glandular trichomes of *Inula viscose* have been found to secrete lipophilic substances, polysaccharides and proteins at different stages during the life span of the trichome and by different organelles (Werker and Fahn 1981). In *Leonotis* (Ascensão *et al.* 1995), both capitate and peltate glands are found on each side of the leaf, predominantly on the abaxial surface concentrating on the intervein areas while the eglandular trichomes are abundant on the veins and leaf margins (Ascensão *et al.* 1995; Corsi and Bottega 1999)

3.3.2 Trichome distribution

There was no distinct pattern in the type and size of the trichomes within and between species. All four types of identified trichomes occur on the plant surfaces examined (Table 5). However, trichome density is variable and *S. stenophylla* has the highest density of peltate trichomes as compared to capitate and eglandular trichomes. This could explain the high percentage of essential oil extracted from this taxon, and because of the size and number, they are expected to contain the bulk of the oil (Maffei *et al.* 1989). An earlier report that *S. stenophylla* produces abundant peltate glandular trichomes compared to capitate and eglandular trichomes (Hoelscher *et al.* 2003) can be verified. The average percentage yield of essential oil from *S. stenophylla* is 0.25%, while in *S. runcinata* it is 0.12% and in *S. repens* 0.09%. Figure 30a shows that *S. runcinata* has the most densely arranged capitate trichomes, while *S. repens* leaf surfaces has more eglandular trichomes compared to glandular trichomes (Figure 30c). In addition, *S. stenophylla* produces some sticky substances when collecting the plant material, which indicates that the plants have the ability to produce taxon-specific phytochemicals in the glandular trichomes (Spring 2000). The taxon-specific phytochemicals are known to be the primary sites of production of bioactive secondary products, which may function as plant growth regulators and defend the plant against insects, pathogens and possibly, other plants (Bisio *et al.* 1998).

There are some difficulties inherent in the study of trichomes. If sampling of data is done so sparsely that variation due to modificatory influences (i.e. phenotypic variation due to environmental conditions and not due to genetic reasons) or developmental processes remains undiscovered, the results could be misleading, although the chemical information itself may be correct (Spring 2000). As the plants mature, the density of trichomes and the relative frequency of different types of trichomes may vary. For instance, the mature stem of *S. stenophylla* can be described as glabrous whereas a young stem will be hairy. Different kinds of trichomes though distinguishable, sometimes intergrade into one another on the structure, for example trichome developmental stages may be mistaken for different types. This also applies to the estimation of the density of the trichomes on a particular surface. In a study by Maffei *et al.* (1989), it was found that during the development of *Mentha* x *piperita* (peppermint) leaves, there was an increase in the total number of peltate trichomes. The number of peltate trichomes on the youngest leaves was greater on the adaxial than on the abaxial surface.

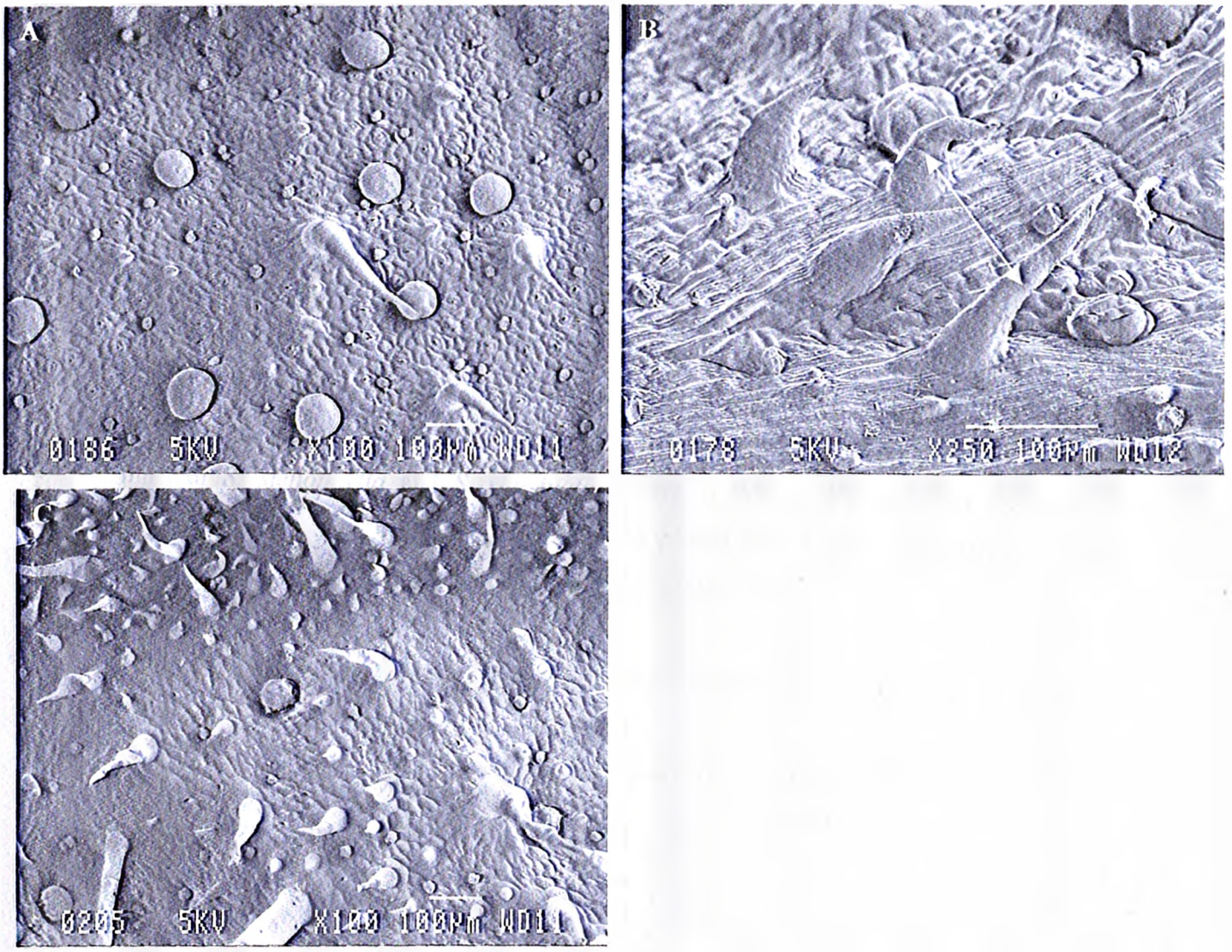


Figure 30. Scanning electron micrographs showing; **A.** capitate trichomes occurring in large numbers on adaxial surface of *S. runcinata* leaf lamina compared to peltate and eglandular trichomes (x 100); **B.** eglandular trichomes arranged along the vein of *S. stenophylla* abaxial side of leaf. Arrows pointing to the trichomes (x 250); **C.** eglandular trichomes occurring in abundance on the adaxial surface *S. repens* leaf (x 100).

surfaces. The trichomes also showed both qualitative and quantitative differences in the composition of the essential oil during the developmental stage.

In spite if all these difficulties, this study agrees with Codd's (1985) key of delimiting *S. stenophylla* and *S. runcinata* on the basis of trichome density. Codd (1985) reported that the stem of *S. runcinata* is covered with a distinct indumentum of short to long eglandular trichomes, while that of *S. stenophylla* is almost glabrous with few scattered eglandular trichomes. Eglandular trichomes are more numerous along the veins on the abaxial side of the lamina in *S. stenophylla* (Figure 30b). However, *S. repens* does not show a very different indumentum cover from that of *S. runcinata*. It was separated from *S. stenophylla* and *S. runcinata* on the basis of bearing long fruiting calyces (7–13 mm) and corollas (10–26 mm), and a rhizomatous root system. The fruiting calyces (4–7 mm) and corollas (7–14 mm) of *S. stenophylla* and *S. runcinata* are much shorter, and the root system is not rhizomatous. This data suggests that *S. stenophylla* and *S. runcinata* are very closely related, but consistently differ only in density of the trichomes.

Table 5. Distribution of trichome types on different surfaces of taxa in the *Salvia stenophylla* species complex.

Taxon	Type of trichome	Adaxial surface of leaf	Abaxial surface of leaf	Stem
Salvia stenophylla	peltate	++	++	+
	capitate	+	+	+
	papillae	+	+	+
	multicellular eglandular	+	+	-
Salvia runcinata	peltate	+	+	+
	capitate	++	++	+
	papillae	+	-	-
	multicellular eglandular	+	+	++
Salvia repens	peltate	+	+	+
	capitate	+	+	+
	papillae	+	-	-
	multicellular eglandular	++	++	++

+ denotes lightly pubescent ++ denotes densely pubescent

CHEMICAL COMPOSITION

3.4.1 Percentage yield of essential oils and non-volatile compounds

There was significant variation in the percentage yield in both the essential oil and methanol extract between different populations of the same taxa and between different taxa (Table 6). *S. stenophylla* from Dordrecht yielded the highest percentage (0.41%) of essential oil followed by Steynsrus and Smithfield samples which yielded 0.34%, while the sample from Sannieshof had the lowest yield (0.14%). The highest essential oil yield at 0.15% in *S. repens* was from the sample collected from George and that of *S. runcinata* collected from Lichtenburg was 0.24%, followed by the sample from Bergville (0.19%). The highest methanol extract with yield of 18.42% was recorded for *S. stenophylla* from Steynsrus.

S. stenophylla yields the highest average percentage of essential oil (0.25%) and non-volatile compounds at 12.8%, as compared to *S. runcinata* yielding 0.19% and 11.68% and *S. repens* yielding 0.07% and 11.6% respectively (Table 7). *S. repens* generally yielded low levels of oil compared to *S. stenophylla* and *S. runcinata* (Figure 31). Quantification of the yield is perhaps underscored due to volatization of some terpenoids in the case of volatile compounds and inadequate extraction in the case of non-volatile compounds. Notably, the essential oils had different colours according to the three *a priori* groups under study. Essential oil extracted from *S. stenophylla* samples had clear yellow oil; *S. runcinata* samples yielded cloudy light yellow oil and *S. repens* produced almost colourless oil.

From the results of the percentage yields, *S. stenophylla* is the most aromatic of the three taxa since it yields the most oil and it has the highest density of peltate glandular trichomes on its surfaces. Its non-volatile compounds are more readily extracted with methanol because of the highest percentage yields observed in the extracts (Table 6). There is a correlation between the percentage yields of essential oil and non-volatile compounds of *S. stenophylla*. This is in agreement with Skoula *et al.* (2000) who stated that the levels of non-volatile compounds and essential oils are positively correlated, for example, *Salvia fruticosa* clones with high percentages of non-volatile compounds were also the ones with the highest levels of essential oils.

Table 6. Localities and percentage yields of essential oil based on wet weight of the raw material and the methanol extracts based on dry weight.

Taxon	Locality	Essential oil yield* (%)	Methanol extract yield++(%)
S. stenophylla	Bethulie	0.20	12.48
S. stenophylla	Biesiesvlei	0.19	12.43
S. stenophylla	Dordrecht	0.41	nd
S. stenophylla	Molteno	0.26	12.04
S. stenophylla	Murraysburg	0.20	nd
S. stenophylla	Sannieshof	0.14	8.98
S. stenophylla	Smithfield	0.34	nd
S. stenophylla	Springfontein	0.19	12.55
S. stenophylla	Steynsrus	0.34	18.32
S. repens	Dordrecht	nd	8.44
S. repens	George	0.15	15.92
S. repens	Indwe	0.05	13.91
S. repens	Knapdaar	0.07	11.69
S. repens	Lady Grey	0.12	8.05
S. repens	Queenstown	0.04	nd
S. runcinata	Bergville	0.19	9.04
S. runcinata	Coligny	0.06	12.6
S. runcinata	Delareyville	0.10	10.68
S. runcinata	Klerkskraal Dam	0.05	9.94
S. runcinata	Koster	0.17	13.9
S. runcinata	Kroonstad	0.14	9.76
S. runcinata	Kuruman	0.08	16.84
S. runcinata	Lichtenburg	0.24	14.4
S. runcinata	Melville Koppies	0.10	nd
S. runcinata	Ventersdorp	0.11	nd
S. runcinata	Vryburg	0.05	12.5

* Based on wet weight (g). ++Calculated from 0.5g dried plant material.

nd=not determined

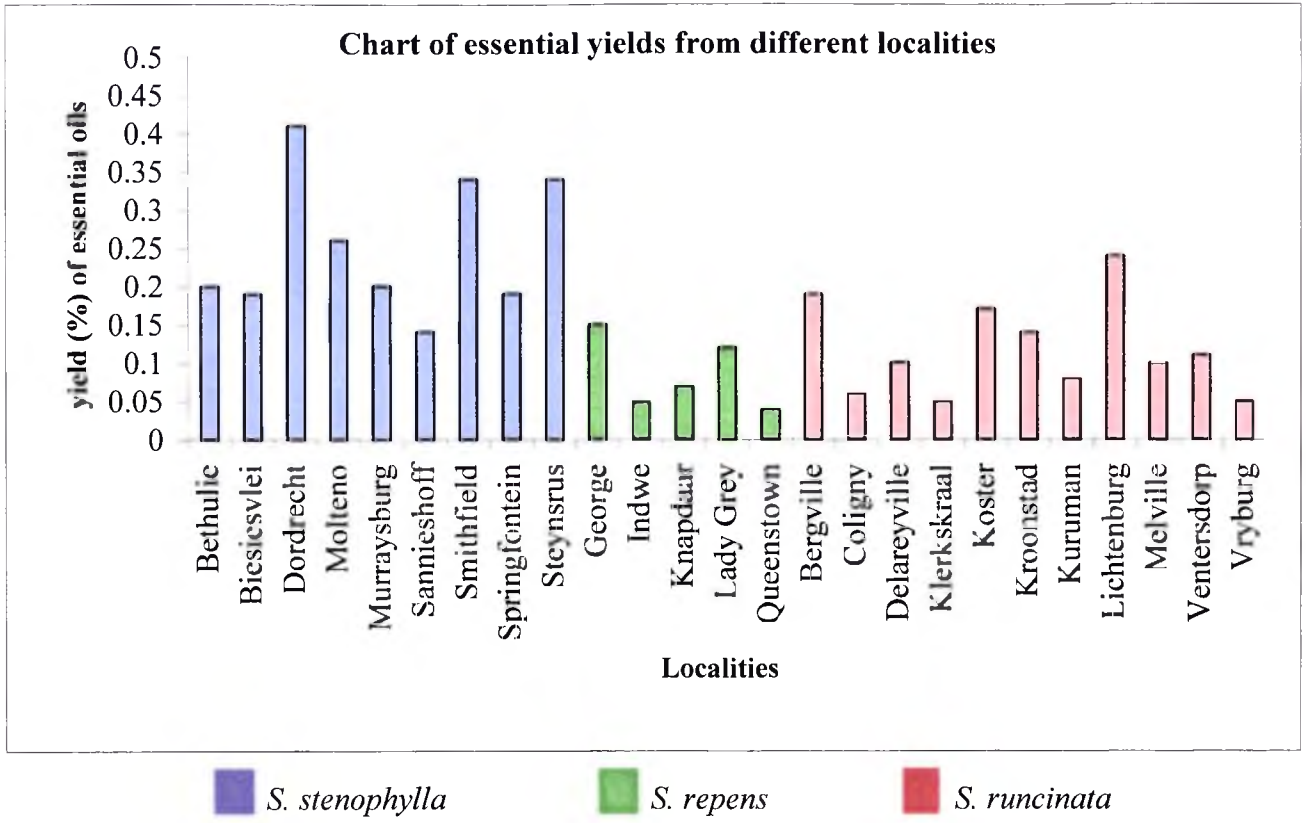


Figure 31. Graph comparing the yields (%) of the essential oils of *S. stenophylla, S. repens* and *S. runcinata*.

Table 7. The means (%) and standard deviations of the essential oil and methanol extract yields of *S. stenophylla, S. runcinata* and *S. repens*.

Taxon	Mean yield of essential oils (%)	Standard deviation	n	Essential oil colour	Mean yield of methanol extracts (%)	Standard deviation	n
S. stenophylla	0.252	0.09	9	yellow	12.8	3.03	6
S. repens	0.086	0.05	5	Colourless to cloudy	11.6	3.41	5
S. runcinata	0.117	0.06	11	Cloudy to light yellow	12.18	2.57	9

3.4.2 Thin layer chromatography

3.4.2.1 Thin layer chromatography (TLC) of essential oils

The three individual plants from Klerkskraal Dam displayed very similar chromatographic profile (Figure 32). This indicated that there is little variation in the essential oil composition of plants within the populations. These results were also confirmed by GC analysis (Figure 33). This led to the harvesting protocol to which, we decided on harvesting plant material from many plants collectively in a population and not individual plants. Two TLC plates were developed and sprayed with vanillin and the other with anisaldehyde to investigate the preferred reagent (Figures 32a and 32b). Both sprays developed the TLC plates well. Vanillin spray developed the prominent compound spots with a khaky to dark green colour while anisaldehyde developed them to bright purple on spraying.

Although the essential oil variation within a population is negligible, there is immense chemical variation between the different populations. Essential oils from Melville Koppies and Bergville (ca. 300 km apart) portrayed similar chemical constitution while Coligny and all Klerkskraal Dam oils (ca. 100 km apart) showed very similar chemical constitution (Figure 32a and 32b). The TLC plate (Figure 34) of all the essential oil samples exhibits immense chemical variation within plants of the same taxon and between those of different taxa. The R_f value of the bisabolol standard was 0.6. The major compounds in the essential oils extracted from Coligny, Klerkskraal Dam, Ventersdorp, Kroonstad, Roossenekal, Kuruman for *S. runcinata*, essential oil from Bethulie, Dordrecht, Smithfield, Steynsrus and

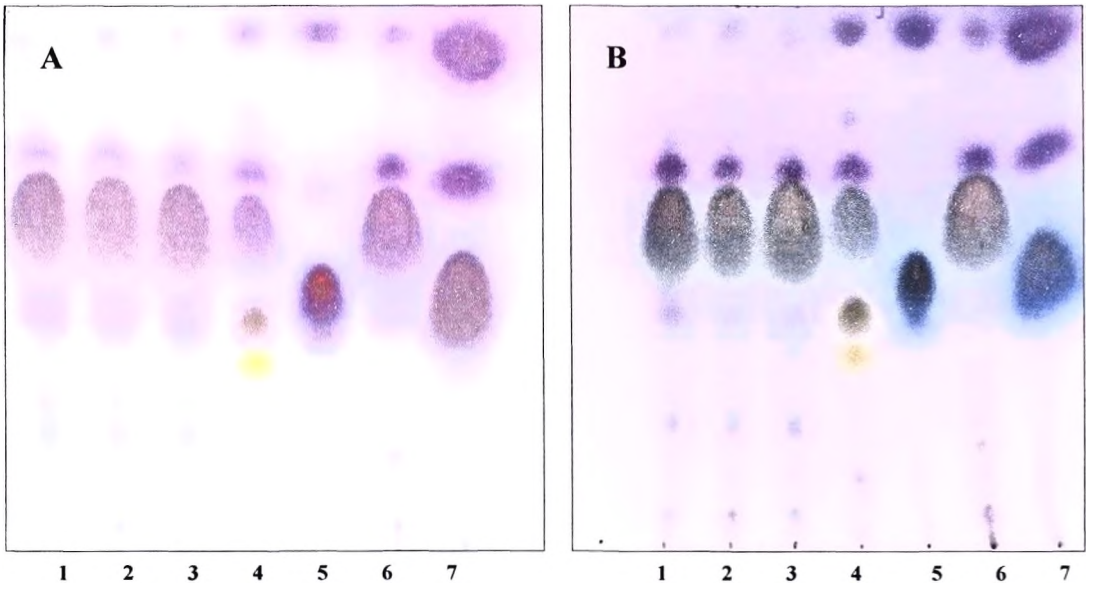


Figure 32. TLC plates of *S. rucinata* collected from same locality (tracks 1, 2 and 3 are essential oils of individual plants a, b and c, while track 4 is for bulk plant material collected from Klerkskraal Dam). Tracks 5, 6 and 7 are essential oil samples from Melville Koppies, Coligny and Bergville respectively. Plate A was sprayed with anisaldehyde and plate B with vannilinic acid.

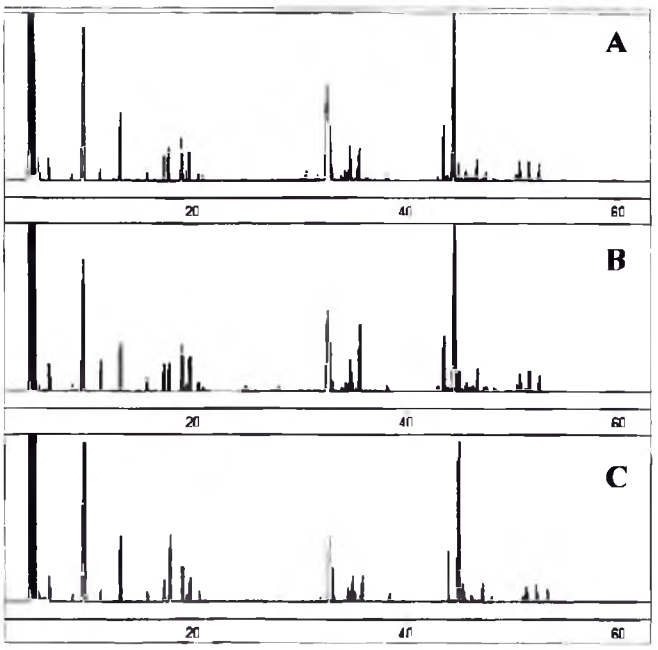


Figure 33. Identical GC profiles for different individual *S. runcinata* (A, B, C) collected from Klerkskraal Dam.

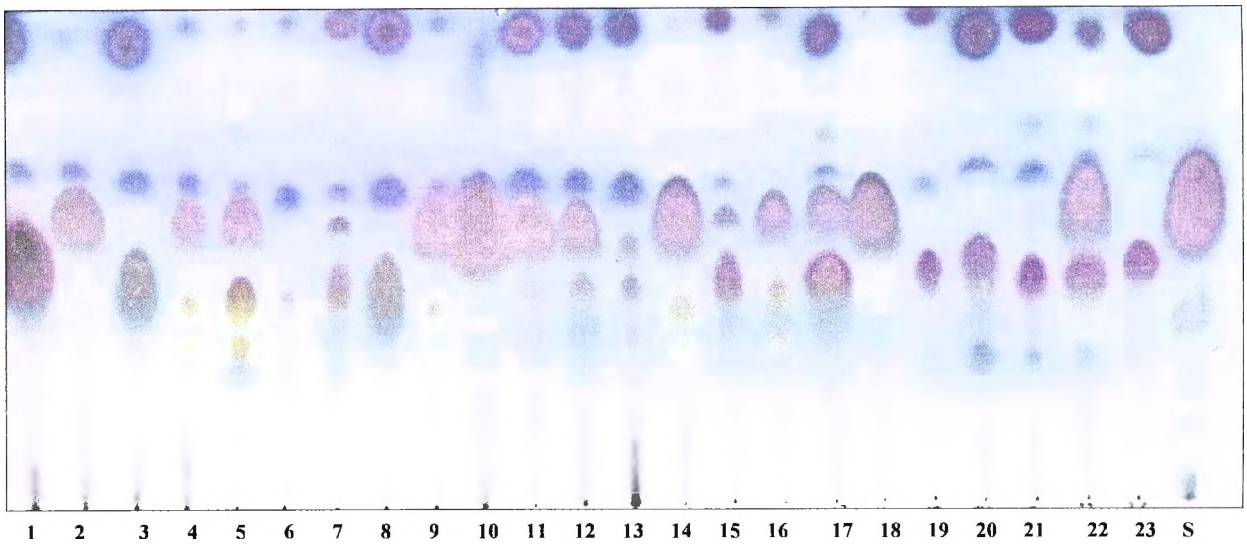


Figure 34. TLC plate of essential oils of the *Salvia stenophylla* complex showing variation within and between the species. S represents bisabolol standard.

Key to the samples;

S. runcinata	***S. stenophylla***	***S. repens***
1. Melville Koppies	14. Smithfield	19. Queenstown
2. Coligny	15. Biesiesvlei	20. Lady Grey
3. Bergville	16. Steynsrus	21. Indwe
4. Klerkskraal Dam	17. Sannieshof	22. Knapdaar
5. Dordrecht	18. Bethulie	23. George
6.Tweeling		
7. Lichtenburg		
8. Koster		
9. Ventersdorp		
10. Kroonstad		
11. Roossenekal		
12. Kuruman		
13. Vryburg		

Sannieshof for *S. stenophylla* and for *S. repens* from Knapdaar had their major compounds with R_f value of 0.6. This implies that the samples from the mentioned localities could contain bisabolol and its derivatives or closely related compounds. Because of the chemical variation portrayed, we were prompted to investigate the variation further using gas chromatography and gas chromatography coupled to mass spectroscopy. However, from the TLC results, two chemotypes can be tentatively identified for *S. stenophylla* and two chemotypes could also be identified in *S. repens*.

Gas chromatography results (Figure 33) also support the TLC results in that the three individual plants (A, B and C) collected from Klerkskraal Dam show a similar GC profile indicating the occurrence of identical chemical composition between plants from the same locality. The three *S. stenophylla* microdistilled plants from Biesiesvlei also display similar GC profiles of the essential oils. However, on identifying the actual compounds by GC/MS, there were slight variations especially in the compounds occurring at high percentages (Figures 36a, 36c and 36e). The essential oil samples from plants B and C have limonene as the major compound while plant D has δ–3-carene.

3.4.2.2 Thin layer chromatography (TLC) of non-volatile compounds

The mobile phase that was selected to develop the plate consisted of toluene/dioxane/acetic acid (90:25:1). The chemical composition of the methanol extracts varied slightly from one population to another (Figure 35), even though there are a number of compounds that seem to be common in all the taxa. The TLC profiles of *S. stenophylla* (samples 6–12) and *S. runcinata* (samples 13–19) are similar, except for the latter sample collected from Lichtenburg. *S. repens* profile is quite different (Samples 1–5) from *S. stenophylla* and *S. runcinata* profiles. The TLC plate was sprayed with DPPH for better visual assessment.

3.4.4 Gas chromatography (GC) and GC coupled to mass spectroscopy

3.4.4.1 *Salvia stenophylla*

A total of 130 compounds were identified from twelve *S. stenophylla* essential oil samples (Table 8). The dominant compounds (Figure 36) of *Salvia stenophylla* include α-bisabolol ranging from 1.8% (Biesiesvlei) to 46.5% (Dordrecht). Comparing the *S. stenophylla* results with those of the literature, some essential oil samples contained lower levels of α-bisabolol, which falls below the ranges as reported (29.8%–41%) by Brunke and Hammerschmidt (1985) and Jequier *et al.* (1980). However, the levels of α-bisabolol go

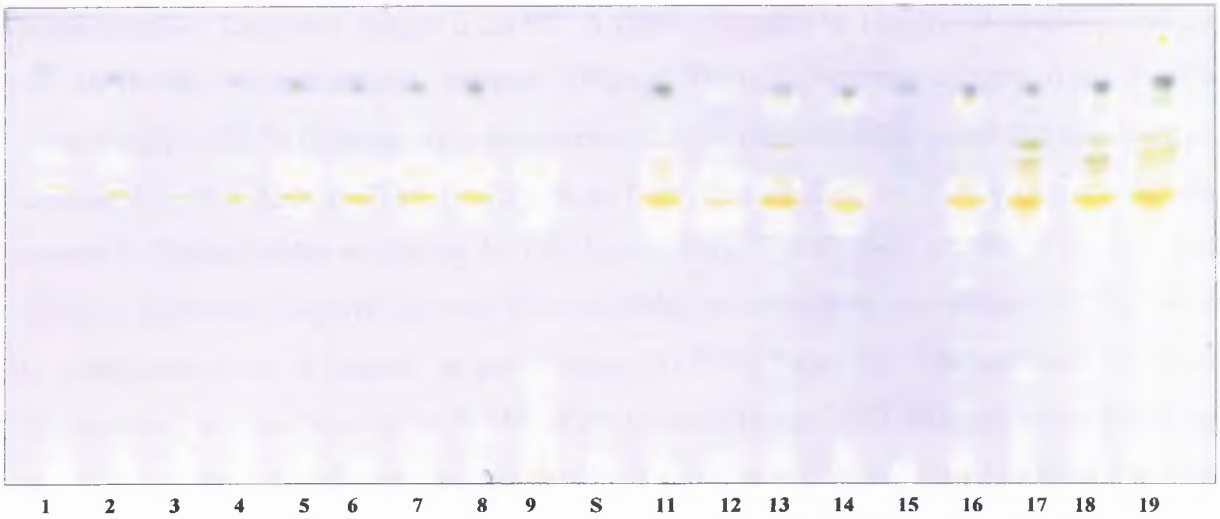


Figure 35. TLC plate of the methanol extracts of the *S. stenophylla* complex exhibiting variation between species. S represents rosmarinic acid and the TLC plate was sprayed with DPPH.

Key to the samples;

S. repens	***S. stenophylla***	***S. runcinata***
1. Lady Grey	6. Springfontein	13. Coligny
2. Knapdaar	7. Molteno	14. Klerkskraal Dam
3. George	8. Roossenekal	15. Lichtenburg
4. Dordrecht	9. Bethulie	16. Kroonstad
5. Indwe	11. Sannieshof	17. Kuruman
	12. Biesiesvlei	18. Delareyville
		19. Vryburg

beyond the highest limit of 41% and it is the highest sesquiterpene in concentration from some samples. Limonene ranges from 0.3 % (Springfontein) to 19.3 % (Biesiesvlei) and up to 38.1% for microdistilled extracts (Figure 36b). δ-3-carene ranges from 0.05% (Smithfield) to 25 % (Steynsrus), γ-terpinene at 20.3% (Smithfield)(Figures 36f and 36g), *p*-cymene (18.4%), (*E*)-nerolidol ranging from 0.3% (Sannieshof) to 53.6% (Bethulie), also occurs in Springfontein sample at 46.7% (Figure 36i). The GC/MS profile of the essential oil from Smithfield displays the peak of α-bisabolol as the highest percentage (19.7%) but it is γ-terpinene that is highest in percentage (20.28%)(Table 8). The essential oil from Murraysburg accumulates up to 20.9% of *cis*-lanceol (Figure 36k). Manool accumulates up to 12.7% in the Springfontein sample, which is significantly higher than has been reported (Jequier *et al.* 1980). It was found to occur only in *S. stenophylla* extracts except for the Dordercht sample, and two *S. runcinata* essential oils (Lichtenburg and Kuruman), which possess the compound. Compounds that were common in all the essential oil samples of *S. stenophylla* include α-pinene, β-pinene, camphene, α-terpinene, limonene, γ-terpinene, *p*-cymene, terpinolene, camphor, linalool, α-terpineol, borneol, (*E*)-nerolidol, α-bisabolol, *cis*-lanceol and manool. It was also noticed that microdistilled samples yielded fewer compounds as compared to hydodistilled samples. For instance, the essential oil from hydrodistilled bulk plant material from Biesiesvlei yielded 73 compounds while the microdistilled individual plants yielded an average of 33 compounds. This implies that different extraction methods yield slightly different chemical composition.

However, like many other studies of volatile compounds of *Salvia* species (Peana *et al.* 1999; Rustaiyan *et al.* 1999), the chemical composition in the *S. stenophylla* complex varies with regard to the predominance of monoterpenes like α– and β–pinene, *p*-cymene, γ-terpinene, δ-3-carene and limonene. Phytol was not detected in *S. stenophylla* and β-caryophyllene was present in four localities (Dordrecht, Sannieshof, Biesiesvlei and Steynsrus), while thymol and isothymol were detected only in Springfontein and Smithfield essential oils. From these results two chemotypes are identified, chemotype I; α-bisabolol, δ-3-carene and limonene-rich chemotype; chemotype II: (*E*)-nerolidol, γ-terpinene, δ-3-carene and *p*-cymen-rich chemotype. This supports the tentative identification of two chemotypes based on TLC results (Figure 34).

Table 8. Essential oil composition of *S. stenophylla* and the relative retention time (RRI).

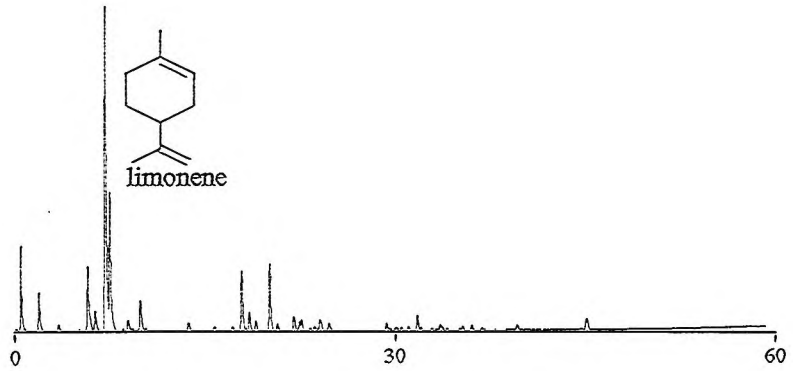
RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L
1000	decane						0.12						
1014	tricyclene	0.13		0.43	0.20	trace		0.24	0.05	0.14	trace		trace
1032	α-pinene	5.79	3.36	5.91	4.54	0.80	3.86	5.04	1.03	2.70	0.60	0.70	2.40
1035	α-thujene						trace		0.86	trace		0.40	
1072	α-fenchene				0.03			0.05		0.02			
1076	camphene	3.10	2.76	6.61	3.73	1.00	0.10	4.27	0.99	3.32	0.60	0.30	0.90
1100	undecane						0.05						
1118	β-pinene	0.44		0.43	0.62	0.30	1.75	0.88	0.47	0.74	0.20	0.40	1.40
1132	sabinene				0.04		0.07	0.06	0.18	0.07	0.10	0.10	0.10
1146	δ-2-carene							0.03		0.01			
1156	δ-3-carene	0.14	10.87	**32.66**	10.16	1.60	11.75	**24.97**	0.05	**18.44**	**19.30**		**18.80**
1174	myrcene			1.99		12.30		2.08	1.19	1.71	7.60	4.40	1.40
1176	α-phellandrene	6.05	2.17	0.10	2.18		0.02			trace			
1182	n-butylbenzene				0.02								
1187	*o* -cymene						0.04			0.07			
1188	α-terpinene	1.83	0.21	0.23	0.49	0.10	0.11	0.34	2.08	0.29	0.30	0.90	0.20
1203	limonene	**38.12**	**30.95**	9.30	**19.34**	0.60	2.00	6.08	0.64	5.29	4.50	0.30	2.80
1205	sylvestrene		0.48	2.56		0.10	0.96	1.93		1.28	2.00		1.40
1213	1,8-cineole					2.00	trace		3.36			2.40	10.90
1218	β-phellandrene	14.46	7.49	3.10	7.84		1.77	3.49		2.91			
1227	7-*epi* -1,2-dehydro-sesquicineole										0.20		
1246	(Z)–β-ocimene	0.08			0.05		0.06	0.02	0.01	0.02	0.30		0.30
1255	γ-terpinene	1.11	trace	0.30	0.39	0.10	0.27	0.51	**20.28**	0.49	0.50	**11.30**	0.60
1265	5-methyl-1,3-heptanone	0.05			trace	trace	0.04						0.20
1266	*(E)*-β-ocimene	trace			trace			0.07			0.30		0.20
1278	*m* -cymen							0.35		0.20			
1280	*p*- cymen	3.19	8.36	5.73	2.42	trace	1.64	1.40	**18.40**	trace	0.50	3.60	0.10
1286	isoterpinolene							0.73		0.76	0.90		0.70
1290	terpinolene	0.08			0.05	0.10	0.01	0.45	0.34	0.54	0.50	0.20	0.50
1327	*(Z)*-3-hexenylacetate												0.30
1348	6-methyl-5-hepten-2-one							0.17	0.23		0.20		
1360	hexanol				trace								
1393	3-octanol	0.04			0.04		0.18	0.03			0.10		
1400	nonanal					trace		0.05	0.02	0.01	trace		
1443	2,5-dimethylstyrene				0.10		0.01	0.21		0.11			
1450	*trans* -linalool-oxide					0.20							
1452	α-*p* -dimethystyrene				0.06		0.04	0.04			trace		
1452	1-octen-3-ol	0.28		0.48	0.10		0.08	0.05	0.12	0.21	0.30	0.20	
1467	6-methyl-1,5-hepten-2-ol						0.01	0.03	0.03		0.10		
1474	*trans* -sabinenehydrate				0.01	trace		0.12	0.29	0.08	0.10	0.30	
1493	α-ylangene				0.02								
1497	α-copaene	0.34		0.37	0.35			0.05					
1532	camphor	6.10	4.94	7.01	4.66	3.60	0.06	4.67	1.75	6.00	0.50	1.50	1.50
1544	α-gujunene	0.19			0.19								
1545	*cis*- α-bergamotene												0.10
1553	linalool	1.55	0.26	2.28	0.51	2.00		1.37	0.40	0.12	0.80	0.10	0.20
1556	*cis* -sabinenehydrate								0.30			0.20	0.10
1568	1-methyl-1,4-acetyl-cyclo-hex-1-ene						0.23	0.02					trace
1568	*trans* -α-bergamotene						0.34	0.30	0.26		0.20		
1568	1-methy-1,4-acetyl-cyclo-hex-1-ol								0.01				
1571	*trans-p* -menth-2-en-1-ol	0.77	0.56		0.55	trace		0.21	0.09	0.18		0.10	0.10
1586	pinocarvone				0.04			0.01					
1589	iso-caryophyllene				0.02								
1594	*trans* -β-bergamotene					trace					0.60		0.30
1610	calarene				0.07								
1611	terpene-4-ol		2.44	2.44		0.30			1.41		0.30	1.30	0.50

RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L
1612	β-caryophyllene	6.44	trace	trace	5.45		1.33	1.51		7.26			
1628	aromadendrene		1.03		1.43		0.02	0.15		1.18		0.10	
1638	*cis* -p-menth-2-en-1-ol	0.55	0.44		0.59			0.15	0.07		0.10	trace	0.10
1639	*trans-p* -mentha-2,8-dien-1-ol						0.05						
1661	alloaromadendrene				0.22			0.05		0.09			
1664	*trans* pinocarveol						0.27						
1664	nonanol					0.10					0.10	trace	
1668	*(Z)*–β-farnescene					trace	0.15	0.08	0.10	0.02	0.70	trace	0.80
1678	*cis -p* -mentha-2,8-dien-1-ol				0.09								
1682	δ-terpineol					0.10			0.07			0.10	0.30
1687	α-humulene	0.66		0.20	1.43		0.12	0.31		1.71		0.10	
1689	*trans* -piperitol										0.10		
1695	*(E)*-β-damascene				0.03								trace
1700	limonen-4-ol								0.01		0.10		
1706	α-terpineol	0.70	0.21	0.83	0.55	0.20		0.75	0.28	0.98	0.30	1.00	1.00
1709	α-terpinylacetate						0.04						
1719	borneol	0.93	1.74	5.56	1.14	0.70	0.31	3.48	0.62	4.81	1.90	0.30	1.30
1738	*p* -mentha-1,5-dien-8-ol										0.10		trace
1740	α-muurolene	0.11			0.21								
1741	β-bisabolene					0.10	0.60	0.41	0.20	0.21	1.20	0.10	11.30
1743	α-cadinene			0.07									
1744	α-selinene				trace								
1755	β-curcumene												0.30
1758	*cis* -piperitol	0.31			0.21						0.10		
1773	δ-cadinene	0.83		0.60	0.54					0.51			
1776	γ-cadinene	0.10		0.40	0.40								
1784	*(E)*-α-bisabolene										0.50		1.00
1796	selina-3(7)11-diene				0.82			trace					
1804	myrtenol	0.77		0.26	trace	0.40		0.38	0.26	1.02			
1805	α-campholene-alcohol						1.96	trace					
1808	Nerol												0.20
1823	*p* -mentha-1(7)5-diene-2-ol				0.15			0.07					
1845	*trans* -carveol	0.03			0.15		0.22	0.02		0.03			
1853	*cis* -calamenene	0.06											
1864	*p* -cymen-8-ol		0.18	1.00	trace		0.09	0.34	0.05	0.24			
1868	(E)-geranylacetone				0.04					0.01			
1882	*cis* -carveol				0.08		0.03						
1889	myrcen-8-ol					0.10							
1896	*cis -p* -mentha-1(7)8-dien-2-ol				0.14								
1921	α-phellandrene-epoxide				0.02								
1932	iso-amylbenzoate				0.03								
1941	α-calacorene-I				0.10					0.01			
1969	*cis* -jasmone					0.10							
2008	caryophyllene-oxide	0.63	0.80	0.70	2.46		1.58	0.36		1.17			
2045	humulene-epoxide I									0.04			
2050	*(E)*-nerolidol	0.08	0.18	0.40	0.53	**53.60**	0.09	0.14	15.11	0.26	0.30	**46.70**	0.20
2071	humulene-epoxide-II	0.12	0.19	0.16	0.81		0.32	0.11		0.37			
2080	cubenol												0.10
2098	globulol		0.36					0.03					
2103	guaiol									3.28		3.50	
2104	viridifloral		9.11		5.74		0.47	0.87		0.30			
2144	spathulenol		0.40		0.50		0.27	0.07		0.28			
2156	α-bisabolol oxide B						0.06	0.13					
2162	bisabolol oxide						5.30	0.79	0.57	0.07			
2170	β-bisabolol												0.10
2181	isothymol								1.07			0.40	
2185	γ-eudesmol											0.20	

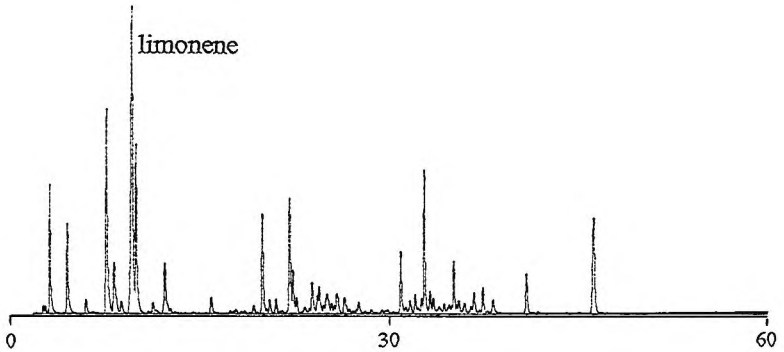
RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L
2187	τ-cadinol	0.05		0.20	0.27			0.11		0.32		0.30	0.50
2198	thymol								0.29			0.20	
2205	Eremoginol											0.10	
2209	τ-muurolol	0.04			0.10								
2221	isocarvacrol								0.55				
2228	pogostol		3.87										
2232	α-bisabolol	0.20		0.43	1.77	4.70	**46.53**	**19.71**	**17.37**	8.17	**37.40**	1.30	**10.40**
2239	carvacrol								0.64			0.20	
2250	α-eudesmol				0.21					0.82		0.60	
2255	α-cadinol	0.04											
2257	β-eudesmol				0.40					0.85		0.40	
2324	caryophylladienol-II	0.12	0.21	0.20	0.77					0.65			
2389	caryophyllenol-I			0.62	0.91								
2392	caryophyllenol-II				0.48		0.36			0.74			0.90
2518	*cis*-lanceol	0.48		0.73	1.85	0.80		0.87	1.30	3.64	3.30	1.00	**20.90**
2622	phytol						0.17						
2676	manool	0.51	3.91	0.73	6.26	12.10		4.48	5.59	10.07	6.00	12.70	3.20
	bp109222	1.21											
	bp109222	0.23											
Total		**99.0**	**97.5**	**95.0**	**95.7**	**98.1**	**94.4**	**95.7**	**99.0**	**94.8**	**93.8**	**98.0**	**98.6**

KEY TO LOCALITIES

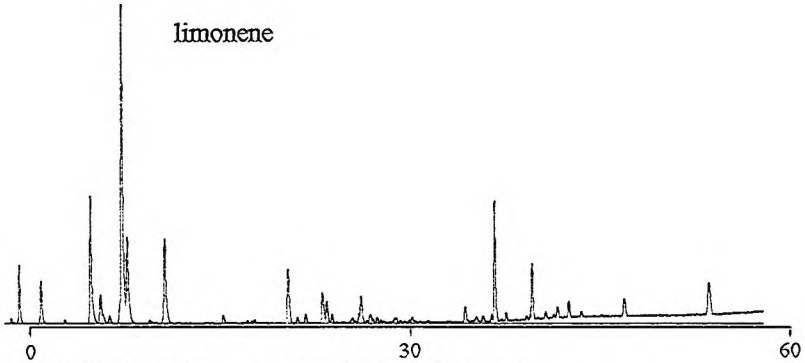
A= Biesiesvlei (micodistilled individual plant)
B= Biesiesvlei (individual plant)
C= Biesiesvlei (microdistilled individual plant)
D= Biesiesvlei (hydrodistilled pooled sample)
E= Bethulie
F= Dordrecht
G= Steynsrus
H= Smithfield
I= Sannieshof
J= Molteno
K= Springfontein
L= Murraysburg



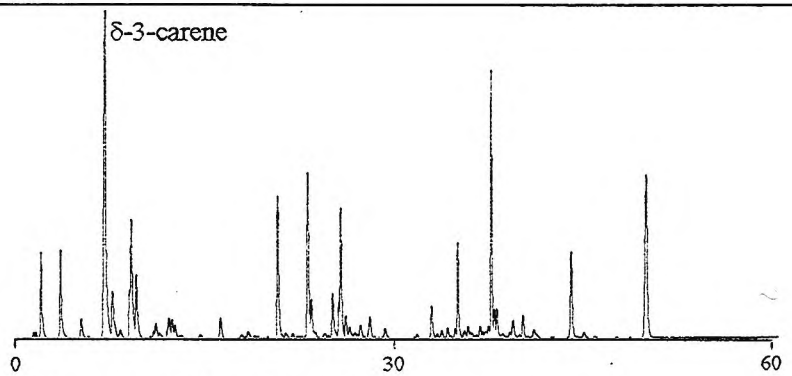
A. Individual plant material (A) from Biesiesvlei



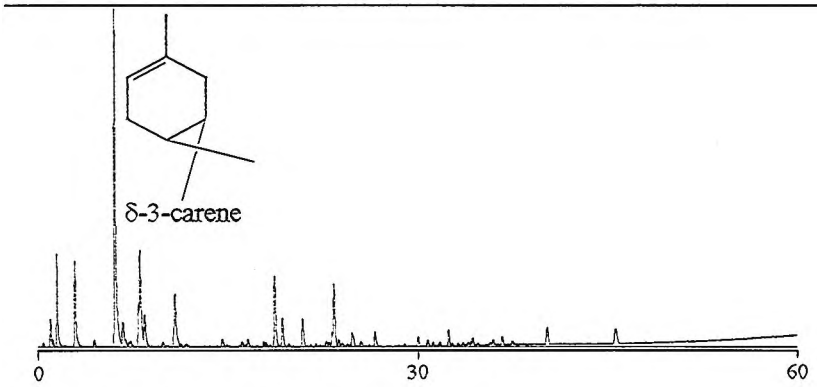
B. Pooled plant material from Biesiesvlei



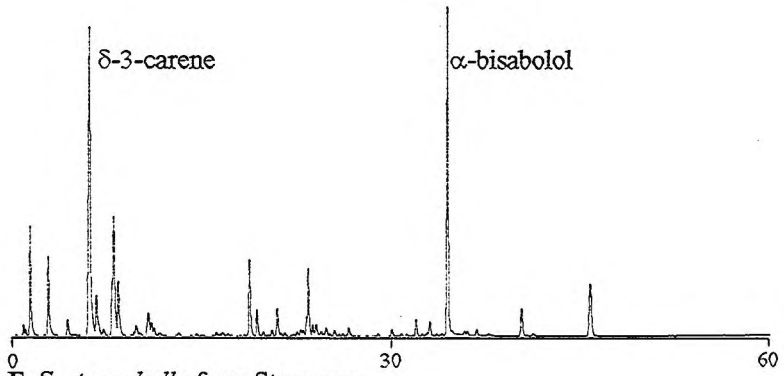
C. Individual plant material (B) from Biesiesvlei



D. *S. stenophylla* from Sannieshof

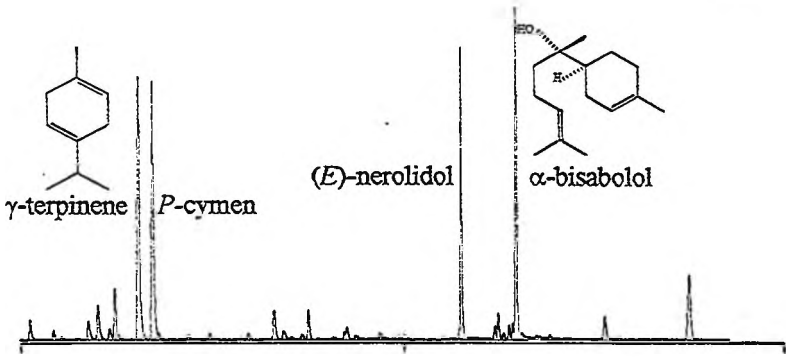


E. Individual plant (C) from Biesiesvlei

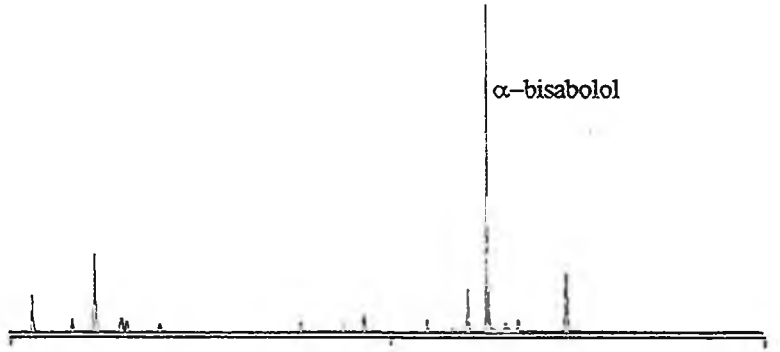


F. *S. stenophylla* from Steynsrus

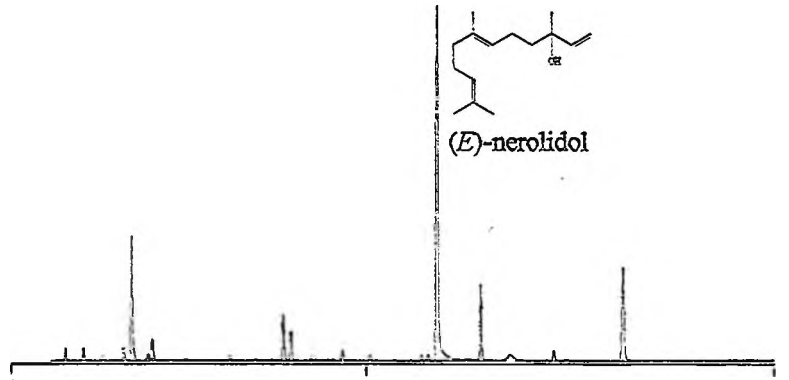
Figure 36. GC/MS profiles of *S. stenophylla*.



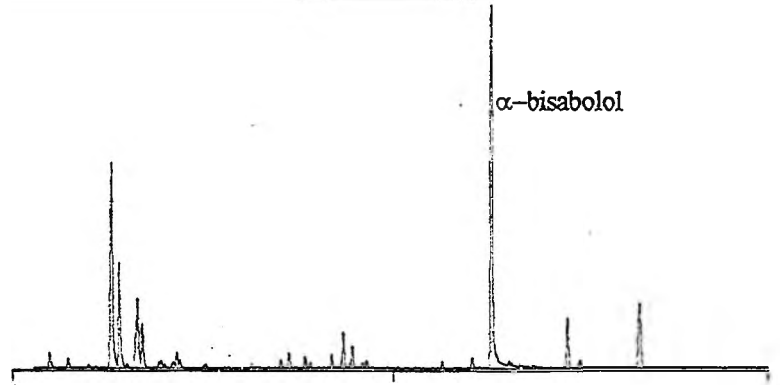
G. *S. stenophylla* from Smithfield



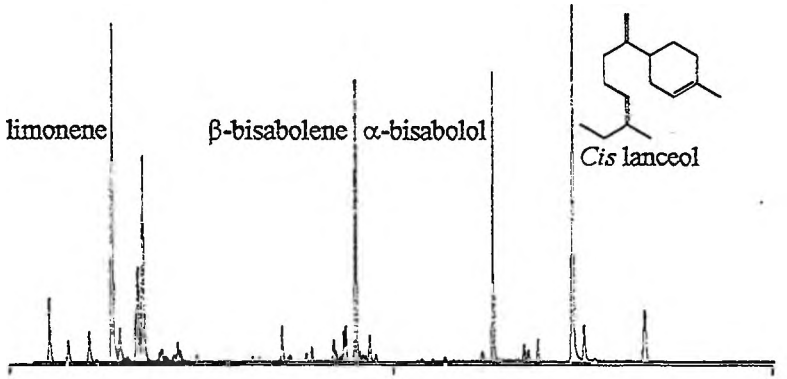
H. *S. stenophylla* from Dordrecht



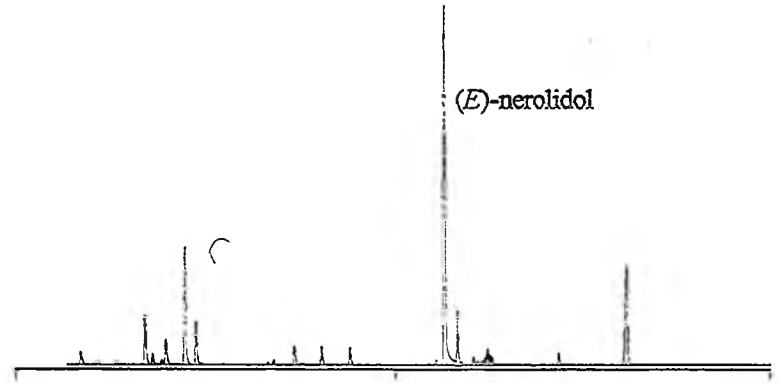
I. *S. stenophylla* from Bethulie



J. *S. stenophylla* from Molteno



K. *S. stenophylla* from Murraysburg



L. *S.stenophylla* from Springfontein

Figure 36. GC/MS profiles of *S. stenophylla.*

3.4.4.2 *Salvia repens*

A total of 109 compounds were recorded and 108 compounds were identified for *S. repens* (Table 9). The most predominant compounds (Figure 37) include; δ-3-carene and α-bisabolol from microdistilled samples collected from Dordrecht at 33% and 24.9% respectively (Figure 37a). Camphor occurs at 12.7% in the microdistilled sample from Queenstown. β-phellandrene occurs at 22.2% in the Lady Grey extract (Figure 37e), β-caryophyllene occurs at 17% (Indwe), 13.6% (Queenstown) and 12.4% (Lady Grey). The sesquiterpene (*E*)-nerolidol accumulates up to 25.2% for the sample collected from Knapdaar, ledol at 23.7% from Queenstown and up to 25.4% for microdistilled samples (Figures 37b and 37c). Quite a few compounds occurred across the taxon, these include limonene, β-phellandrene, γ-terpinene, *p*-cymene, 1-octen-3-ol, borneol, α-campholene alcohol and caryophyllene oxide. Two chemotypes predominantly consisting of sesquiterpenes can be retrieved from this data, these being chemotype III; β-phellandrene, β-caryophyllene and ledol-rich chemotype and chemotype IV; α-bisabolol and (*E*)-nerolidol-rich chemotype. This supports the tentative chemotypes identified based on TLC results (Figure 34).

3.4.4.3 *Salvia runcinata*

A total of 170 compounds were identified. The chemical compounds that occurred in high percentages in *S. runcinata* include (*E*)-nerolidol ranging between 0.004%-72.6%. It was the major essential oil constituent from Coligny (72.6%), Kroonstad (70.4%), Delareyville (56%), Ventersdorp (39.1%), Roossenekal (38.7%), Kuruman (32.1%) and Klerkskraal Dam (32%) (Figures 38e, 38f, 38g, 38h, 38i, 38j and 38k). Other predominant compounds include α-bisabolol (38%) and 41.1% from Ventersdorp and Klerkskraal Dam (individual plant) respectively. The essential oil of bulk plant material from Klerkskraal Dam yielded only 0.3% of the α-bisabolol. This pattern of compounds was not observed in the Kuruman sample and individual plant essential oil components. However, what was observed in both cases is that the essential oil from individual samples recorded more compounds than the oil obtained from a 'pool' of plant material in a population. For instance, individual plant essential oil from Kuruman yielded 61 compounds while the oil from the bulk plant material yielded 58 compounds. This also applies to the individual plant essential oil from Klerkskraal Dam yielding 73 compounds and the bulk sample yielded 47 compounds (Table 10). Other compounds that were in high percentages include α-pinene (45%) and β-pinene

Table 9. Essential oil composition of *S. repens* and the relative retention times (RRI).

RRI	COMPOUND	A	B	C	D	E	F
1014	tricyclene		0.20				
1032	α-pinene	3.80	6.50	0.80	4.47	**10.86**	
1035	α-thujene	1.80					
1076	camphene		4.00	0.10	4.61	0.22	
1100	decane						0.33
1100	undecane	0.10					0.17
1118	β-pinene	2.70	3.00	0.60	2.50	2.69	
1132	sabinene	0.00	0.20				
1145	ethyl benzene						0.06
1155	1-undecene			0.10			
1159	δ-3-carene		0.10			**33.03**	
1174	myrcene	1.00	2.30	0.70	trace		
1176	α-phellandrene				1.46	0.21	
1183	*p*-mentha-1(7)8-diene	0.50					
1188	α-terpinene		0.20	0.10		0.10	
1203	limonene	5.80	9.80	3.20	2.86	5.15	0.03
1205	sylvestrene					2.67	
1218	β-phellandrene	**13.20**	**22.20**	8.10	**12.83**	3.42	0.38
1246	(*z*)-β-ocimene	0.20	2.70	1.40	1.43		0.05
1255	γ-terpinene	0.20	0.50	0.50	0.36	0.42	0.01
1265	5-methyl-3-heptanone	0.10			0.46		
1266	(*E*)-β-ocimene	0.10	1.50	0.40	0.31		trace
1278	*m*-cymen					0.72	
1280	*p*-cymen	0.50	0.70	0.50	0.43	0.66	0.04
1282	*cis*-allo ocimene		0.10	0.10			
1290	terpinolene	0.10	0.30	0.10			
1327	(*Z*)-3-hexenyl acetate		0.10				
1348	6-methyl-5-hepten-2-one			0.20			
1393	3-octanol	0.10	0.10	0.10		0.25	0.02
1443	2,5-dimethylstyrene					0.07	
1452	1-octen-3-ol	0.20	0.50	0.20	1.56	0.10	0.37
1452	α-*p*-dimethylstyrene					0.06	
1467	6-methyl-5-hepten-2-ol	trace	0.10	0.20			
1474	*trans*-sabinene hydrate	0.10	0.30				0.01
1495	bicycloelemene	0.10					
1497	α-copaene						0.14
1532	camphor	4.80	6.90	0.80	**12.67**		3.43
1544	α-gujunene		0.20	0.10			0.62
1549	α-cubebene						0.01
1549	β-cubebene	trace					
1553	linalool	0.30	0.20	0.10			0.07
1556	*cis*-sabinene hydrate		0.20				
1562	isopinocamphone	0.10					
1568	1-methyl-4-acetyl cyclohex-1-ene					0.14	
1571	*trans-p*-menth-2-en-1-ol	0.20	0.20	0.20			0.19
1586	pinocarvone	0.20	0.10				0.04
1589	isocaryophyllene						0.07
1594	*trans*-β-bergamotene			0.30			
1597	bornyl acetate	3.00	0.50	0.10			
1611	terpene-4-ol					0.94	
1612	β-caryophyllene	**17.00**	12.40	4.40	8.39		**13.60**
1628	aromadendrene	1.70	0.90	0.50	0.82		2.62
1638	*cis-p*-menth-2-en-1-ol		0.20				
1648	myrtenal	0.10					
1650	γ-elemene		0.50				
1661	alloaromadendrene	0.60	0.50		5.25		12.43
1668	(*Z*)-β-farnescene			0.40			
1687	α-humulene	4.80	3.20	1.40	1.37		3.27

RRI	COMPOUND	A	B	C	D	E	F
1700	limonen-4-ol					0.13	
1704	γ-curcumene			0.10			
1719	borneol	0.60	1.50		2.05	0.46	1.18
1727	7-*epi*-1,2-dehydro sesquicineole			0.60			
1730	δ-guaiene	2.20					
1741	β-bisabolene			0.60			
1748	piperitone	0.60					
1755	β-curcumene			0.60			
1755	bicyclogermacrene	1.60	0.80				
1773	δ-cadinene		0.30	0.40			0.28
1776	γ-cadinene						trace
1786	Ar-cucumene			0.40			
1804	myrtenol						0.02
1805	α-campholene alcohol	1.90	2.30	1.30	2.04	1.59	1.50
1845	*trans*-carveol	0.20	0.20	0.10		0.19	0.20
1853	*cis* calamenene						0.02
1854	germacrene B	0.10	0.60				
1864	*p*-cymen-8-ol					0.53	
1900	*epi*-cubebol			0.10			0.01
1941	α-calacorene-I						0.09
1953	palustrol						1.90
1969	*cis* jasmone			0.10			
2008	caryophyllene oxide	6.60	2.00	2.10	2.26	0.64	9.38
2033	*epi*-globulol	0.30					
2050	(*E*)-nerolidol	2.00		**25.20**			0.17
2057	ledol		0.60		**25.39**		**23.67**
2071	humulene epoxide-II	1.20	0.50	0.80		0.11	1.72
2098	globulol	0.90	0.20				0.72
2103	guaiol			2.50			
2104	viridiflorol	0.90	3.50			0.07	
2144	rosifoliol	0.20					
2144	spathulenol	1.50	1.00	0.50	2.04		2.76
2156	α-bisabolol oxide B			0.30			
2162	bisabolol oxide					3.37	
2170	β-bisabolol	1.70		8.50			
2187	τ-cadinol		0.20	0.70			
2209	τ-muurolol		0.10				0.06
2228	pogostol	6.50					1.51
2232	α-bisabolol		0.70	19.00		**24.87**	
2250	α-eudesmol		0.10				
2255	α-cadinol		0.30				0.06
2257	β-eudesmol			1.00			
2300	dibenzofuran						0.05
2324	caryophylladienol-II	1.00	0.30			0.11	5.08
2389	caryophyllenol-I	1.10	0.90	3.50			1.15
2392	caryophyllenol-II	0.50	0.10	0.20			1.63
2518	*cis*-lanceol	0.30	0.30	2.40		3.52	3.79
2545	(*Z*)-nuciferol			0.20			
2622	phytol	0.30					0.66
2740	Anthracene						0.70
	bp 120						0.01
Total		**90.0**	**91.2**	**96.1**	**91.1**	86.4	**96.3**

KEY

A= Indwe

B= Lady Grey

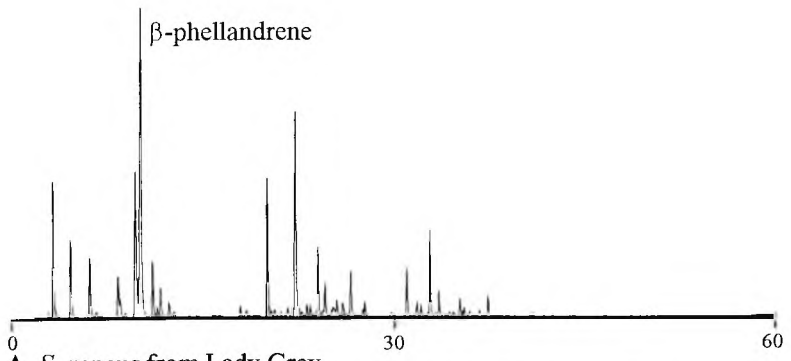
C= Knapdaar

D= Queenstown (microdistilled pooled sample)

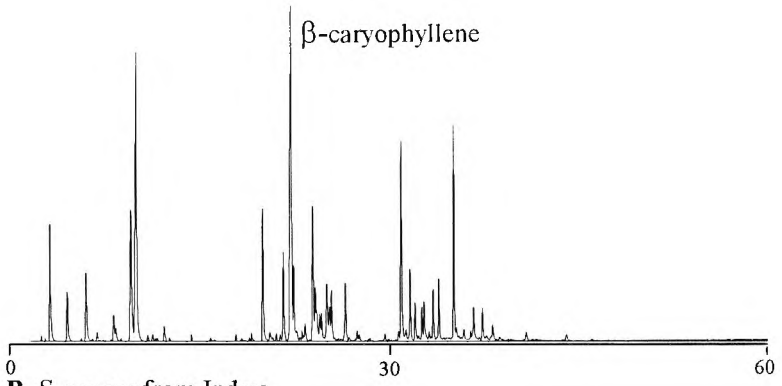
E= Dordrecht (microdistlled pooled sample)

F= Queenstown (hydrodistilled pooled sample)

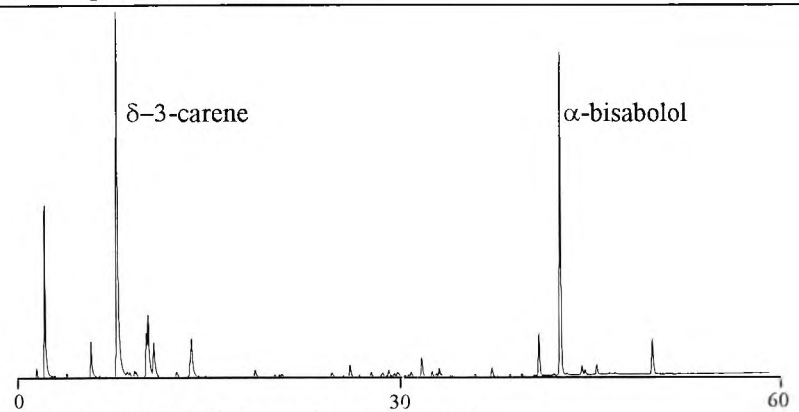
bp= base peak for unidentified compound



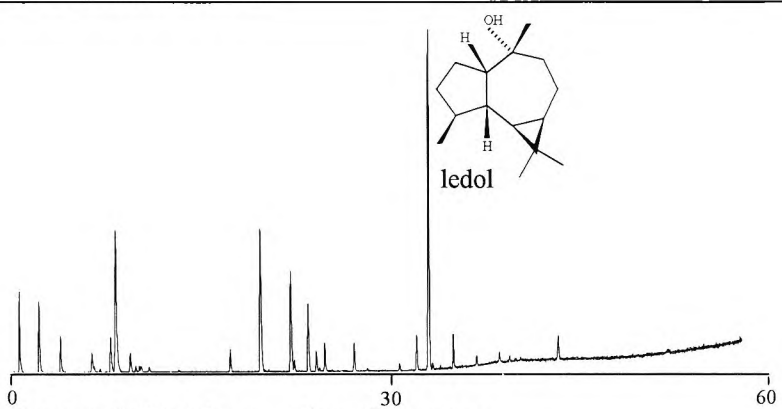
A. *S. repens* from Lady Grey



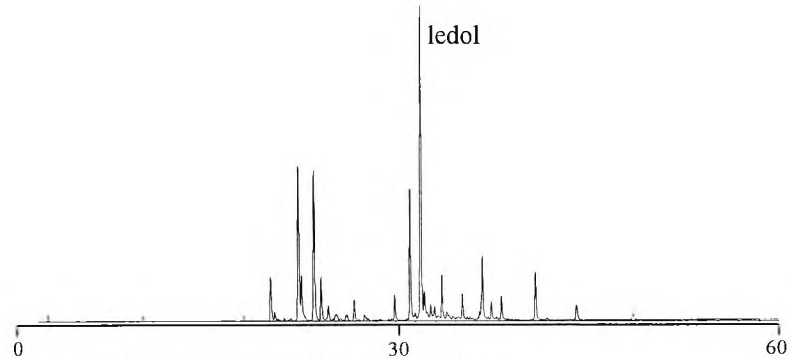
B. *S. repens* from Indwe



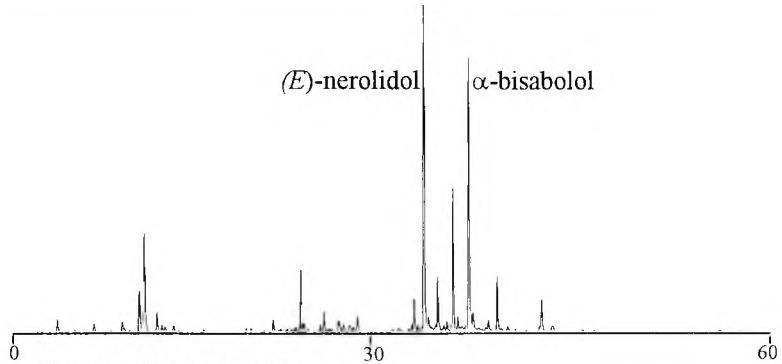
C. Microdistilled *S. repens* from Dordrecht



D. Microdistilled *S. repens* from Queenstown



E. Hydrodistilled *S. repens* from Queenstown



F. *S. repens* from Knapdaar

Figure 37. GC/MS profiles of *S. repens.*

Table 10. Essential oil composition of *S. runcinata* and the relative retention time (RRI).

RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1000	decane			0.05	0.08	0.02		0.09					0.14		0.17	
1014	tricyclene					0.01	0.02	0.02	0.19		0.07	0.02	0.02		0.05	0.03
1032	α-pinene	45.29	1.60	0.08	0.66	0.84	4.19	1.77	5.07		2.30	8.46	3.45	9.14	11.35	16.23
1072	α-fenchene								0.03						0.02	0.03
1076	camphene	1.24	0.04		0.25	0.33	0.68	0.55	3.69		2.31	0.62	0.78	0.12	1.50	0.79
1100	undecane			0.02	0.02			0.03					0.06		0.07	
1118	β-pinene	15.52	1.28	0.09	0.41	0.18	1.96	0.82	0.64	0.01	0.97	3.26	1.48	2.34	3.14	5.72
1132	sabinene	0.14					0.04	0.04	0.05		0.02	0.02		0.08	0.05	0.11
1145	ethylbenzene						0.00	0.02								
1146	δ-2-carene								0.01							
1159	δ-3-carene		0.21	0.04	0.11	0.06		0.27	7.05		0.55	0.14	0.02	0.19	0.11	0.11
1174	myrcene	0.63	0.28		0.12	6.19	0.21	0.29	trace		0.39	9.74	0.02	8.70	0.63	0.78
1176	α-phellandrene								3.00							
1177	β-terpinene												0.05	0.20		
1182	n-butyl benzene	0.14							0.02						0.04	0.17
1188	α-terpinene		0.01			0.03	0.03	0.03	0.67		0.04	0.12	0.01	0.08	0.10	0.13
1203	limonene	0.78	0.48	0.07	0.31	0.28	0.40	0.55	24.09	0.11	0.90	0.53	0.33	0.92	1.55	1.34
1205	sylvestrene															
1213	1,8-cineole	0.18	0.82		0.44	0.44	0.31	1.95		0.20			0.29	2.06	2.07	3.67
1218	β-phellandrene		trace	0.23	trace		trace	trace	10.44		1.73	0.54	trace		trace	
1244	amylfuran							0.01								
1246	(*Z*)-β-ocimene	0.68	0.38	0.16	0.40	0.11	0.03	0.40	0.04	0.07	1.11	0.22	0.02	10.41	2.98	2.91
1255	γ-terpinene	0.19	0.06		0.03	0.04	0.08	0.08	0.01	0.03	0.16	0.13			0.32	0.37
1265	5-methyl-3-heptanone		trace				0.14	trace			0.40		0.10			
1266	(*E*)-β-ocimene	0.39	0.64	0.28	0.69	0.18	0.04	0.76	0.51	0.20	0.90	0.47	0.05	3.31	1.67	1.46
1280	*p*-cymen	0.19	0.17		0.02	0.24	0.04	0.20	2.87	0.06	0.39	0.13	0.10		0.46	0.48
1290	terpinolene		0.03	0.01	0.06		0.08	0.06	0.17	0.03	0.06	0.07	0.01	0.30	0.15	0.18
1327	(*Z*)-3-hexenylacetate															0.01
1348	6-methy-5-hepten-2-one					0.02		0.03			0.11					
1348	6-methyl-3,5-heptadien-2-one										0.04					
1360	hexanol							0.01			0.03					0.01
1382	*cis* allo ocimene							0.01			0.03			0.45	0.10	0.14
1391	(*Z*)-3-hexen-1-ol					0.01				0.01						0.25
1391	(*Z*)-3-hexanol				0.01											
1393	3-octanol						0.00	0.07	0.03		0.03	0.01	0.01	0.05		0.02
1400	nonanal		0.04	0.09	0.07	0.05	0.03	0.01		0.05	0.09	0.03	0.10		0.02	0.04
1429	perillen															
1443	2,5-dimethylstyrene								0.06					0.05		

RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1450	*trans*-linalool oxide		0.02			0.02		trace		0.02		0.01	0.03			
1452	α-*p*-dimethylstyrene								0.02							0.03
1452	1-octen-3-ol		0.50	0.18	0.07		0.60	0.40	0.17	0.38	0.88	0.07	0.18	0.40	0.03	0.30
1466	α-cubebene		0.01				0.06				0.01	0.21	0.50			0.14
1467	6-methyl-5-hepten-2-ol															
1474	*trans* sabinene hydrate		0.03	0.01			0.20	0.10	0.15	0.05	0.05			0.15	0.10	0.06
1478	*cis* linalool oxide			0.01		0.02		0.05		0.03			0.01			
1493	α-ylangene						0.01		0.02			0.03	0.02			
1497	α-copaene		0.01				0.16	0.11	0.32		0.13	0.69	1.75			
1498	(*E*)-β-ocimeneepoxide													0.02		
1499	α-campholene aldehyde	2.63													0.02	0.04
1532	camphor	2.31	0.60	0.14	2.22	0.51	1.75	1.97	4.74	1.56	6.04	1.35	0.51		0.08	0.08
1541	benzaldehyde			0.02		0.01										
1544	α-gujunene						0.10		0.19			0.50	0.80	0.12	0.10	0.16
1553	linalool		0.54	0.26	0.17	0.49	0.27	0.31	0.73	0.26	0.75	0.34	0.52	0.99	0.02	0.13
1556	*cis* sabinene hydrate						trace								0.15	
1562	octanol							0.01		0.04	0.05					
1568	1-methyl-4-acetyl cyclohex-1-ene							0.19								
1568	1-methyl-4-acetyl cyclohex-1-ol			0.09												
1571	*trans p*-menth-2-en-1-ol		0.03				0.00		0.78	0.02		0.02	0.01		0.02	0.04
1586	pinocarvone	1.24			0.02				0.10		0.05	0.06				0.30
1589	aristolene		0.06												0.23	
1589	isocaryophyllene		0.86	0.10	0.71	0.51	0.35	0.64	0.06		0.30	0.33	0.57	0.02	0.38	0.34
1594	*trans* β-bergamotene			0.43				0.57								
1597	bornyl acetate										0.10					
1604	thymol methylether									0.04						
1610	calarene														0.11	
1611	terpene-4-ol	0.65														
1612	β-caryophyllene		31.90	6.73	23.37	7.65	29.05	11.39	4.82	9.11	5.84	16.20	14.43	20.42	10.30	11.34
1626	2-methyl-6-methylene-3,7-octadien-2-ol					0.02										
1628	aromadendrene		1.12						1.12			2.12		3.13	3.19	2.73
1638	*cis p*-menth-2-en-1-ol								0.70							
1638	β-cyclocitral			0.04		0.12		0.08		0.12	0.16		0.10			
1639	*trans p*-mentha-2.8-dien-1-ol															
1648	myrtenal	1.83														
1661	allo aromadendrene		0.08						0.07			0.16		0.39	trace	
1663	phenyl acetaldehyde					0.02										
1664	nonanol			0.12	0.19	0.22				0.16	0.19		0.25			
1668	(*Z*)-β-farnescene			0.51		0.04		0.41		0.05	0.05					

RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1670	*trans* pinocarveol	4.78											0.01		0.81	1.29
1677	*epi* zonarene											0.08	0.13			
1678	*cis p* -mentha-2,8-dien-1-ol								0.08							
1681	(*Z*)-3-hexanyltiglate															
1682	δ-terpineol															0.03
1683	*trans* verbenol	4.66														
1687	α-humulene		9.14	1.64	6.04	1.81	8.48	2.71	1.26	2.34	1.35	4.25	3.71	6.16	2.78	
1695	(*E*)-β-farnescene							0.15								
1704	γ-muurolene											0.38	0.97			
1706	α-terpineol	0.26	0.21	0.41	0.14	0.16	0.68	0.17	0.54	0.29				0.11	0.21	0.35
1719	borneol	0.25	0.20		0.10	0.10	0.57	0.30	1.04	0.43	2.01	0.47	0.20	0.72	5.35	3.41
1725	verbenone	1.43														
1726	germacrene D						0.13			0.08						
1737	(*Z.E*)-α-farnescene									0.06						
1740	α-muurolene						0.24					3.18	0.30			
1741	β-bisabolene			0.53				0.89								
1742	β-selinene		0.13		0.08											
1743	α-cadinene															
1744	α-selinene				0.06											
1755	α-farnescene			0.01												
1755	bicyclogermacrene				0.05		0.03					0.58		0.35	0.02	0.12
1755	sesquicineole							0.46								
1758	*cis* piperitol								0.32							
1758	(*E.E*)-a-farnescene									0.02						
1766	1-decanol		0.03	0.05	0.05		0.02	0.01		0.12		0.02	0.03			
1773	δ-cadinene		0.04	0.03				0.03	0.56		0.17	1.42	2.92			
1776	γ-cadinene						0.60	0.09	0.40		0.36	1.00	3.10			
1783	β-sesquiphellandrene			0.26				0.28								
1786	Ar-cucumene									0.01						
1796	selina-3(7)11-diene								0.66							
1799	cadina-1,4-diene											0.15	0.33			
1804	myrtenol	2.25		0.05					trace					0.40		0.01
1805	α-campholene alcohol		0.03		0.06					0.05	0.26				0.42	0.92
1810	3(7)-guaiadiene						0.01					0.11	0.26			
1830	2,6-dimethyl-3(*E*)5(*E*)-7-octatriene-2-ol													0.13		
1838	(*E*)-β-damascenenone							0.03						0.01		0.03
1845	*trans* -carveol	0.66		0.01				0.01	0.11		0.04		0.01	0.36	0.13	0.20
1853	*cis* -calamenene		0.02				0.02	0.06	0.22		0.07	0.35	1.42			
1864	*p* -cymen-8-ol		0.03					0.02		0.01	0.03			0.03	0.03	0.05

RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1670	*trans* pinocarveol	4.78											0.01		0.81	1.29
1677	*epi* zonarene											0.08	0.13			
1678	*cis p* -mentha-2,8-dien-1-ol								0.08							
1681	(*Z*)-3-hexanyltiglate															
1682	δ-terpineol															0.03
1683	*trans* verbenol	4.66														
1687	α-humulene		9.14	1.64	6.04	1.81	8.48	2.71	1.26	2.34	1.35	4.25	3.71	6.16	2.78	
1695	(*E*)-β-farnescene							0.15								
1704	γ-muurolene											0.38	0.97			
1706	α-terpineol	0.26	0.21	0.41	0.14	0.16	0.68	0.17	0.54	0.29				0.11	0.21	0.35
1719	borneol	0.25	0.20		0.10	0.10	0.57	0.30	1.04	0.43	2.01	0.47	0.20	0.72	5.35	3.41
1725	verbenone	1.43														
1726	germacrene D						0.13			0.08						
1737	(*Z.E*)-α-farnescene									0.06						
1740	α-muurolene						0.24					3.18	0.30			
1741	β-bisabolene			0.53				0.89								
1742	β-selinene		0.13		0.08											
1743	α-cadinene															
1744	α-selinene				0.06											
1755	α-farnescene			0.01												
1755	bicyclogermacrene				0.05		0.03					0.58		0.35	0.02	0.12
1755	sesquicineole							0.46								
1758	*cis* piperitol								0.32							
1758	(*E.E*)-a-farnescene									0.02						
1766	1-decanol		0.03	0.05	0.05		0.02	0.01		0.12		0.02	0.03			
1773	δ-cadinene		0.04	0.03				0.03	0.56		0.17	1.42	2.92			
1776	γ-cadinene						0.60	0.09	0.40		0.36	1.00	3.10			
1783	β-sesquiphellandrene			0.26				0.28								
1786	Ar-cucumene									0.01						
1796	selina-3(7)11-diene								0.66							
1799	cadina-1,4-diene											0.15	0.33			
1804	myrtenol	2.25		0.05					trace					0.40		0.01
1805	α-campholene alcohol		0.03		0.06					0.05	0.26				0.42	0.92
1810	3(7)-guaiadiene						0.01					0.11	0.26			
1830	2,6-dimethyl-3(*E*)5(*E*)-7-octatriene-2-ol													0.13		
1838	(*E*)-β-damascenenone							0.03						0.01		0.03
1845	*trans* -carveol	0.66		0.01				0.01	0.11		0.04		0.01	0.36	0.13	0.20
1853	*cis* -calamenene		0.02				0.02	0.06	0.22		0.07	0.35	1.42			
1864	*p* -cymen-8-ol		0.03					0.02		0.01	0.03			0.03	0.03	0.05

RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1868	(*E*)-geranylacetone					0.02		0.02	0.01	0.02	0.03			0.01	0.02	0.02
1878	2,5-dimethoxy-*p*-cymene									0.38	0.19					
1882	*cis*-carveol			0.01					0.07				0.01		0.03	0.05
1889	myrcen-8-ol											0.22				
1896	*cis*-*p*-mentha-1(7)8-dien-2-ol								0.13							0.02
1900	*epi*-cubebol						0.15	0.02			0.05	0.16	0.94			
1932	isoamylbenzoate								0.02							
1941	α-calacorene-I							0.03	0.10		0.02	0.09	0.40			
1949	*trans*-jasmone		0.11	0.06	0.09	0.09	0.10	0.13		0.07	0.09			0.08		
1957	cubebol						0.18					0.11	0.80			
1969	*cis*-jasmone			0.06	0.04	0.13		0.15		0.13	0.15			trace	0.01	
1984	α-calacorene-II							0.02			0.05	0.04	0.14			
2001	*iso*-caryophyllene-oxide		0.22		0.12					0.01		0.06		0.18		
2008	caryophyllene-oxide	1.37	5.08	1.97	8.01	1.22	0.10	6.86	1.64	6.67	3.61	5.01	6.39	9.15	5.49	4.41
2033	*Epi*-globulol											0.11			0.29	
2045	humulene-epoxide-I		0.12				0.01		0.03			0.11				
2050	(*E*)-nerolidol			39.09	31.96	70.38	0.00	6.80	0.38	72.63	56.84		38.73	8.00	32.09	24.28
2057	ledol											0.18				
2071	humulene-epoxide-II	1.29	1.12		1.80		0.25	1.36	0.60			1.00	1.46	2.14	1.59	1.21
2080	cubenol											0.21				
2081	humulene-epoxide-III		0.07													
2088	1-*epi*-cubenol						0.04					0.22	0.60			
2096	Elemol						0.08									
2098	globulol		0.34									0.38		0.24	0.77	0.52
2103	guaiol						26.02		3.31							
2104	viridifloral															
2131	Hexa-hydro-farnesylacetone			0.11				0.08								
2144	rosifoliol		0.09				2.66					0.19		0.15	0.35	0.24
2144	spathulenol		0.43		0.29				0.42			0.51		1.01	1.28	0.92
2156	α-bisabolol oxide B							1.65								
2162	bisabolol oxide			1.21												
2162	α-bisabolol oxide							1.82								
2185	γ-eudesmol		3.73		1.26		1.29	0.14				3.20				
2187	τ-cadinol								0.24		0.10		2.08			
2209	τ-muurolol								0.06				1.24			
2219	δ-cadinol										0.10		0.59			
2219	bulnesol						1.82									
2232	α-bisabolol			37.98	0.29			41.05	0.89							
2247	*trans*-α-bergamotol													0.04		

RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
2250	α-eudesmol		12.99		5.74		4.59		0.37			10.58				
2255	α-cadinol										0.10		1.66			
2256	*epi* -α-bisabolol							2.07								
2257	β-eudesmol		12.53		5.54		4.86		0.46			11.63				
2316	caryophylladienol-I		0.17				0.30									
2324	caryophylladienol-II		1.26	0.41	2.38	0.18		1.40	0.61	0.41	0.56	1.68	1.84	0.28	1.29	0.88
2384	farnesyl-acetone															
2389	caryophyllenol-I				0.54	0.44		0.80	0.79	0.69	0.24	0.58	0.91	0.98	1.34	0.94
2392	caryophyllenol-II		0.71	0.36	1.39	0.63		1.05	0.45	0.26	0.52	1.11	1.68	1.12	1.16	0.77
2518	*cis* -lanceol							1.47	2.11							
2524	abietatriene															
2622	phytol			0.17	0.05	0.03	0.01			0.14	0.09	0.04		0.03	0.07	0.01
2655	benzylbenzoate								0.01							
2676	manool								6.40							3.32
2740	anthracene													0.39		
	bp45166								0.55					1.40		
	bp109					0.04				0.74	1.39					
	bp109															0.28
	bp109					0.04	0.95				trace					
	bp120						0.72									
	bp161222		5.45									3.39				
	bp161222		3.12													
Total		**91.0**	**99.1**	**94.1**	**96.5**	**93.9**	**95.7**	**96.4**	**97.5**	**98.1**	**95.6**	**99.4**	**99.5**	**97.5**	**96.8**	**94.9**

KEY TO LOCALITIES

A= Smithfield
B= Bergville
C= Ventersdorp
D= Klerkskraal Dam (pooled sample)
E= Kroonstad
F= Melville Koppies
G= Klerkskraal Dam (individual plant)
H= Lichtenburg
I= Coligny
J= Delareyville
K= Koster
L= Roossenekal
M= Vryburg
N= Kuruman (pooled sample)
O= Kuruman (individual plant)
M= Vryburg
bp= base peak of unidentified compounds

RRI	COMPOUND	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
2250	α-eudesmol		12.99		5.74		4.59		0.37			10.58				
2255	α-cadinol										0.10		1.66			
2256	*epi* -α-bisabolol							2.07								
2257	β-eudesmol		12.53		5.54		4.86		0.46			11.63				
2316	caryophylladienol-I		0.17				0.30									
2324	caryophylladienol-II		1.26	0.41	2.38	0.18		1.40	0.61	0.41	0.56	1.68	1.84	0.28	1.29	0.88
2384	farnesyl-acetone															
2389	caryophyllenol-I				0.54	0.44		0.80	0.79	0.69	0.24	0.58	0.91	0.98	1.34	0.94
2392	caryophyllenol-II		0.71	0.36	1.39	0.63		1.05	0.45	0.26	0.52	1.11	1.68	1.12	1.16	0.77
2518	*cis* -lanceol							1.47	2.11							
2524	abietatriene															
2622	phytol			0.17	0.05	0.03	0.01			0.14	0.09	0.04		0.03	0.07	0.01
2655	benzylbenzoate								0.01							
2676	manool								6.40							3.32
2740	anthracene													0.39		
	bp45166								0.55					1.40		
	bp109					0.04				0.74	1.39					
	bp109															0.28
	bp109					0.04	0.95				trace					
	bp120						0.72									
	bp161222		5.45									3.39				
	bp161222		3.12													
Total		**91.0**	**99.1**	**94.1**	**96.5**	**93.9**	**95.7**	**96.4**	**97.5**	**98.1**	**95.6**	**99.4**	**99.5**	**97.5**	**96.8**	**94.9**

KEY TO LOCALITIES

A= Smithfield
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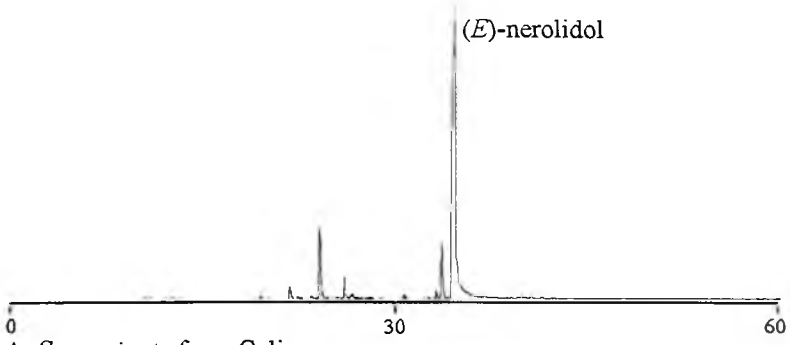
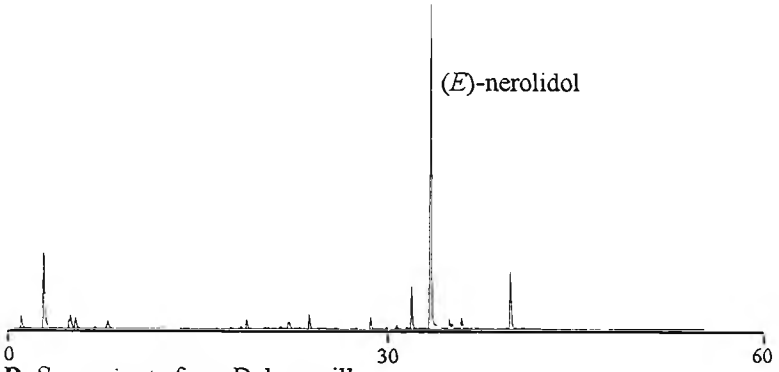
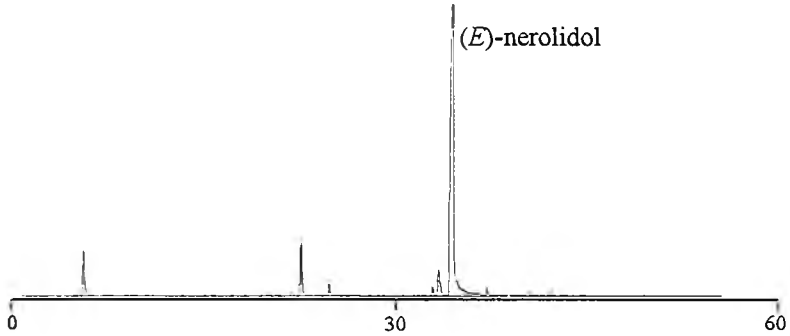
L= Roossenekal
M= Vryburg
N= Kuruman (pooled sample)
O= Kuruman (individual plant)
M= Vryburg
bp= base peak of unidentified compounds



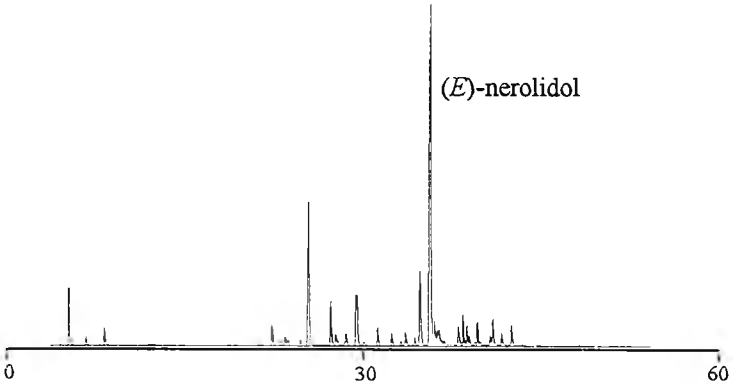
A. *S. runcinata* from Coligny



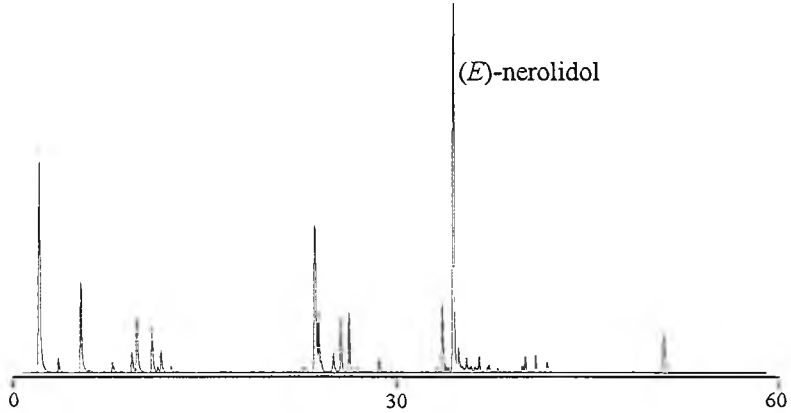
B. *S. runcinata* from Delareyville



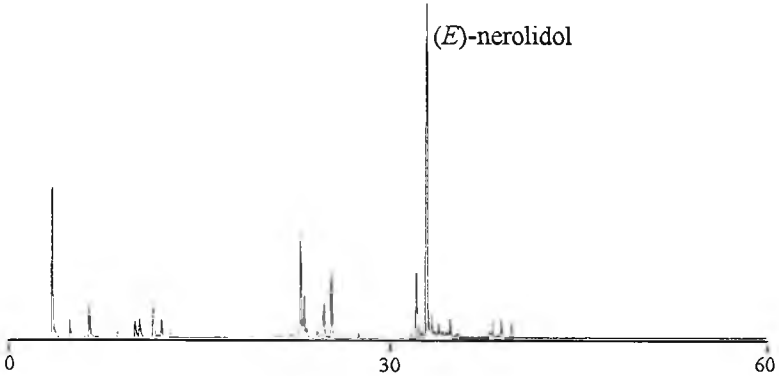
C. *S. runcinata* from Kroonstad



D. *S. runcinata* from Roossenekal

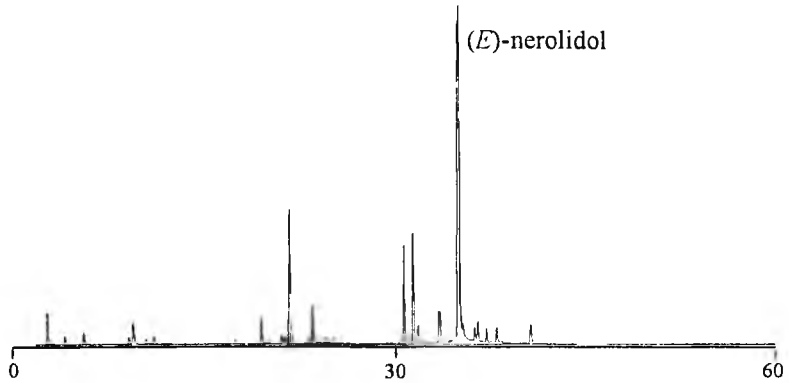


E. *S. runcinata* oil from an individual plant from Kuruman

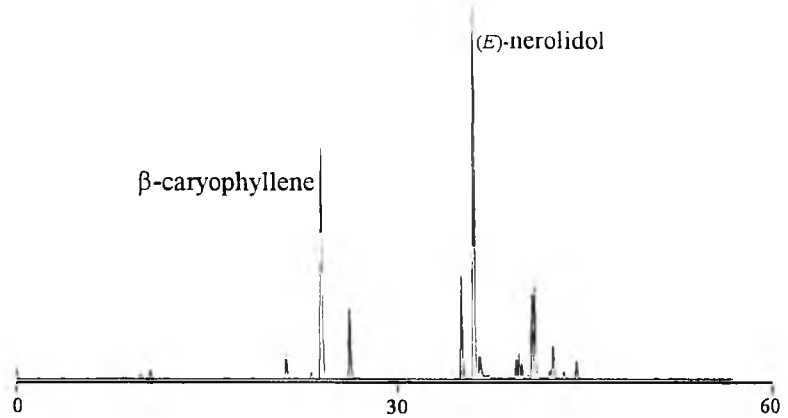


F. *S. runcinata* oil from bulk plant material from Kuruman

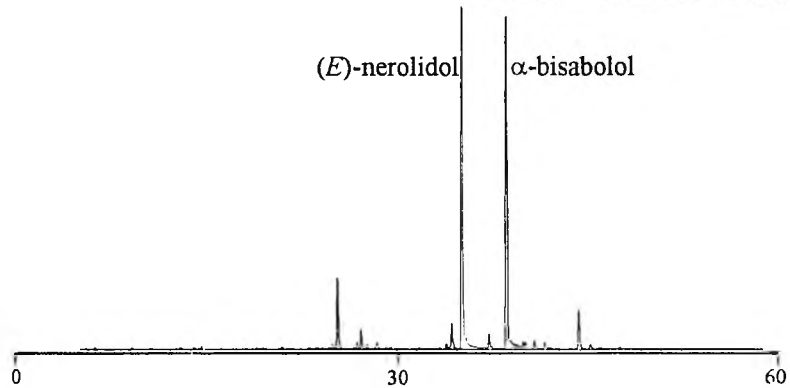
Figure 38. GC/MS profiles of *S. runcinata.*



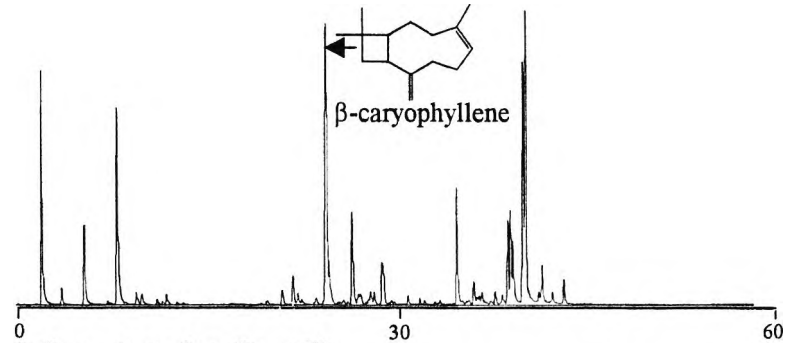
G. *S. runcinata* oil from an individual plant from Klerkskraal Dam



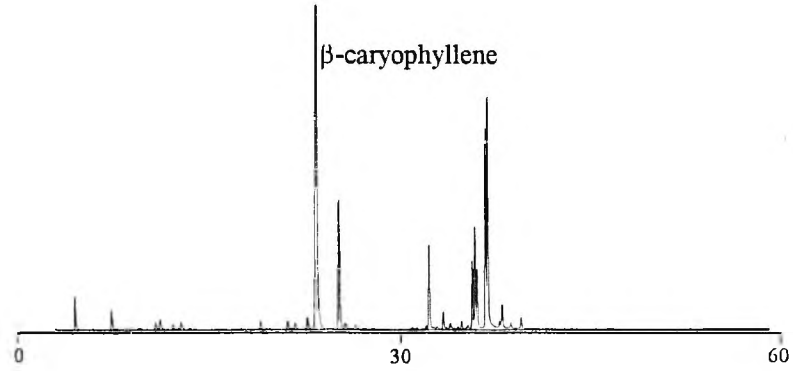
H. *S. runcinata* oil from bulk plant material from Klerkskraal Dam



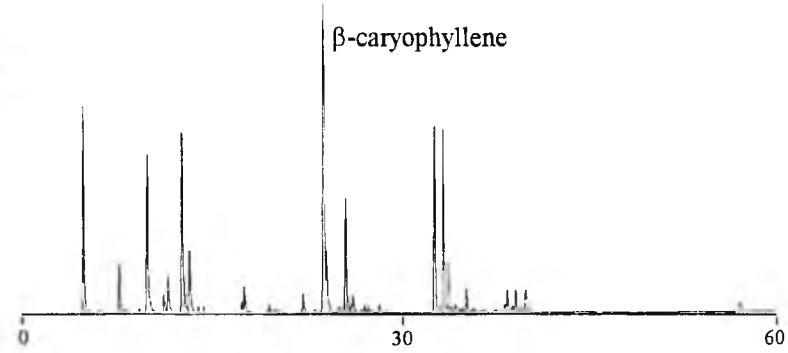
I. *S. runcinata* from Ventersdorp



J. *S. runcinata* from Bergville

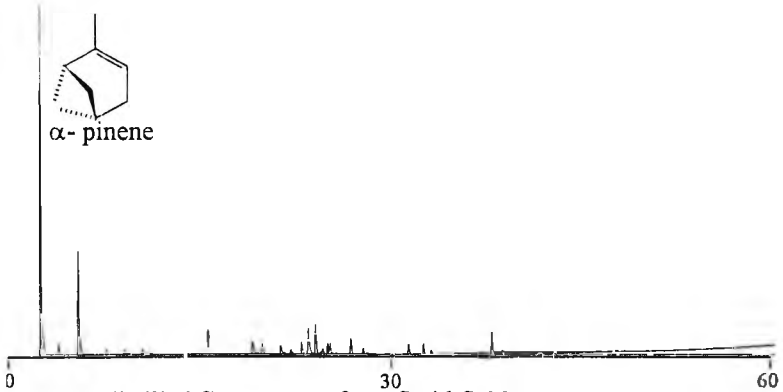


K. *S. runcinata* from Koster

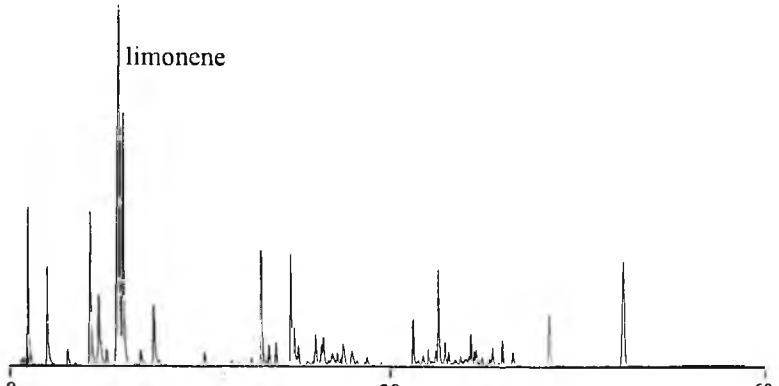


L. *S. runcinata* from Vryburg

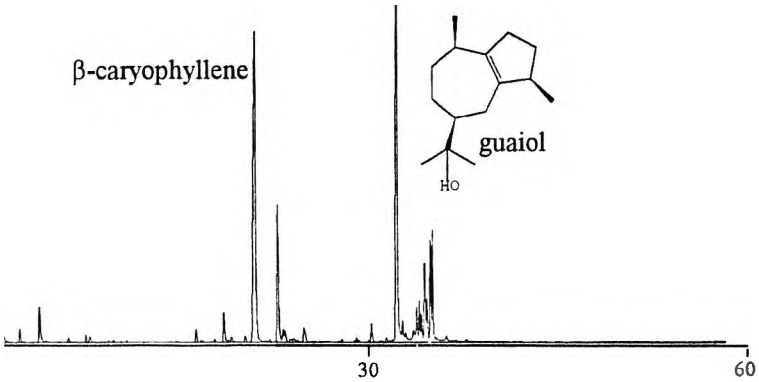
Figure 38. GC/MS profiles of *S. runcinata*.



M. Microdistilled *S. runcinata* from Smithfield



N. *S. runcinata* from Lichtenburg



O. *S. runcinata* from Melville Koppies

Figure 38. GC/MS profiles of *S. runcinata.*

(15%) (Smithfield), guaiol (26%) (Melville Koppies). Limonene occurred at 24.1% in Lichtenburg sample. There are only four compounds that were abundant across the taxon, these being: β-pinene, limonene, caryophyllene oxide and β-caryophyllene, which does not occur only in the Smithfield microdistilled essential oil (Figure 38a).

From these findings, three chemotypes can be identified, chemotypes V; (*E*)-nerolidol-rich type (24%–72%), chemotype VI; β-caryophyllene-rich and a α-pinene-rich type (16%–45%) with some extract containing β-pinene, and thirdly chemotype VII, α-bisabolol and (*E*)-nerolidol rich type (38%–41%) Of the three chemotypes, sesquiterpenes predominates in chemotypes V and VI. *S. repens* and *S. runcinata* share a chemotype, which is composed of α-bisabolol and (*E*)-nerolidol (Figure 39).

3.4.5 The species complex and phenetic analysis

A total of 214 different compounds were identified from 33 different essential oil extractions of the three taxa. The results showed variability in the chemical composition of the essential oil samples of the three taxa. Chemical composition of the oils varied not only from one taxon to another, but also among populations of the same taxon. From the different localities, different percentages of some compounds were accumulated. In general, the differences in chemical composition of the taxa are both quantitative and qualitative.

3.4.5.1 Cluster analysis

The quantitative data analysis of essential oils (Figure 40) was poorly resolved. Two major clusters are recognized at the highest level of dissimilarity 9.54. The first cluster (A) comprises nine OTUs and the second cluster (B) has 16 OTUs. All the OTUs assigned to the *a priori* taxa are scattered into the groups indicating variation in chemical composition among the populations of the same taxon, even between those from very close localities, as was the case for the samples collected from Delareyville, Vryburg and Ventersdorp. This indicates the existence of chemical polymorphism. However, at 8.70 level split of dissimilarity, four clusters A_1, A_2, B_1 and B_2 can be identified. These groups are associated with a common chemical compound. For instance group A_1 OTUs have in common high concentrations (above 5%) of β-phellandrene and limonene. This group consists of four OTUs from all the three *a priori* taxa; two *S. repens* from Indwe and Lady Grey, *S.*

The chemotypes identified in *S. stenophylla*, *S. repens* and *S. runcinata*.

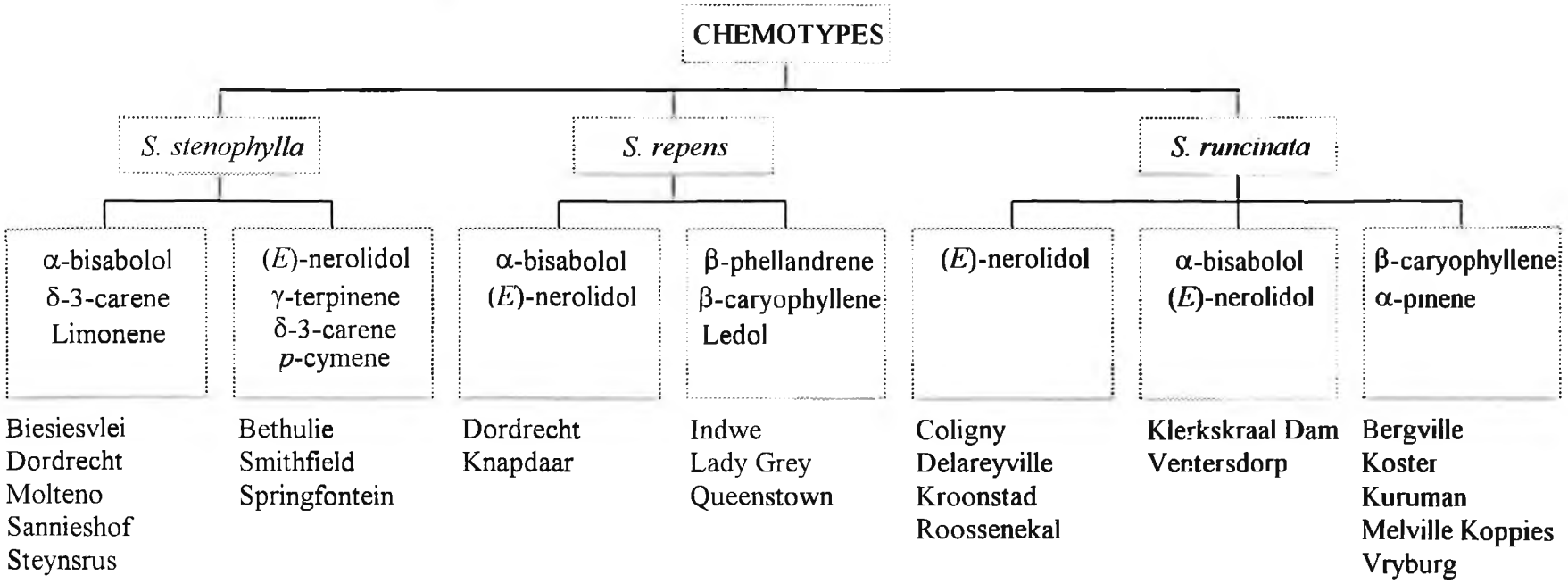


Figure 39. Chart of six chemotypes identified in the *S. stenophylla* species complex.

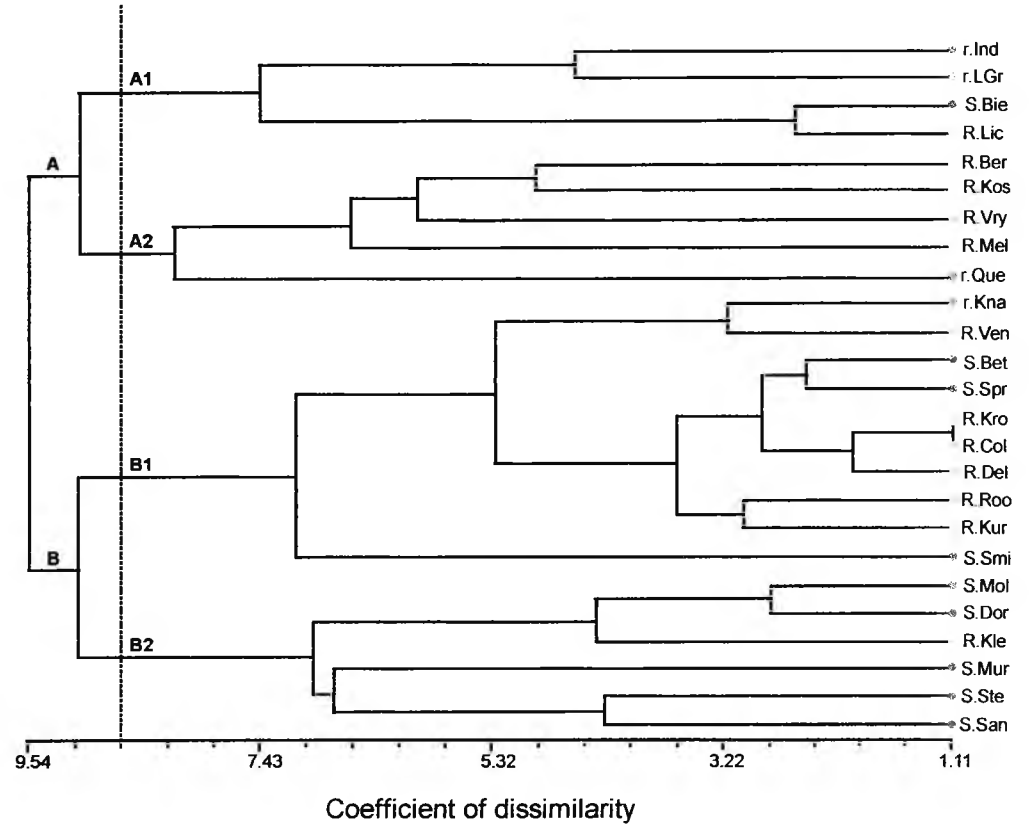


Figure 40. Dendrogram of the quantitative data matrix.

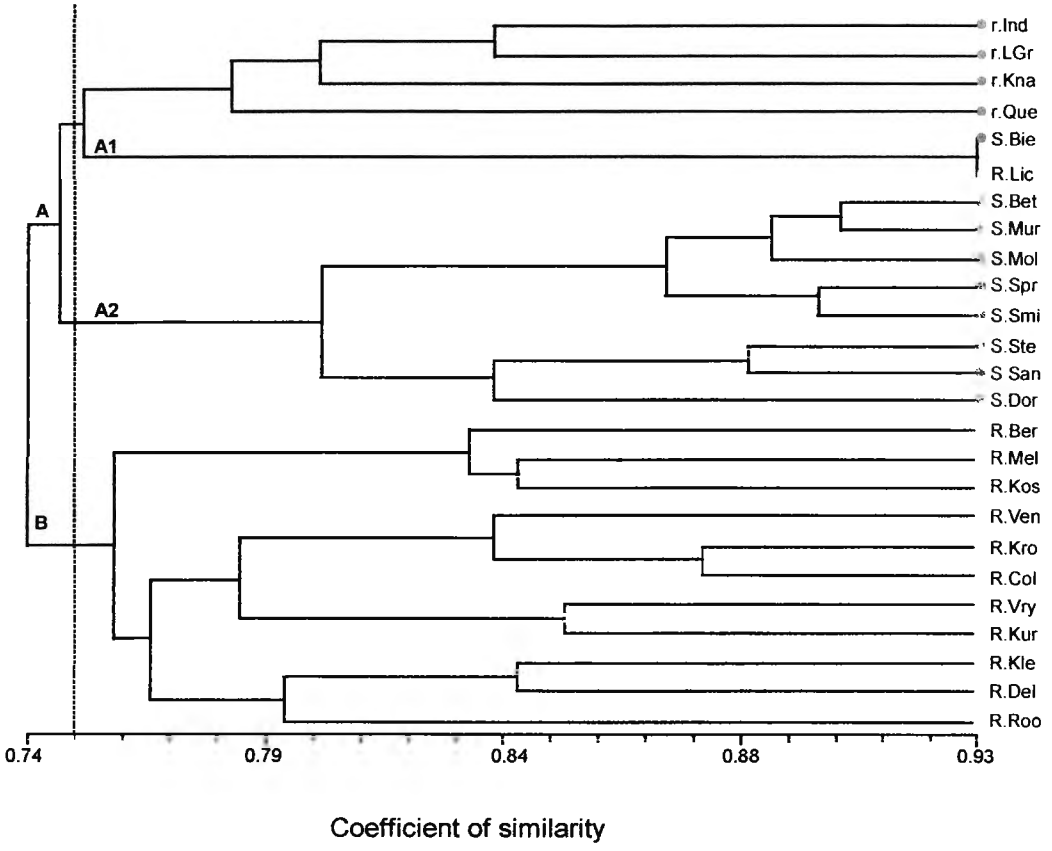


Figure 41. Dendrogram of the qualitative data matrix.

stenophylla from Biesiesvlei and *S. runcinata* from Lichtenburg. Cluster A_2 is dominated with *S. runcinata* taxa and *S. repens* collected from Queenstown, although it branches off from the other OTUs. The taxa in this group have β-caryophyllene in common. Group B_1 is comprised of taxa having more than 15% of (*E*)-nerolidol. *S. runcinata* samples from Kroonstad and Coligny indicate that they have exactly the same chemical compounds (characters) that cluster the OTUs into their groups according to similarity. These two samples have the highest level of (*E*)-nerolidol at 70.38% (Kroonstad) and 72.63% (Coligny). α-bisabolol rich (over 8%) taxa are united in group B_2. This cluster consists of six OTUs, five of which are *S. stenophylla* and one *S. runcinata* from Klerkskraal Dam. The cophenetic correlation coefficient of this analysis is, r = 0.899 indicating good fit. According to Rolf (1993) an 'r' value of greater than 0.9 indicates very good fit, between 0.8–0.9 indicates good fit, while 0.7–0.8 indicates poor fit of the clustering matrix with the original similarity matrix.

The phenogram based on the qualitative data set (41) retrieved two major clusters (A and B) at 0.74 level split. Cluster A is dominated by *S. stenophylla* and *S. repens* taxa, while cluster B is dominated by *S. runcinata* OTUs. There are two distinct subclusters (A_1 and A_2) within cluster A. Cluster A_1 consists of samples of *S. repens* from Indwe, Lady Grey, Knapdaar, and *S. stenophylla* from Biesiesvlei and *S. runcinata* from Lichtenburg. However, this cluster is dominated by *S. repens* OTUs. The second subcluster A_2 unites most *S. stenophylla* OTUs. Cluster B contains *S. runcinata* from Bergville to *S. runcinata* from Roossenekal. These taxa are all of the same species collected between December 2001 and March 2002. Notably the terminal ends of the clusters are characterized by taxa that occur in close proximity geographically. For instance, in cluster A_1, all the *S. repens* OTUs occur in the same geographical area, except for the *S. stenophylla* and *S. runcinata* from Biesiesvlei and Lichtenburg respectively. In cluster A_2 the OTUs occur in the eastern part of South Africa. The correlation coefficient of this data set was, however, very low with r = 0.752 indicating poor goodness of fit.

The combined matrix (quantitative and qualitative data) generated a similar dendrogram as that of the quantitative dendrogram (Figure 40). This result reveals the strength of the quantitative data which is further supported by the correlation coefficient which was r = 0.905 indicating very good fit. It is however, evident that phenetic analysis involving

quantitative characters may not be able to produce absolute boundaries between the OTUs (in this case members of the *S. stenophylla* species complex). Rather the cluster analysis of the combined data set strongly supports erratic chemical composition of the species complex.

3.4.5.2 Principal component analysis (PCA)

The principal component analysis plot for quantitative characters does not show any defined groups (Figure 42), as it only assesses relationships within a single set of interdependent variables. This is regardless of any relationships existing to variables outside the data set (McGarigal *et al.* 2000). The first three components x, y and z explain 20 9%, 20 6% and 26.1% respectively of the total variance). This was substantially more and unresolved than for the principal coordinates analysis (PCOORD) (Figure 43), which was applied to the combined data matrix, demonstrating the importance of the binary characters in the analysis for the resolution of the taxa in three-dimension. The three-dimension diagram (Figure 43) derived from the PCOORD display two apparent groups in the X-axis of which the smaller groups constitute three *S. stenophylla* OTUs (Biesiesvlei, Sannieshof and Steynsrus), one *S. runcinata* (Lichtenburg) and *S. repens* (Lady Grey). The second group has a representation of all the taxa. The Z-axis defines three groups, with the majority of the taxa occurring in one, the second group having four *S. runcinata* OTUs and the third group has three OTUs. The first three axes account for 37.66% of the total variance. However, the two groups were clearly defined along the first principal axis (X), which accounts for 17.53% of the total variation. *S. repens* overlaps with most of the *S. runcinata* with one of its OTUs grouping with *S. stenophylla*. This could imply that *S. repens* is phenetically similar to *S. runcinata*, which is contrary to the cluster analysis. Characters (major compounds that are above 5%) in the first principal axis that had eigenvectors more than 1.0 include tricyclene, camphene, β-pinene, δ-3-carene, limonene, β-phellandrene, *p*-cymene, camphor, isocaryophyllene, β-caryophyllene, aromadendrene, α-humulene, α-terpineol, *p*-cymen-8-ol, *cis* carveol, caryophyllene-oxide, humulene epoxide, spathulenol, α-eudesmol, β-eudesmol, τ-cadinol, α-bisabolol, caryophylladien and caryophyllenol-II. The second principle axis contributes 16.23% of character variation and the third axis contributes 3.9%.

The variability in yield and chemical composition of the essential oils in the Lamiaceace is attributed to the contribution of genetic and phenotypic variation as well as extrinsic factors

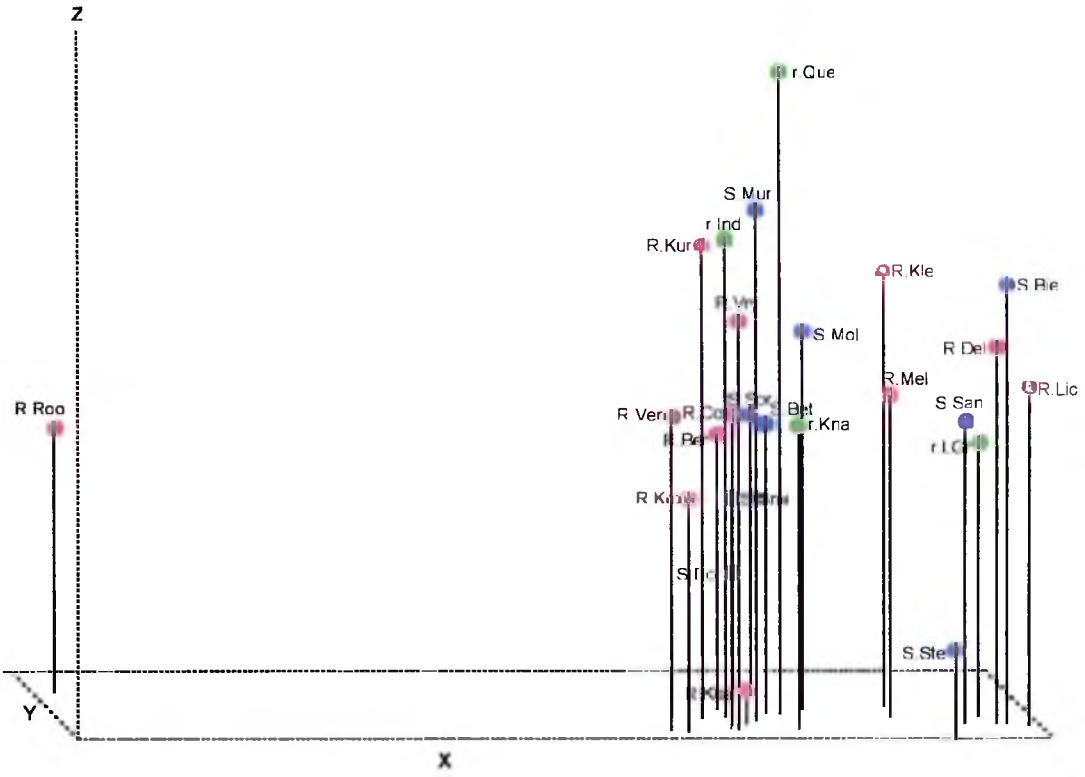


Figure 42. Three dimension plot of PCA of the quantitative data matrix

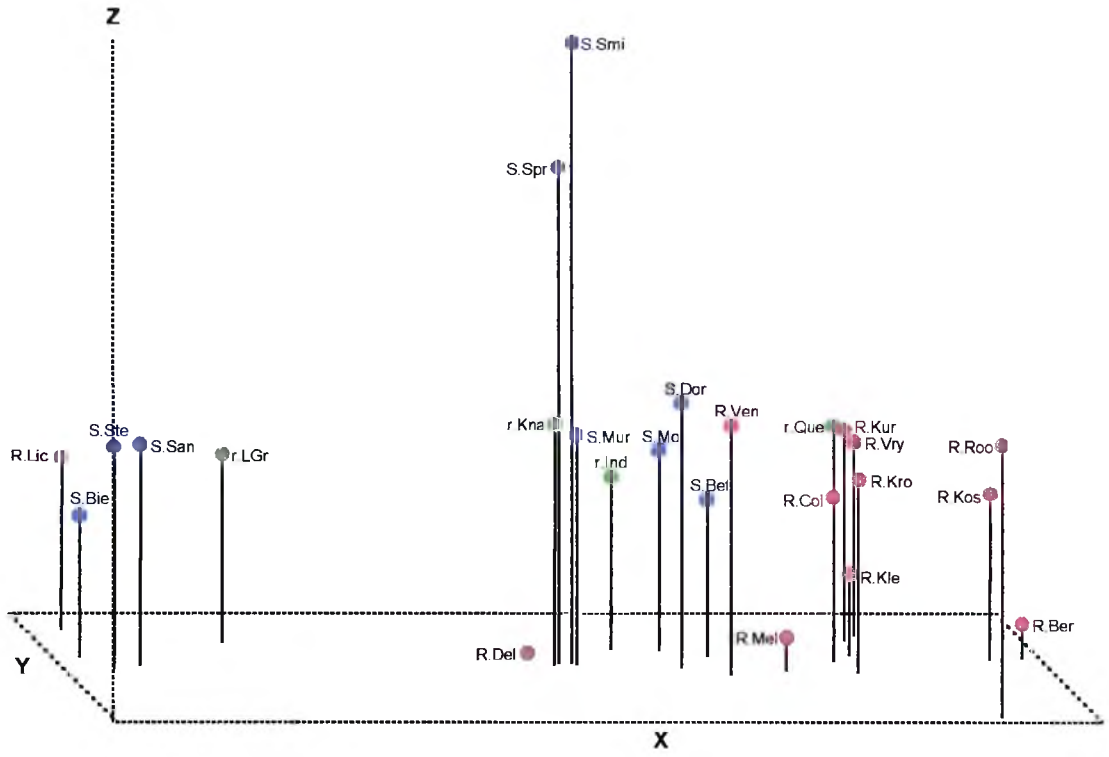


Figure 43. Three dimension plot of PCOORD of the combined data matrix.

like soil and climate (Palic *et al.* 1982; Skoula *et al.* 2000; Yayi *et al.* 2001). This can be noted in the qualitative dendrogram (Figure 41) where the taxa collected in the same geographical area emerge from the same branch of similarity as in the case of *S. stenophylla* from Biesiesvlei and *S. runcinata* from Lichtenburg. However, *S. stenophylla* from Sannieshof (crossing Harts River), a similar habitat as that of Biesiesvlei clusters with the rest of the *S. stenophylla* OTUs (Figure 41). Morphological observations of the three taxa (Biesiesvlei, Lichtenburg and Sannieshof OTUs) suggest that the plants from Biesiesvlei are very similar to those from Sannieshof and yet quite different from the Lichtenburg plants. *S. stenophylla* from Biesiesvlei and *S. runcinata* from Lichtenburg show no degree of dissimilarity (Figure 40). The clustering of these two taxa can only be attributed to the same environmental and climatic conditions. However, the clustering of these two taxa with *S. repens* is strange and environmental differences seem not to have any influence on the essential oil composition of this cluster (A_1). Indwe, Lady Grey, Knapdaar and Queenstown are in the Free State and Eastern Cape, mostly a temperate plateau with cool rainy summers and cold dry winters, while Biesiesvlei and Lichtenburg are in the North-West Province a rather semi-arid interior plateau with hot rainy summers and cool dry winters (Vlok and van der Merwe 1994). Since the plants are of different species and they were exposed to different environmental conditions, their genetic make-up and quantity of chemical compounds should have been different.

The genetic make-up determines the particular enzymes which catalyse the synthesis of characteristic compounds. The differences in the relative composition of say limonene found in *S. stenophylla* from Biesiesvlei (19.34%), compared to other populations (Sannieshof - 5.29%, Smithfield - 0.64%, Steynsrus - 6.08%) may suggest modulation by the environment in terms of region and the season of harvesting. Change in environmental conditions such as temperature induce particular interconversions at the precursors level and enhance the activity of some of the coexisting isoenzymes, with different optimal temperatures, that compete for the same substrate (Gil *et al.* 2000). Therefore, a variation in chemical composition of plants from different localities is expected and thus great plasticity occurs within the taxon. However, the results of the cluster analysis for qualitative data are remarkably consistent with the *a priori* assignments of collections to the different taxa according to the delimitation of Codd (1985). This implies that the enzymes involved in the synthesis of the chemical compounds responsible for the clustering of the taxa in each of them are different but specific to the taxon (Agnese *et al.* 1999). In addition, Hoelscher *et*

al. (2003) reports that one enzyme (recombinant synthase) catalyses the divalent metal ion-dependent conversion of geranyl diphosphate to (+)-3-carene and to lesser amounts of limonene, myrcene, 4-carene and β-phellandrene, suggesting that this single enzyme is responsible for the production of all the monoterpenes in *S. stenophylla* essential oil. This strongly supports the use of qualitative data to chemically group members of the *S. stenophylla* species complex. In addition, the observation of the similarities in the essential oil compositon of the *S. runcinata* plant material collected from Coligny and the cultivated plant material xxxx. However, it is also accepted that the definition of infraspecific chemotypes may well be the result of the presence or absence of a particular biosynthetic pathway and not the single presence or absence of a particular compound (Kokkini 1992). Thus reliable chemical characters are those that are exclusive to specialist groups and primary compounds that are always present, and those typical of higher taxonomic levels have little value in separating taxa (Grace *et al.* 2002).

Many studies have, however, shown how genetic and phenotypic variation (differences in ecological conditions) contribute to differences in the concentration of volatile terpenes (monoterpenes) and the magnitude of these differences varies between populations because natural populations exhibit heterogeneity and hence are composed of plants with different chemotypes (Palić *et al.* 1982; Echerrigaray *et al.* 2001). This has been observed in the major components of *Hyssopus officinalis* oil (hyssop oil), which are said to be biosynthetically related (Lawrence 1992). Yayi *et al.* (2001) attributed the chemical composition variation of *Ocimum basilicum* of different regions to different times of harvesting. In *Thyme vulgaris*, genetic constitution (RAPD analysis) and environmental conditions influenced the yield and composition of volatile oils (Echeverrigaray *et al.* 2001). However, in the same study, it was found that variation in wild *Salvia fruticosa* plants from three populations was accompanied by population-related differences in genetic profiles generated by RAPD analysis. While clary sage oil (*Salvia sclarea*) was found not to have any infraspecific chemical variations like most Lamiaceae species (Lawrence 1992). The only major difference between the oils from different populations was in the linalool/linaly acetate contents and the main reason for this difference was ascribed to the state of the plant material on distillation. Venskutonis and Matte Larsen (1996) report a reduction in the total content of essential oils after drying the plant, except for β-caryophyllene and thymol, which increased in concentration. For instance, in the USA, the

plant material is distilled a few hours after collection, whereas in France and USSR, the harvested plant material is air-dried prior to distillation (Lawrence 1992). The excess moisture, which is associated with processing of fresh plant material, causes the major constituent, linalyl acetate, to hydrolyse to linalool like in the case of the formation of sylvestrene from car-3-ene. This according to Banthorpe (1998) is as a result of an increased pH of the plant material during distillation due to the absence of a buffer. This was observed in our samples that were stored in the freezer awaiting microdistillation. The total essential oil yield dropped in the microdistilled samples in comparison to the hydrodistilled samples.

Our data could thus suggest that *S. runcinata* collected from Lichtenburg could be identified as a hybrid of *S. stenophylla* or vice versa. While Biesiesvlei sample has 75 compounds identified, Lichtenburg sample has 70 compounds. Hybrids generally have an 'additive' pattern of secondary chemical compounds (Briggs and Walters 2000), which was exhibited in the chemical compositions of the two samples. The presence and absence of a chemical compound is therefore a reliable taxonomic character, unlike the quantity of the chemical compounds, which is greatly influenced by environmental factors. This has been demonstrated by the quantitative and combined character phenograms (Figures 40 and 41). The *S. stenophylla* complex presents a taxonomic problem because the morphological characters used to delimit the species overlap and so is the distribution which is also continuous. Furthermore, the failure of the cluster analysis to produce a coherent or consistent classification of the quantitative and combined data is not surprising because cluster analysis can distort relationships among clusters (Sneath and Sokal 1973; Cormack 1971 as cited by Shaw 1998; Nowak 2002). This is exhibited in the loss of the close relationship between the taxa. Since it seeks clear divisions within the dataspace, it is not suited to partitioning data such as the results presented here which approximate to a smooth multivariate continuum. However, McGarigal *et al.* (2000) states that variables can either be continuous or categorical because there is no consensus on the validity of mixed data sets. The qualitative data matrix, however, omits a lot of information ($r = 0.752$) that the quantitative data carries, but it is the most natural approach of generating the data matrix for analysis.

3.4.6 High performance liquid chromatography

The results of the HPLC/UV/MS analyses show some degree of chemical variation of the *S. stenophylla* species complex. Five compounds (Figure 44) were identified; these include

carnosic acid, carnosol, isocarnosol, oleanolic acid and rosmarinic acid. The two standards (rosmarinic acid and carnosic acid) were identified by co-injection at 22.179 and 36.027 retention time (Rt) respectively (Figures 46A and 46H).

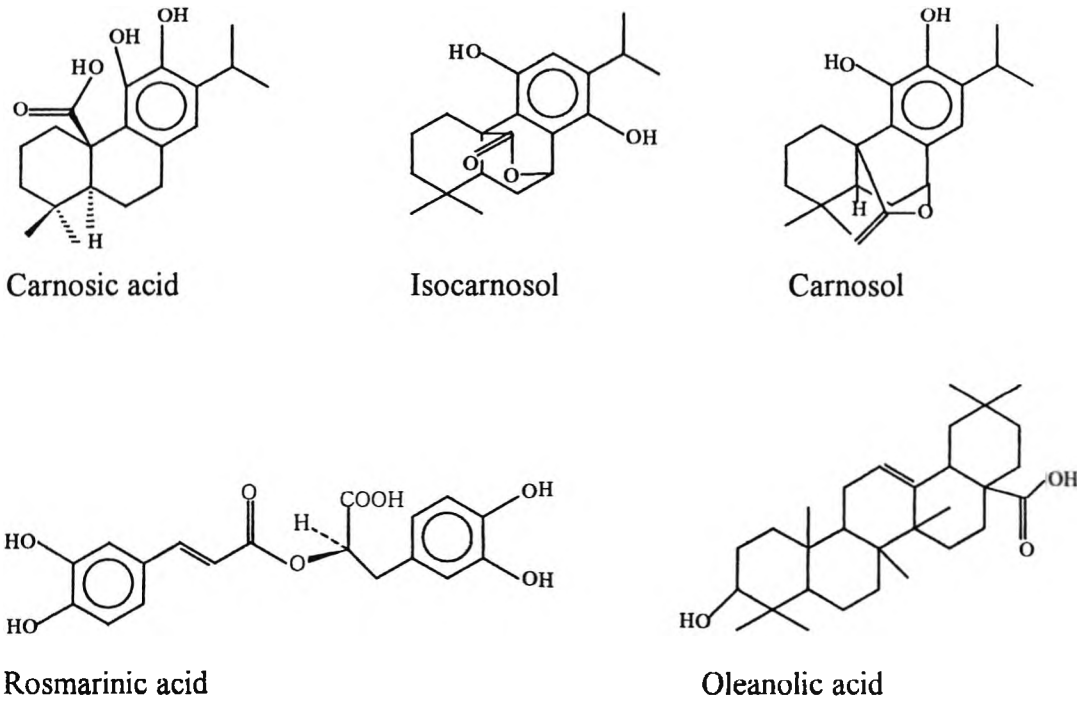


Figure 44. Five compounds identified in the methanol extracts of *S. stenophylla*, *S. runcinata* and *S. repens*.

For the nine crude extracts, rosmarinic acid occurred in all the taxa examined (Figure 45), more in *S. runcinata* and *S. repens* (except for the extract obtained from Klerkskraal Dam and George). The carnosic acid concentration was high in *S. stenophylla* samples from Molteno and Sannieshof, while the Biesiesvlei sample was devoid of carnosic acid, but accumulated high concentrations of compounds at 30.5 and 33.6 retention time, and at medium concentration of compounds at 31.5 and 36.8 (carnosol derivatives) retention times. *S. repens* samples accumulated between low to high concentration of carnosic acid and only one *S. runcinata* sample from Roossenekal and Vryburg accumulates the compound at low concentration. *S. stenophylla* (Biesiesvlei) and *S. runcinata* (Klerkskraal Dam) only have the carnosol derivatives with retention times 36.77 and 34.03 respectively (Table 11). A compound predicted to be oleanolic acid (UV and MS) was also detected at retention time 37.93, and it occurred in medium concentration in *S. stenophylla* and *S. repens* samples. The compounds that have been identified are reported to have strong antioxidant properties, which concur with the TLC-DPPH results, which portrayed having additional antioxidant

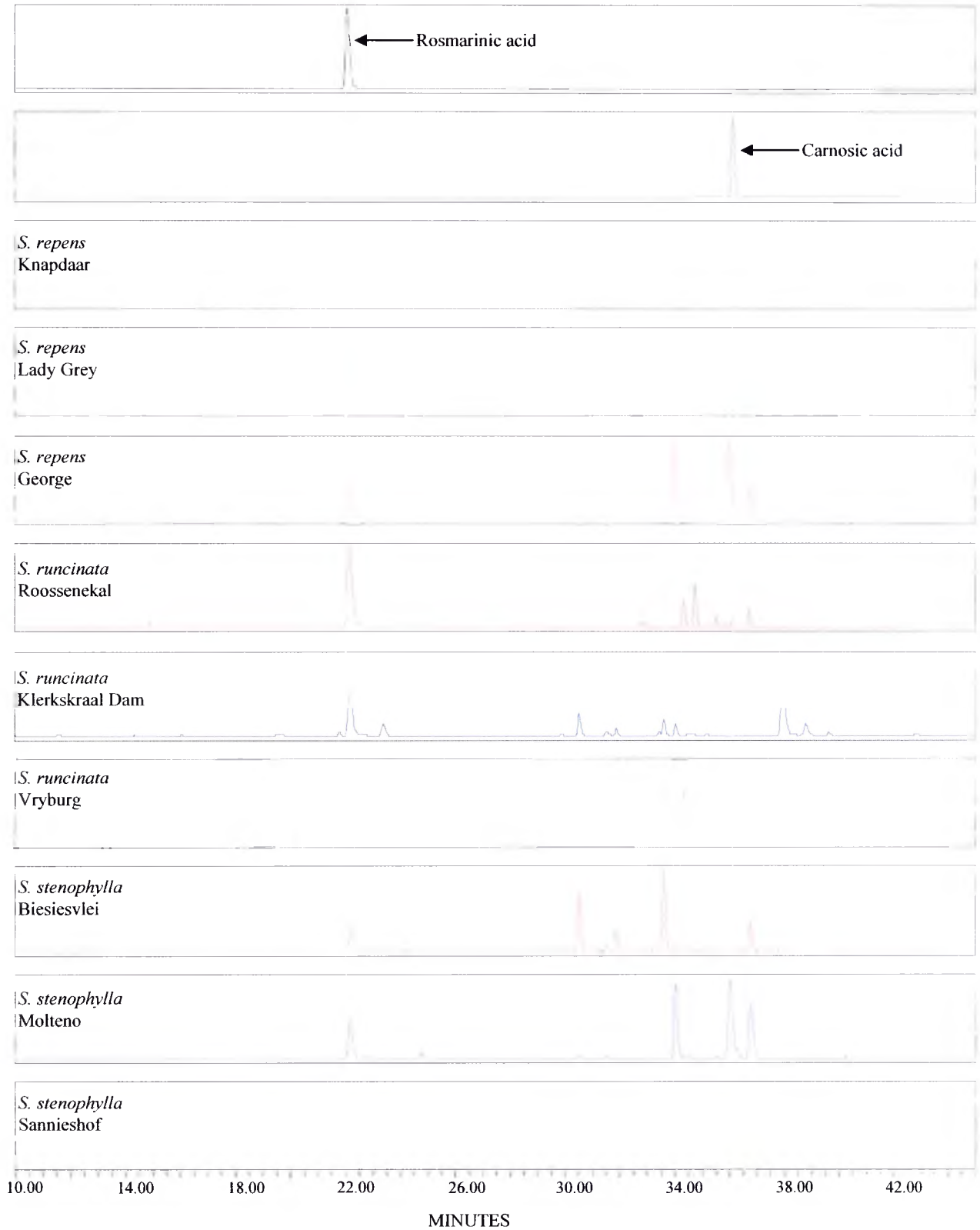


Figure 45. HPLC/UV chromatograms of *S. stenophylla, S. repens* and *S. runcinata* and HPLC profiles of rosmarinic acid and carnosic are also shown.

Table 11. HPLC/UV/MS results showing the chemical variation existing between extracts of *S. stenophylla*, *S. repens* and *S. runcinata*.

Retention time		**22.18**	**24.77**	**30.51**	**31.51**	**33.61**	**34.03**	**34.73**	**36.03**	**36.77**	**37.93**
UV (λmax)		324 327	327	205 287	215 283 333	207 286	282 287	280 425	282	282 285	232
Taxon	Locality						Cd			Cd	
S. stenophylla	Biesiesvlei	++		+++	++	+++				++	
S. stenophylla	Molteno	++	+	+	+		+++		+++	+++	++
S. stenophylla	Sannieshof	++		+	+	+	++		+++	++	++
S. repens	Knapdaar	+++	++				++		+	+++	++
S. repens	George	++					+++		+++	++	
S. repens	Lady Grey	+++					++		+	+++	++
S. runcinata	Klerkskraal Dam	++		++		+	+				+
S. runcinata	Roossenekal	+++					++	++	+	++	+
S. runcinata	Vryburg	+++					+++	++	+	+	+
Standard	Rosmarinic acid	✓									
Standard	Carnosic acid								✓		

\+ = low concentration ++ = medium concentration +++ = high concentration cd = carnosol derivative

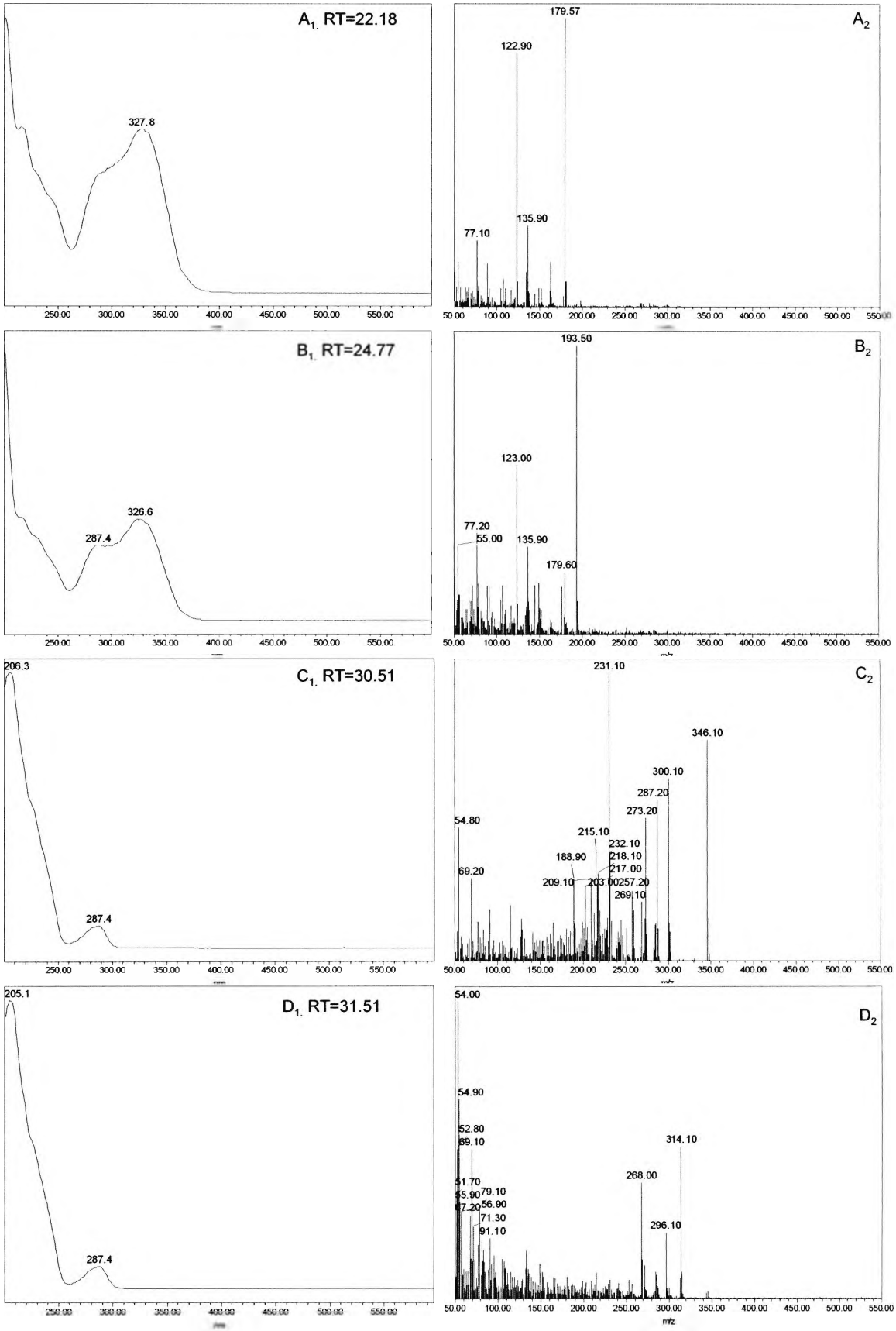


Figure 46. UV spectra and their corresponding MS data for compounds listed in Table 11.

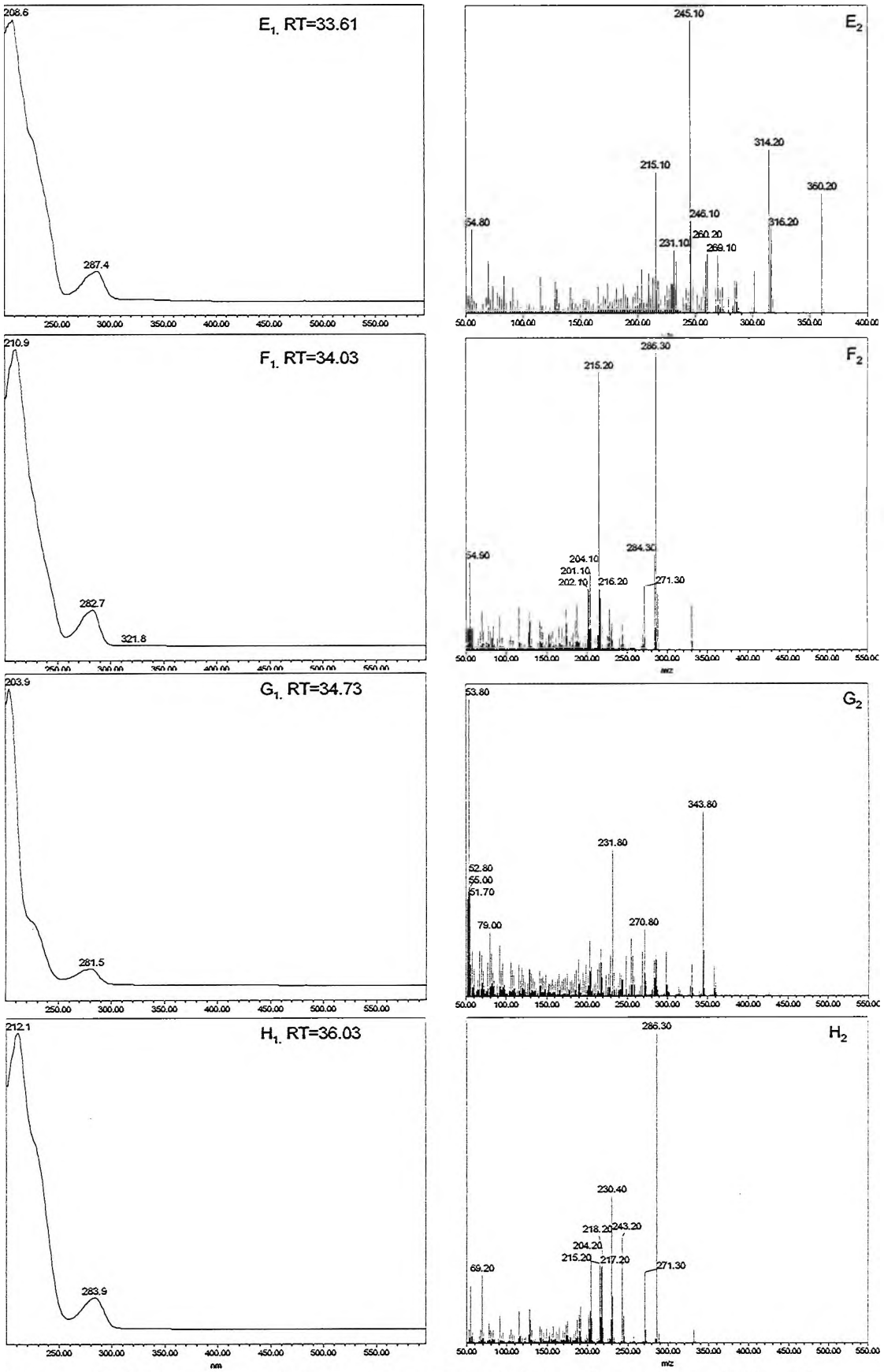


Figure 46. UV spectra and their corresponding MS data for compounds listed in Table 11.

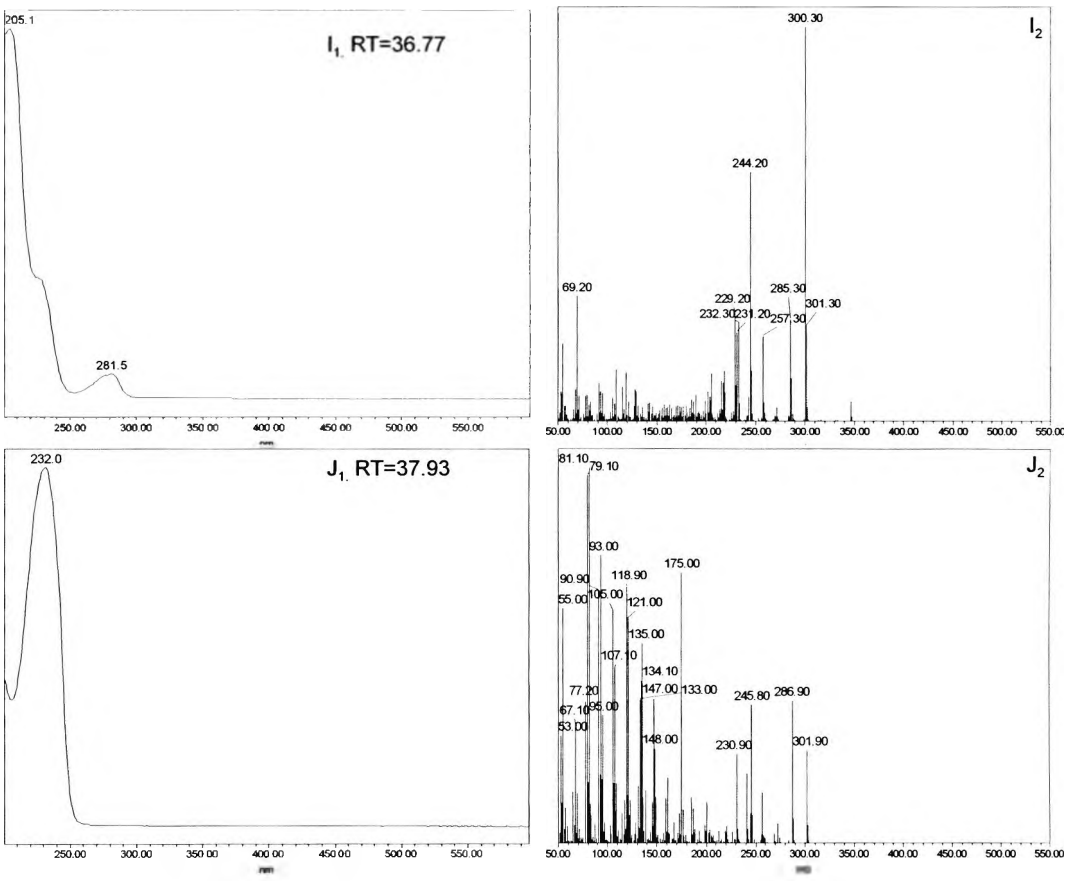


Figure 46. UV spectra and their corresponding MS data for compounds listed in Table 11.

compounds apart from rosmarinic acid. The occurrence of both carnosic acid and other carnosol derivatives could be as a result of incomplete biosynthethic processes of the compounds' conversion to stable compounds. For instance carnosic acid can enzymatically be converted to carnosol because it has very unstable moieties, which readily convert to carnosol (Munné-Bosch *et al.* 2000). Carnosic acid is the starting element of the 'carnosic acid cascade', a series of chemical reactions that involve the transformation of carnosic acid into carnosol, then rosmanol, and finally galdosol. With each of these transformations, free radicals are quenched. Carnosic acid is a precursor of phenolic diterpenes featuring γ- and δ-lactone structures *in vitro* (Wenkert *et al.* 1965 as cited by Munné-Bosch *et al.* 2000). Other studies that have reported isolating and identifying carnosic acid have always identified the presence of carnosol and it derivatives. For example; in *Salvia officinalis* where ursolic acid was the major compound (Baricevic *et al.* 2001), carnosic acid and carnosol were shown to be the major phenolic diterpenes in leaves of *Rosmarinus officinalis* and *Salvia officinalis* (Munné-Bosch *et al.* 2000). Carnosol and isocarnosol among other abietane diterpenes have also been isolated from the roots of *Salvia officinalis* and aerial parts of *Salvia lanigera* Poir, deoxocarnosol has been isolated from the roots of *Salvia canariensis* (Nuñez and De Castro 1992; Rodríguez-Hahn *et al.* 1992). Other species are reported to have the final compound in the 'carnosic acid cascade', galdosol and such include *Salvia lavandulifolia* Vahl. and *S. canariensis* L (Rodríguez-Hahn *et al.* 1992). The occurrences of carnosic acid and other carnosol derivatives explain why *S. stenophylla* and *S. repens* have more antibacterial activity than *S. runcinata*. In addition to rosmarinic acid and carnosol and/or isocarnosol, other non-volatile compounds were found in the HPLC chromatogram of the examined taxa. The retention times of the compounds are listed in Table 11 and the corresponding UV and MS profiles of the compounds are shown in Figure 46.

The presence and absence of a particular compound seems to follow a predictable pattern, except for a few taxa like *S. stenophylla* from Biesiesvlei and *S. runcinata* from Klerkskraal Dam. Unlike the essential oils, the distribution of non-volatile diterpenes is constant from population to population in the sampled plants displaying chemical homogeneity. There are however, some factors that cause quantitative chemical variations. Nuñez and De Castro (1992) pointed out that predation pressure among aromatic plants is one factor among others that contribute to the synthesis of different related compounds. Other factors include;

(a) Seasonal variation or variations in growing conditions from specimen to specimen, such as solar radiation and water content. The lower concentration of rosmarinic acid in *S. stenophylla* as compared to *S. runcinata* and *S. repens* could be because the samples collected occur in areas with seasonal low precipitation and high solar radiation. Munné-Bosch *et al.* (2000) reports how the highest concentrations of the major diterpenes carnosic acid and carnosol were found during winter and the lowest concentration during summer. High water content increased the concentration of carnosic acid while increased solar radiation and temperature reduced the concentration.

(b) Changes in the chemical contents in also associated with the way the plant material is treated prior and during storage.

(c) Compound deterioration due to long-time storage. In this study, the non-volatile compounds were extracted from plant material that dried progressively in uncontrolled conditions. Pedersen (2000) reports how in Verbenaceae, the concentration of rosmarinic acid was 90% of the total compounds in freshly collected specimens and only 50% in dried specimens under controlled conditions. In most studies, the plant material is freeze-dried or nitrogen dried to reduce the likelihood of temporal changes that may occur.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLPS)

3.5.1 DNA extraction

Extraction from 20 plants out of a total of 30 yielded high concentrations of DNA. Evidence for the success of the extractions is shown in Figure 47 by the well-defined bands of high molecular weight DNA, which were visible on the 1% agarose/ethidium bromide gel. The remaining ten extracts did not yield similar quantities of DNA (Figure 47b). However, AFLP banding patterns are not particularly sensitive to the initial concentration of the template DNA (Vos *et al.* 1995; Zhu *et al.* 1998 as cited by Qamaruz-Zaman *et al.* 1998).

3.5.2 AFLP fingerprinting

As a result of the initial primer trial one primer combination (EcoR1-ACT/ Tru9-CAA) was applied to all the samples under study. The selection of primer pairs is usually based on lack of background "noise" and the presence of variation between the taxa being studied (Fay *et al.* 2002).

The resulting binary matrix consisted of 307 characters ranging in size from 50 to 500 base pairs. AFLP profiles were successfully generated from 13 of the 30 taxa (four *S. stenophylla*, six *S. repens* and three *S. runcinata*). Time constraints limited the completion of the matrix for all 30 samples. Of the 307 bands scored 197 were unique (autoapomorphic) and no bands were shared by all 13 individuals.

3.5.2.1 UPGMA analysis

From the UPGMA dendrogram (Figure 48), two major clusters can be identified. One cluster (A), is comprised of the *S. repens* individuals, except for the sample collected from Indwe. These samples occur in the same geographical area. The other cluster (B) is characterized by *S. runcinata* and *S. stenophylla* samples. Within this cluster, *S. runcinata* individuals (1 and 2) from Coligny cluster together, then *S. stenophylla* individual (1) from Sannieshof clusters with Biesiesvlei individual (2), while *S. runcinata* (Bergville) clusters with *S. stenophylla* (1) from Biesievlei. Individuals that occur in the same geographical area also characterize cluster B. However, the clustering of *S. stenophylla* (Biesiesvlei) with *S. runcinata* (Bergville) is more difficult to explain because their clustering is not supported by their geographic location. *S. repens* was clearly the most distinct taxon in the analysis.

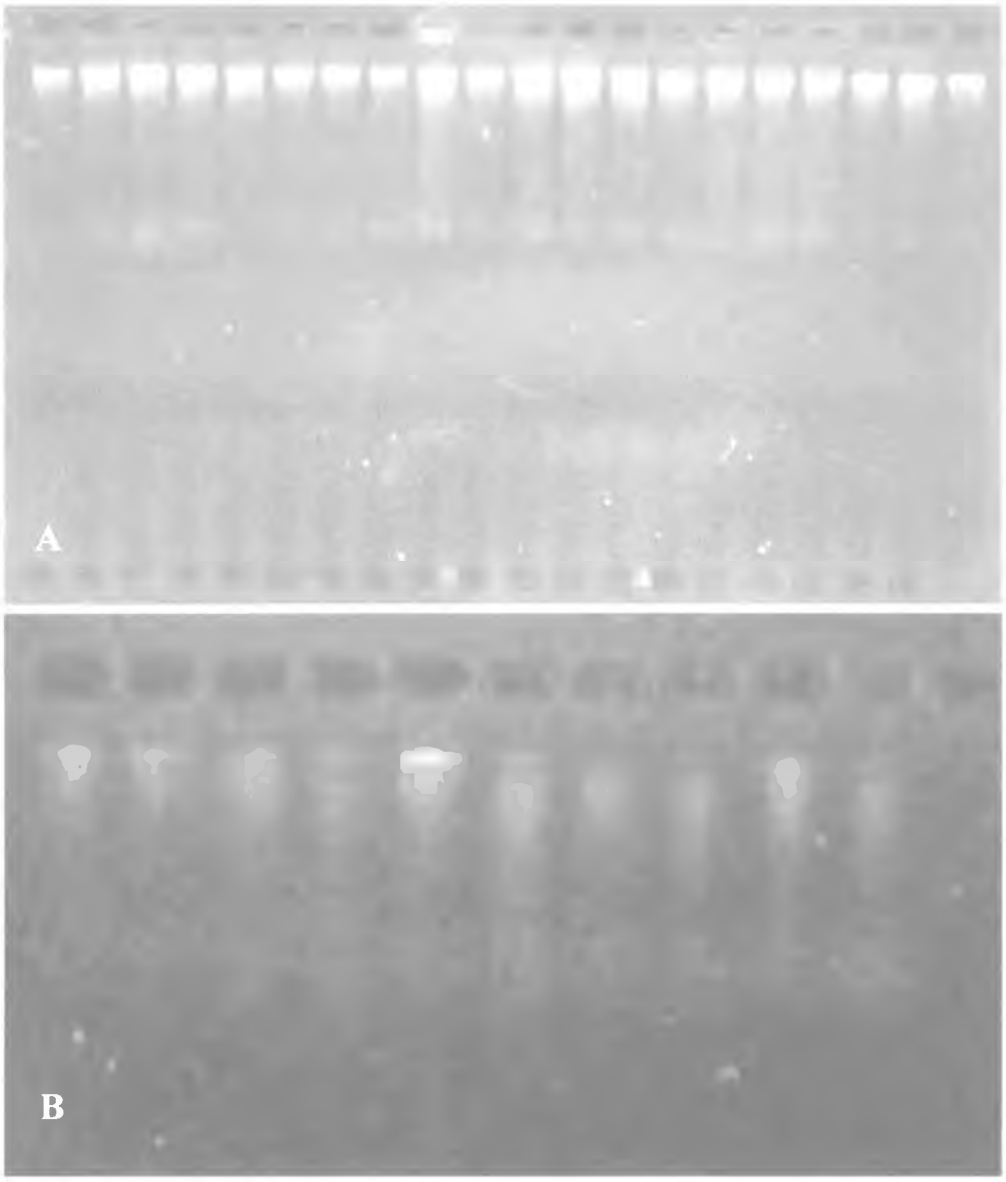


Figure 47. **A**. Bands of high molecular weight DNA extracted from 20 plant samples. **B.** Bands of high molecular weight DNA from 10 samples.

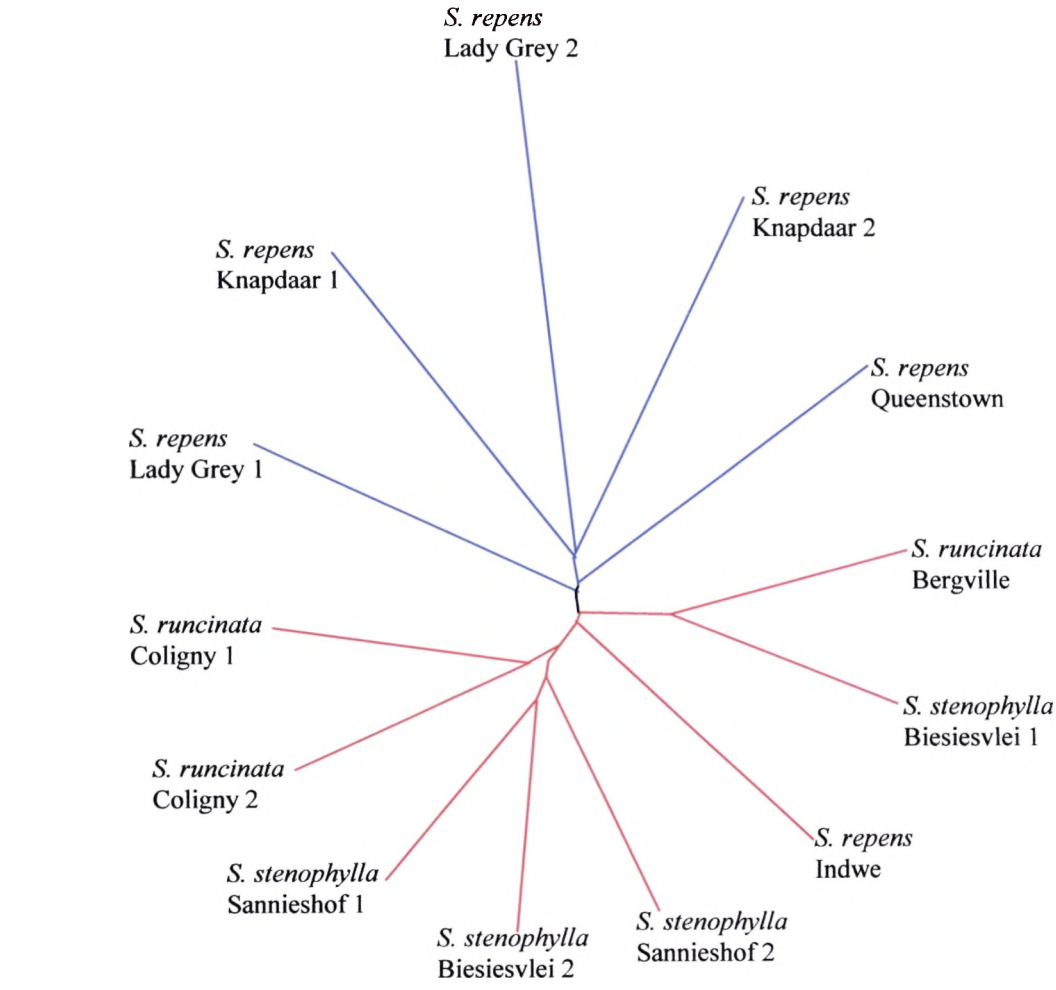


Figure 48. AFLPs unweighted pair group method using arithmetic averages (UPGMA) dendrogram.

_____ = cluster A

_____ = cluster B

3.5.2.2 Neighbour joining (NJ) analysis

Like the UPGMA analysis, two major clusters were also retrieved in this analysis even though different constituents characterized them (Figure 49). Cluster A comprises seven individuals of *S. repens* (except for the individual from Knapdaar), *S. runcinata* (Bergville) and *S. stenophylla* (Biesiesvlei). Cluster B is characterised by a mixture consisting of *S. repens* (Knapdaar), the two Coligny individuals of *S. runcinata* and *S. stenophylla* from Biesiesvlei and Sannieshof. The dendrogram does not show a systematic pattern of taxa relatedness, for instance, it shows that samples collected from the same locality are polyphyletic as in the case of *S. stenophylla* samples (1 and 2) collected from Biesiesvlei and *S. repens* 1 and 2 collected from Knapdaar. There is a preliminary indication that the level of genetic variability is much higher in the *S. repens* individuals.

The AFLP technique is proficient at revealing diversity at and below the species level and providing an effective means of covering a large portion of the genome in a single assay (Qamaruz-Zaman *et al.* 1998). *S. repens* individuals are coming out as a defined unit in both analyses while individuals of *S. stenophylla* and *S. runcinata* are intermixed. This pattern may suggest that hybridization is occurring among individuals of these two species, which is feasible due to their close proximity geographically. In this respect our molecular data set is congruent with the morphological characters data and Codd's (1985) conclusion that *S. runcinata* is the closest ally to *S. stenophylla*. This is, however, contrary to the results observed in the dendrogram generated by qualitative essential oil data, which suggests that *S. repens* is most similar to *S. stenophylla*. The possibility of hybridization among individuals of *S. stenophylla, S. repens* and *S. runcinata* is supported by the erratic chemical variation existing between the species and our inability to find species-specific markers for each of these taxa. Rather the large number of markers in common among the individuals of these species has resulted in their intermixing in the dendrograms generated and presented here. However, the qualitative chemical evidence suggested that *S. runcinata* represents a more coherent taxonomic entity, and this is not supported by the AFLP data. Instead the AFLP data represents *S. repens* to be a more distinct unit genetically. Ideally this study would benefit from more detailed sampling, both of individuals and further AFLP markers (which could be generated with the use of alternative primer combinations). However, the preliminary evidence presented here suggests that species delimitation of *S. stenophylla* and *S. runcinata* is problematic. Based upon chemical and genetic data the three taxa may not deserve status as distinct species. It would appear that to bioprospect efficiently

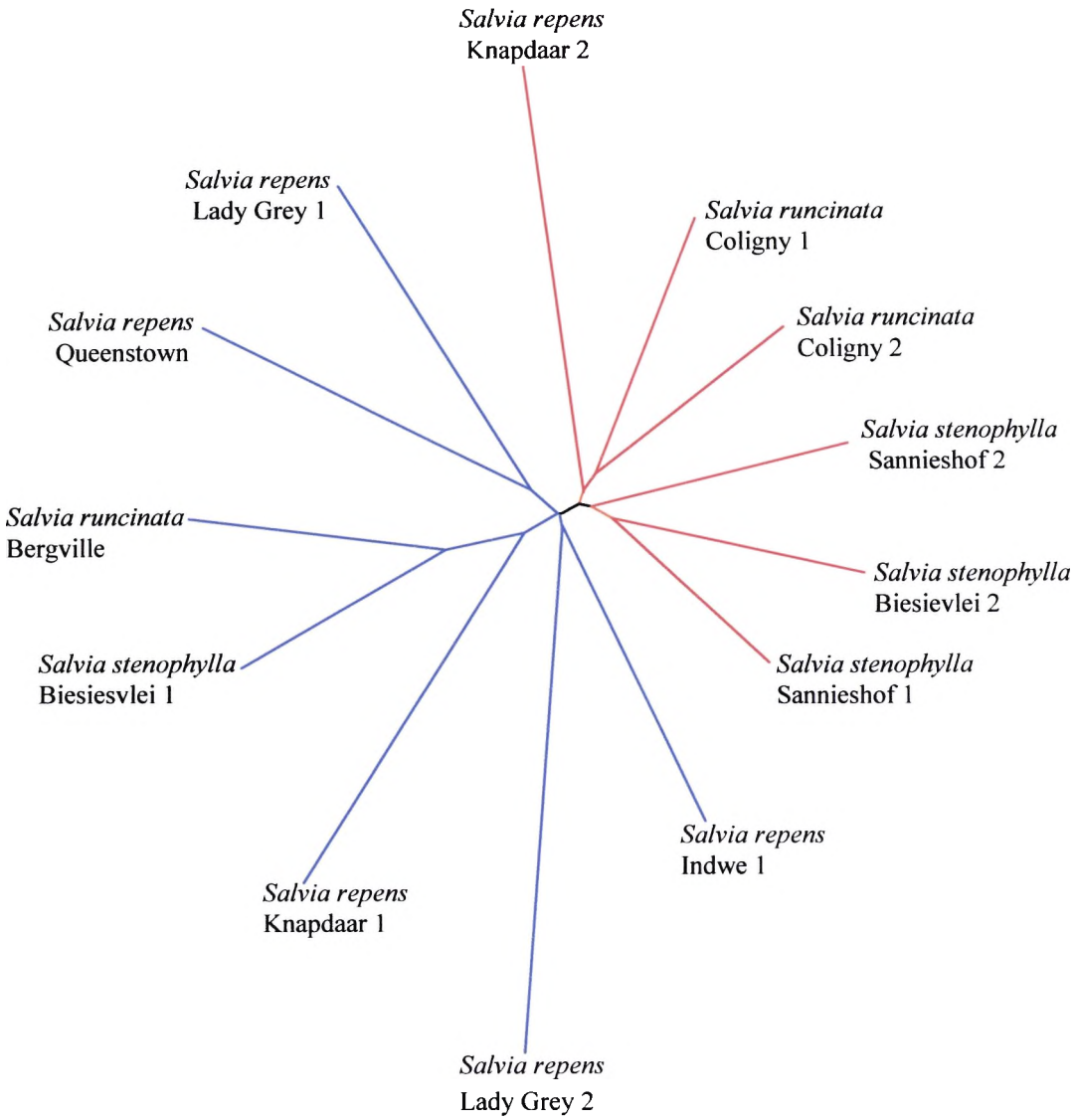


Figure 49. AFLPs Neighbour Joining (NJ) dendrogram.

_____ = cluster A

_____ = cluster B

from this complex of species the taxonomy of the group requires further attention to solve the problems of species delimitation.

BIOLOGICAL ACTIVITY

3.6.1 Antimicrobial activity

3.6.1.1 Disc diffusion

3.6.1.1.1 Volatile compounds

All gram-negative bacteria, yeasts and the moulds showed resistance to the methanol and essential oil extracts of *Salvia stenophylla*, *S. repens* and *S. runcinata*; hence we did not include the results in the Tables 12 and 13. The essential oils showed either very poor (up to 1 mm inhibition) or no activity against all the microorganisms that were screened, with an exception of three *S. runcinata* essential oils collected from Klerkskraal Dam, Koster and Vryburg and three *S. stenophylla* essential oils collected from Dordrecht, Sannieshof and Smithfield (Table 12). These had an activity of 2 mm against *B. cereus* (ATCC 11778) and *B. subtilis* (ATCC 6051).

Essential oils are very complex mixtures of compounds, which constitute mainly monoterpenes, sesquiterpenes (Shane *et al.* 1999; Svoboda and Hampson 1999) and diterpenes (Nakatsu *et al.* 2000) with the general formula $(C_5H_8)n$. These constituents have been shown to have antimicrobial activity in terms of partial or complete inhibition of growth to bactericidal activity (Shane *et al.* 1999). It is worth noting that biological activities could be due to single compounds in a very complicated concert of synergistic or antagonistic activities (Buchbauer 1993; Svoboda and Hampson 1999; Dorman and Deans, 2000; Nakatsu *et al.* 2000). Thus testing and evaluating of the antimicrobial activity of essential oils is quite difficult because of many factors (Janssen *et al.* 1987). With regard to this statement, essential oils of *S. stenophylla, S. runcinata* and *S. repens* had very little or no activity at all against the microorganisms that were screened. The *S. stenophylla* species complex oils contain more monoterpenes such as; α-pinene, β-pinene, γ-terpene and limonene (hydrocarbon terpenes), δ-3-carene and *p*-cymene, and these are reported to exhibit little activity (Shane *et al.* 1999). However, the single compounds in highest concentration are sesquiterpenes (α-bisabolol, (*E*)-nerolidol and ledol) for some samples. Nakatsu *et al.* (2000), reviews how essential oils rich in hydrocarbons obtained from *Salvia gilliessi*, *Xylopia aromatica* and *X. longifolia* are active against fungi and bacteria. Most of the essential oil components have low water solubility but are highly antiseptic (Shane *et al.* 1999). The test method used for assessment of antimicrobial activity is often the disc

diffusion method, which is highly dependent on water solubility and the ability of test components to diffuse through agar (Southwell *et al.* 1993 as cited by Shane *et al.* 1999). Thus, it would be expected that compounds of lower water solubility such as essential oils would show less activity even if solubility did not affect their activity in other situations. These compounds have lower H-bonding capacity and are less soluble in water such that they do not dissolve and diffuse through the seeded agar. However, these two molecular properties do not entirely account for all the trends of inactivity (Shane *et al* 1999). Janssen *et al.* (1987) adds that the hydrophobic nature of essential oils and some plant extracts prevents the uniform diffusion of the compounds through the agar medium, and because of this inability to donate a lone pair of electrons in a hydrogen bond situation, the compounds were completely inactive against gram-negative bacteria. Shane *et al.* (1999) also states that geometric isomerism of the compounds has a large effect on antibacterial activity as in the case of geraniol and nerol.

A common feature of terpenoids is isomerism and pairs of isomers can be isolated from either the same plant extract or different plant extracts. This complicates the isolation and identification process of the compounds because terpenoids are mostly alicyclic and the stereochemistry of the cyclic terpenoids is highly involved and often difficult to determine (Harbone 1998). For example an isomer of α-bisabolol, (+)-*epi*- α-bisabolol occurs in *S. stenophylla*, while other isomers have been isolated in different plant species, like (-)-*epi* α-bisabolol occurs in *Myoporum crassifolium*, (-)-α-bisabolol occurs in *Matricaria chamomilla* and (+)-α-bisabolol accumulates in *Populus balsamifera* buds (Brunke and Hammerschmidt 1985). From a practical point of view, it must be remembered that both isomerization and structural rearrangement within the molecule may occur quite readily under relatively mild conditions and artifact formation is always possible during isolation procedures (Banthorpe 1998).

Different functional groups have an effect on antibacterial activity. For instance, an alcohol functional group is crucial for activity against *E. coli*, *S. aureus* and *C. albicans* (Knobloch *et al.* 1989). Viljoen *et al.* (2002) showed that alcohols are generally more active than ketones and aldehydes against a number of organisms, including *S. aureus*. It is also reported that α-isomers are inactive relative to β-isomers and *trans*-isomers are more active than the corresponding *cis*-isomers (Dorman and Deans, 2000; Viljoen *et al.* 2002).

The presence of sesquiterpenes could also contribute to the inactivity of essential oils as they may not possess antimicrobial properties and yet they are in high concentration as single compounds. A large percentage of *S. runcinata* essential oils contain (*E*)-nerolidol in high concentration. Nerolidol was found to have active antifungal properties after being isolated and identified from *Ocotea usambarensis* Engl. (Lauraceae) by TLC bioautographic assay against *Cladosporium cucumerinum*. Other studies of *Salvia* species have, however, shown conflicting results on the activity of essential oils on gram-positive and gram-negative bacteria. For instance, the essential oil of *Salvia ringens* (Tzakou *et al.* 2001) showed activity against gram-negative bacteria (*E. coli, Enterobacter cloacae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans, Candida vaginalis* and *Torulopsis glabrata*), but were inactive against gram-positive bacteria (*S. aureus* and *S. epidermidis*). This activity was attributed to monoterpenes (α-pinene and 1,8-cineole) that were in high concentration. Brandi *et al.* (1996) reports that oils rich in carvacrol (oregano and winter savory), 1,8-cineole (rosemary) and thujone (sage) were only able to delay the bacterial growth. In *Origanum scabrum* (Aligianns *et al.* 2001) strong antibacterial activity was exhibited against both gram-positive and gram-negative bacteria except for *Pseudomonas aeruginosa*, which has a high degree of resistance to many antimicrobial agents (Izzo *et al.* 1995). This activity is attributed to the main constituent of the essential oil, which is carvacrol (74.86%). Pure standards of carvacrol were also tested on cultures used on the test samples, and the results showed a similar activity. Jalšenjak *et al.* (1987) attributed the antibacterial activity to α- and β-thujone, compounds that were present in high concentration in sage oil. In another study (Ulubelen *et al.* 2000) of *Salvia viridis*, a diterpene (1-oxoferruginol) compound was found to be active against the gram-positive bacteria that were tested. In some genera, such as *Abies* (Fir), essential oils rich in monoterpenes were more active against yeasts than bacteria (Bağcı and Dığrak 1996).

There seems to be a trend that compounds in high concentration are also the active agents against microorganisms. This, however, is not the case because their action could be a result of the combined effect of more than one compound where one compound together with another contributes to increased antimicrobial activity. The inactive compounds might influence resorption, rate of reactions and bioavailabilty of the active compounds (Svoboda and Hampson 1999). Thus the chemical constituents may have synergistic effects. This agrees with Chalchat *et al.* (1997) who pointed out that correlating biological activity to

chemical composition should be tentative, because the involvement of less abundant compounds should be considered. The presence of trace components, even the unidentified, contributes say to the odour (manool at approximately 13% contributes to the woody odour of *S. stenophylla*), flavour and possibly the biological activity (Venskutonis *et al.* 1996; Svoboda and Hampson 1999) and to the whole constituent and nature of the essential oil. There is considerable variability on the effect of essential oils against microorganisms due to low solubility and high volatility in addition to their chemical composition which is determined by factors such as geographic origin, agronomic conditions, seasonal and climatic changes, harvest period, plant tissues and extraction techniques (Biavati *et al.* 1996).

Table 12. Antibacterial activity of essential oils measured by size of the inhibition zones in millimeters.

Essential oil (taxon)	Locality	Zone of inhibition (in mm from disc edge)					
		B. cereus ATCC 11778	*B. subtilis* ATCC 6051	*S. aureus* ATCC 25923	*S. aureus* ATCC 6538	*S. epidermidis* ATCC 2223	MRSA clinical strain
S. stenophylla	Bethulie	r	r	r	r	r	1
S. stenophylla	Biesiesvlei	<1	1	r	<1	r	r
S. stenophylla	Dordrecht	2	2	r	<1	r	r
S. stenophylla	Sannieshof	2	nd	r	<1	r	r
S. stenophylla	Smithfield	2	1	<1	<1	r	<1
S. stenophylla	Steynsrus	nd	nd	r	<1	r	r
S. repens	George	r	r	<1	<1	r	1
S. repens	Indwe	r	r	r	<1	r	1
S. repens	Knapdaar	r	r	<1	r	r	r
S. repens	Lady Grey	r	r	r	r	r	<1
S. repens	Queenstown	<1	r	r	r	r	r
S. runcinata	Bergville	1	1	<1	<1	<1	1
S. runcinata	Lichtenburg	1	<1	r	<1	r	r
S. runcinata	Coligny	1	1	<1	r	<1	<1
S .runcinata	Delareyville	nd	nd	1	<1	<1	<1
S. runcinata	Klerkskraal Dam	2	<1	<1	r	r	<1

Essential oil (taxon)	Locality	Zone of inhibition (in mm from disc edge)					
		B. cereus ATCC 11778	*B. subtilis* ATCC 6051	*S. aureus* ATCC 25923	*S. aureus* ATCC 6538	*S. epidermidis* ATCC 2223	MRSA clinical strain
S. runcinata	Koster	2	nd	<1	<1	r	1
S. runcinata	Kroonstad	<1	nd	<1	r	r	<1
S. runcinata	Kuruman	<1	nd	r	r	r	r
S. runcinata	Melville Koppies	1	<1	r	<1	<1	<1
S. runcinata	Roossenekal	1.5	1	r	r	<1	<1
S. runcinata	Ventersdorp	<1	<1	nd	nd	nd	nd
S. runcinata	Vryburg	1	2	r	r	<1	r
Neomycin 30 µg		6	5	6	5	6	5

*nd = not determined r = resistant

3.6.1.1.2 Non-volatile compounds

The methanol extracts of *Salvia stenophylla* inhibited growth of the gram-positive bacteria to a larger extent than *S. runcinata* and *S. repens*, which had variable activity (Table 13). The methanol extract of *S. stenophylla* from Sannieshof exhibited the highest antimicrobial properties against *Staphylococcus aureus* (ATCC 6538) with inhibition zone of 7 mm (Figure 50), while the essential oil produced an inhibition zone of <1 mm. In comparison to the control (Neomycin, 30 µg), the Sannieshof extract was more or equally active against all the test organisms (Figures 50a, 50b and 50d). The sample from Biesiesvlei was the second most active, while the sample from Bethulie was the least active against all the bacteria that were screened.

The methanol extract of *S. repens* collected from George recorded a high antibacterial activity of 5mm against *S. aureus* (ATCC 25923 and 6538), while that from Lady Grey showed high activity of 5 mm against *B. cereus* (Figure 51c). *S. repens* essential oil and methanol extract from Indwe exhibited no activity against all the microorganisms except for *S. aureus* (ATCC 6538) (Figure 52b), with activity of <1 mm for essential oil (Table 12) and *S. aureus* (ATCC 25923) with activity of <1 for the methanol extract (Table 13).

S. runcinata from Bethlehem showed the highest inhibition zone of 5 mm compared to the other *S. runcinata* samples, while other populations of *S. runcinata* showed little or no

activity at all against *B. cereus* (ATCC 11778). However, *S. runcinata* samples collected from Roossenekal and Vryburg showed considerable activity against all the gram-positive bacteria. The extract from Koster had no activity against almost all the microorganisms. There were different degrees of antibacterial activity for the methanol extracts depending on the taxa and the taxon's locality (Table 13). 1999). *Staphylococcus aureus* (ATCC 25923 and 6523) were the most sensitive organisms to the methanol extracts while *B. cereus* and *B. subtilis* were more sensitive to the essential oils and the rest were quite resistant. The methanol extracts of *S. stenophylla* and some of *S. runcinata* and *S. repens* showed remarkable antimicrobial activity against all the gram-positive bacteria screened, which are consistent with the pattern of *in vitro* inhibition emerging from other studies in which it was found that plant extracts and essential oils readily inhibit gram-positive bacteria rather than gram-negative bacteria (Izzo *et al.* 1995; Brandi *et al.* 1996; Meyer and Dilika 1996; Janssen *et al.* 1997). Baratta *et al.* (1998) however, states that the microorganisms' identity of being gram-positive or gram-negative has little influence on the sensitivity to the plant extracts, even though gram-negative bacteria tend to be more resistant than gram-positive bacteria (Rabe and van Staden 1997). The antibacterial properties of the methanol extracts in the present study could be attributed to the occurrence of rosmarinic acid, carnosic acid and carnosol, compounds that are reported to have strong antibacterial activity as well as antioxidant properties. For example, Lee *et al.* (1999) in their study on *Salvia miltiorrhiza* Bunge found that the ability of the compounds to generate superoxide radicals also inhibit the growth of bacteria by non-selective inhibitory action on DNA, RNA and protein syntheses. They report the antibacterial activity of the non-volatile terpenoids (Cryptotanshinone and dihydrotanshinone I) extracted and isolated from the roots (Dan Shen) of *Salvia miltiorrhiza*. While the site of action and some aspects of the mode of action of several terpenoids have been studied, actual structure-activity relationships of the terpenes are not well researched. It is known that carbonylation of terpenoids increases their bacteriostatic activity but not necessarily their bactericidal activity (Dorman and Deans, 2000). It has also been shown that terpenoids have different antiseptic potency, depending on their solubility in water (Shane *et al.* 1999).

Table 13. Antibacterial activity of methanol extracts measured by size of inhibition zones in millimeters.

Methanol extracts (taxon)	Locality	Zone of inhibition (in mm from disc edge)					
		B. cereus ATCC 11778	*B. subtilis* ATCC 6051	*S. aureus* ATCC 25923	*S. aureus* ATCC 6538	*S. epidermidis* ATCC 2223	MRSA clinical strain
S. stenophylla	Bethulie	2	2	2	3	4	4
S stenophylla	Biesiesvlei	6	6	6	5.5	6	5
S. stenophylla	Molteno	5	4	5	4	5	4
S. stenophylla	Sannieshof	6	6.5	5	7	5	4.5
S. stenophylla	Springfontein	3	6	5	5	4	3
S. stenophylla	Steynsrus	5	5	5	5	2	3
S. repens	George	4	4	5	5	4	3
S. repens	Indwe	r	r	<1	r	r	r
S. repens	Knapdaar	2	2	2	1	1	1
S. repens	Lady Grey	5	4	2	1	2	3
S. repens	Queenstown	<1	1	r	1	r	<1
S. runcinata	Bergville	<1	<1	r	<1	r	r
S. runcinata	Bethlehem	5	3	<1	<1	<1	<1
S. runcinata	Coligny	r	2	<1	<1	r	<1
S. runcinata	Delareyville	2	1	<1	<1	<1	<1
S. runcinata	Klerkskraal Dam	3	3	1	2	1	1
S. runcinata	Koster	r	r	r	r	r	r
S. runcinata	Kroonstad	2	4	1	1	<1	1
S. runcinata	Kuruman	<1	1	2	2	<1	<1
S. runcinata	Lichtenburg	1	3	r	1	<1	<1
S. runcinata	Roossenekal	2	4	3	4	2	1
S. runcinata	Vryburg	3	2	2	3	1	2
Neomycin 30 µg		7	6.5	6	5	6	6

r = resitant

3.6.1.2 Minimum inhibition concentration (MIC) assay

The results indicate that *S. stenophylla* extracts have good activity compared to *S. runcinata* because they exhibit the lowest concentration inhibiting bacterial growth (Figure 54). The MIC of *S. stenophylla* is 0.098 mg/ml against *S. aureus* (ATCC 25923), *B. cereus* (ATCC 11778) and *B. subtilis* (ATCC 6051). The MIC for *S. repens* is also 0.098 mg/ml only

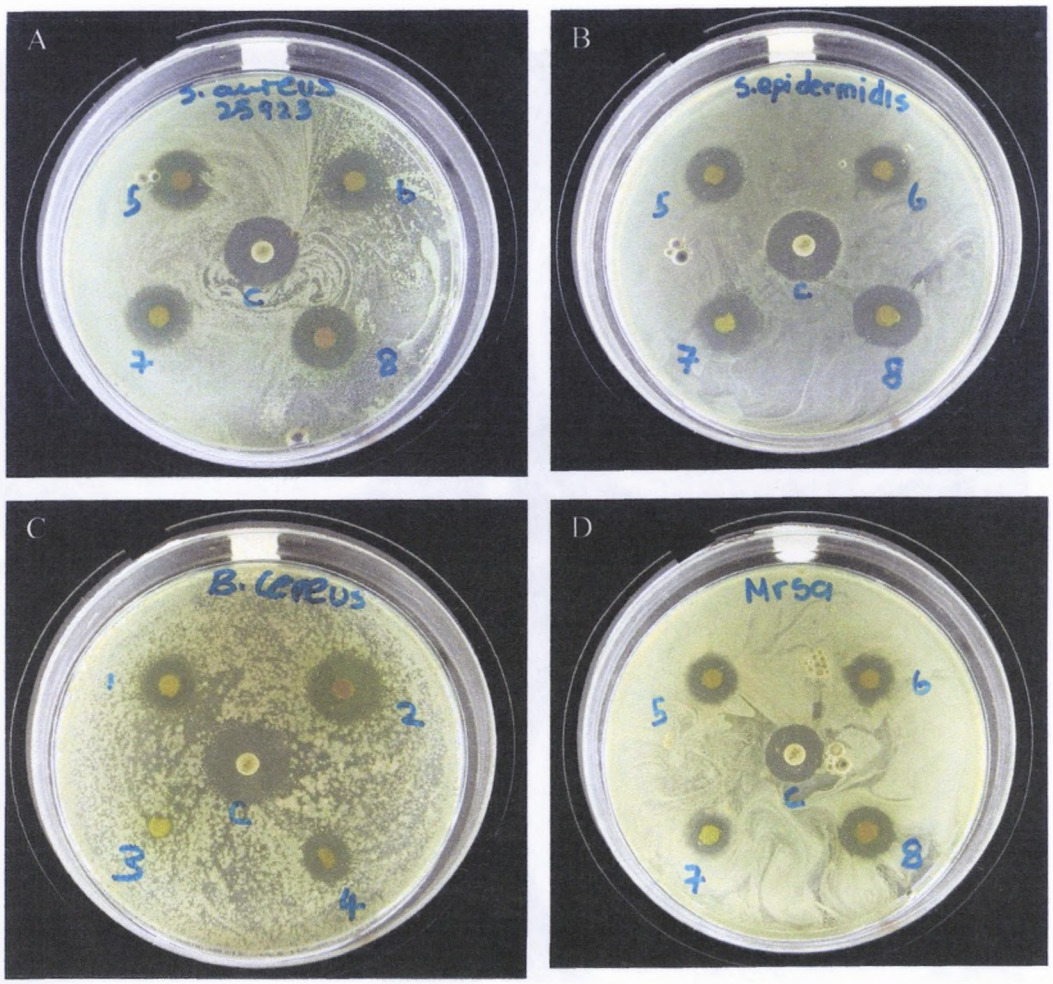


Figure 50. Plates showing activity against different gram (+) bacteria. **A.** *Staphylococcus aureus* (ATCC 25923); **B.** *Staphylococcus epidermidis* (ATCC 2223); **C.** *Bacillus cereus* (ATCC 11778); **D.** methicilline resistant *Staphylococcus aureus* (MRSA).

Reference codes;

1. *S. repens* (Knapdaar)
2. *S. repens* (Lady Grey)
3. *S. repens* (Indwe)
7. *S. repens* (George)

4. *S. stenophylla* (Bethulie)
5. *S. stenophylla* (Molteno)
6. *S. stenophylla* (Springfontein)
8. *S. stenophylla* (Sannieshof)

C. neomycin 30 µg

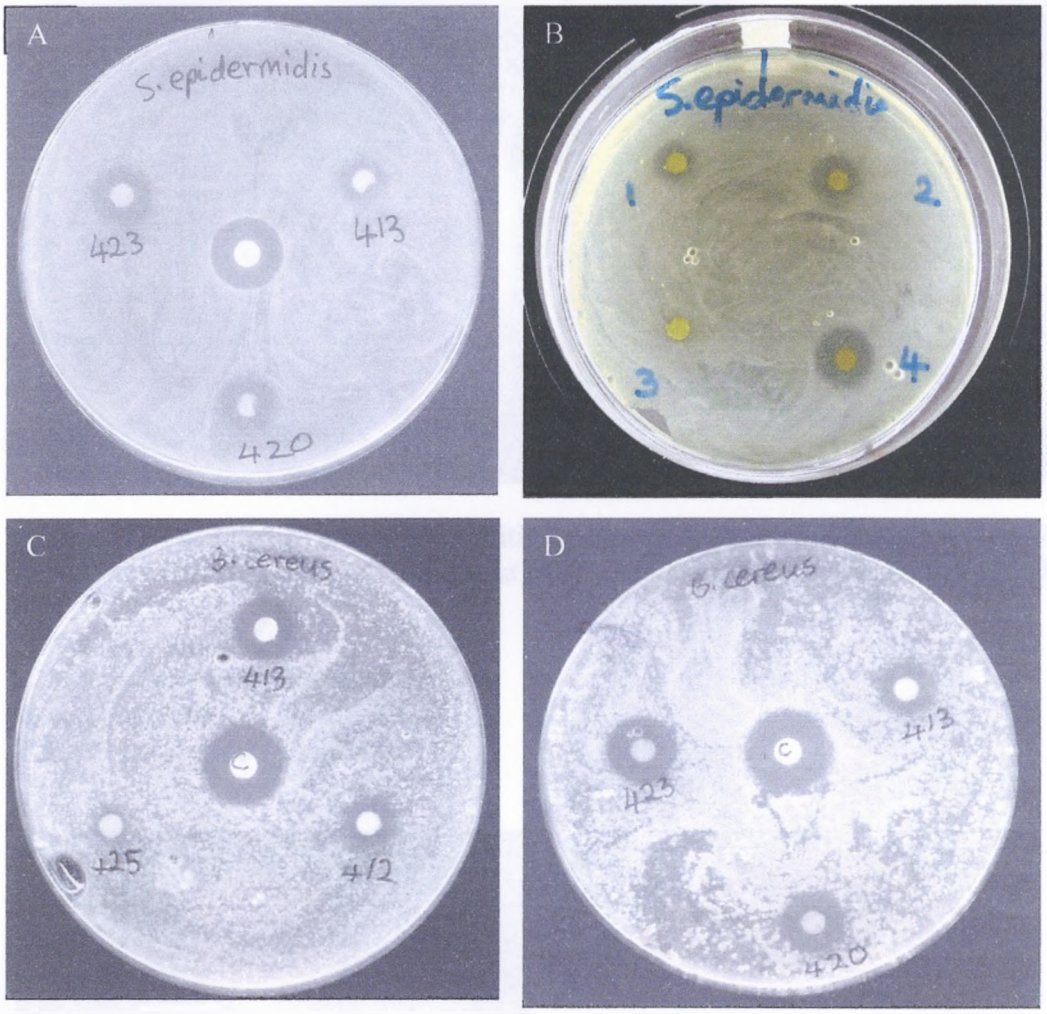


Figure 51. Plates showing activity against different gram (+) bacteria. **A and B** *Staphylococcus epidermidis* (ATCC 2223); **C and D** *Bacillus cereus* (ATCC 11778).

Reference codes;

1. *S. repens* (Knapdaar)
2. *S. repens* (Lady Grey)
3. *S. repens* (Indwe)
4. *S. stenophylla* (Bethulie)

412. *S. repens* (Queenstown)
413. *S. stenophylla* (Biesiesvlei)
425. *S. runcinata* (Delareyville)

420. *S. stenophylla* (Steynsrus)
423. *S. stenophylla* (Sannieshof)
C neomycin 30 µg

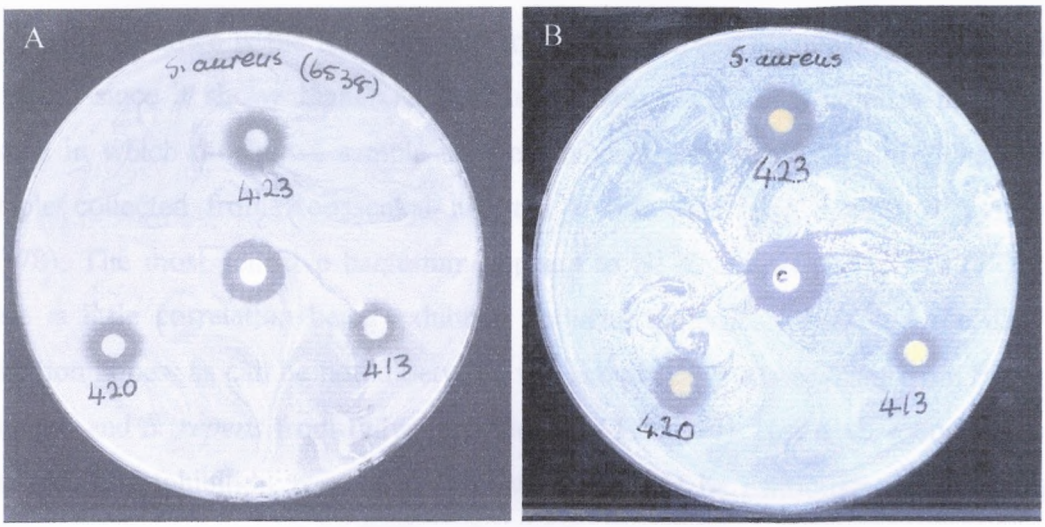


Figure 52. Petri dishes showing inhibition zones of *S. stenophylla* against the two strains of *Staphylococcus aureus*. **A.** *S. aureus* (ATCC 6538); **B.** *S. aureus* (ATCC 25923).

Reference codes;
413. *S. stenophylla* (Biesiesvlei)
420. *S. stenophylla* (Steynsrus)
423. *S. stenophylla* (Sannieshof)

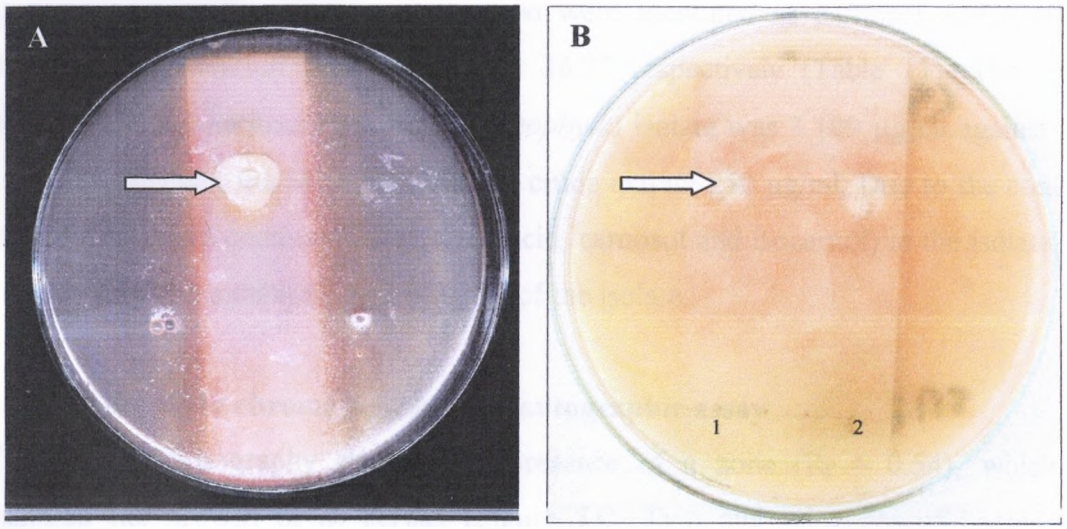


Figure 53. TLC bioautographic assay plates of *S. stenophylla*. The clear zones (arrows) show inhibition of the bacterium, *Bacillus cereus* (ATCC 6538); **A.** methanol extract collected from Biesiesvlei; **B.** methanol extracts *S. stenophylla* collected from Sannieshof, 1 denotes point of application of the isolate and 2 for the crude extract.

against *B. cereus* (ATCC 11778) for samples collected from George and Knapdaar. The sample from Indwe is the least active among the *S. repens* samples, against all the bacteria screened since it shows higher MIC values. These results also confirm the disc diffusion results in which the Indwe sample showed minimal inhibition (<1 mm). The *S. runcinata* sample collected from Roossenkal had the lowest MIC also against *B. cereus* (ATCC 11778). The most sensitive bacterium appears to be *B. cereus* (ATCC 11778). However, there is little correlation being exhibited between the MIC values and the disc diffusion inhibition zones, as can be noted between the activity of *S. stenophylla* from Biesiesvlei and Molteno and *S. repens* from Indwe and George (Table 14). However, associations between disc diffusion inhibition zones and MIC values should be cautiously made because of the differences in state (liquid for MIC and solid for disc diffusion) of the media (Janssen *et al.* 1987).

3.6.1.3 Column chromatography

Material of *S. stenophylla* collected from Sannieshof revealed the presence of three compounds at 34.03, 36.03 and 36.77 retention time (Figure 55). One compound was carnosic acid at Rt =36.03 and the two were identified as carnosol and/or isocarnosol derivatives at retntion times 34.03 and 36.77 respectively (Table 11). The MIC value obtained for the methanol extract *S. stenophylla* isolate was 3.109 µg/ml against *S. aureus* (ATCC 6538) and the MIC value for the crude extract 250 µg/ml. Due to the concentration of the very active compounds (carnosic acid, carnosol and isocarnol) in the isolate, the MIC value is very low exhibiting high potency of the isolate.

3.6.1.4 Thin layer chromatography-bioautographic assay

The TLC bioautography showed the presence of a zone (R_f = 0.54), which strongly inhibited the growth of *B. cereus* (Figure 53). Two merged zones of bacterial growth inhibition were observed after the plates were sprayed with 0.2 mg/ml INT solution. Bacterial growth was observed over the whole plate as a maroon coloration, except in the zones containing the active antibacterial compounds. The TLC plate on which carnosic acid was applied showed similar results. The spot of inhibition of carnosic acid is identical in R_f value to that of the *S. stenophylla* isolate and crude extract (Figure 53b). Rosmarinic acid did not exhibit any bacterial inhibition, which implies that the antibacterial activity in *S. stenophylla* is a result of carnosic acid and/or other carnosol derivatives. The HPLC/UV/MS

Table 14. Minimum inhibitory concentration (MIC) (mg/ml) in comparison to the inhibition zones (mm) of disc diffusion.

Taxon	Methanol extracts	*S. aureus* (ATCC 25923)		*S. aureus* (ATCC 6538)		*S. epidermidis* (ATCC 2223)		*B. cereus* (ATCC 11778)		*B. subtilis* (ATCC 6051)	
		MIC (mg/ml)	*I.Z(mm)	MIC (mg/ml)	*I.Z(mm)	MIC (mg/ml)	*I.Z(mm)	MIC (mg/ml)	*I.Z(mm)	MIC (mg/ml)	*I.Z(mm)
S. stenophylla	Biesiesvlei	nd	6	0.78	5.5	0.2	6	0 39	6	0.098	6
S. stenophylla	Molteno	0.098	5	0.2	4	0.78	5	0.098	5	nd	4
S. stenophylla	Sannieshof	0.098	5	0.2	7	3.13	5	0.098	6	0 39	6.5
S. stenophylla	Springfontein	0.2	5	0.2	5	3.13	4	0.098	3	nd	6
S. repens	Indwe	6.25	<1	6.25	r	6.25	r	1.56	r	nd	r
S. repens	George	0.2	5	0.39	5	3.13	4	0.098	4	nd	4
S. repens	Knapdaar	0.39	2	0.2	1	3.13	1	0.098	2	nd	2
S. repens	Lady Grey	0.39	2	0.78	1	3.13	2	0.39	5	nd	4
S.runcinata	Bethlehem	nd	<1	3.13	<1	3.13	<1	6.25	5	1.56	3
S.runcinata	Klerkskraal Dam	nd	1	1.56	2	3.13	1	3.13	3	1.56	3
S.runcinata	Kroonstad	nd	1	6.25	1	6.25	<1	3.13	2	3.13	4
S.runcinata	Lichtenburg	nd	r	12.5	1	6.25	<1	3.13	1	6.25	3
S.runcinata	Roossenekal	0.2	3	0.39	4	3.13	2	0.098	2	nd	4

*I.Z = inhibition zones measured from the disc edge nd = not determined because plant extract was not available at the time of testing.

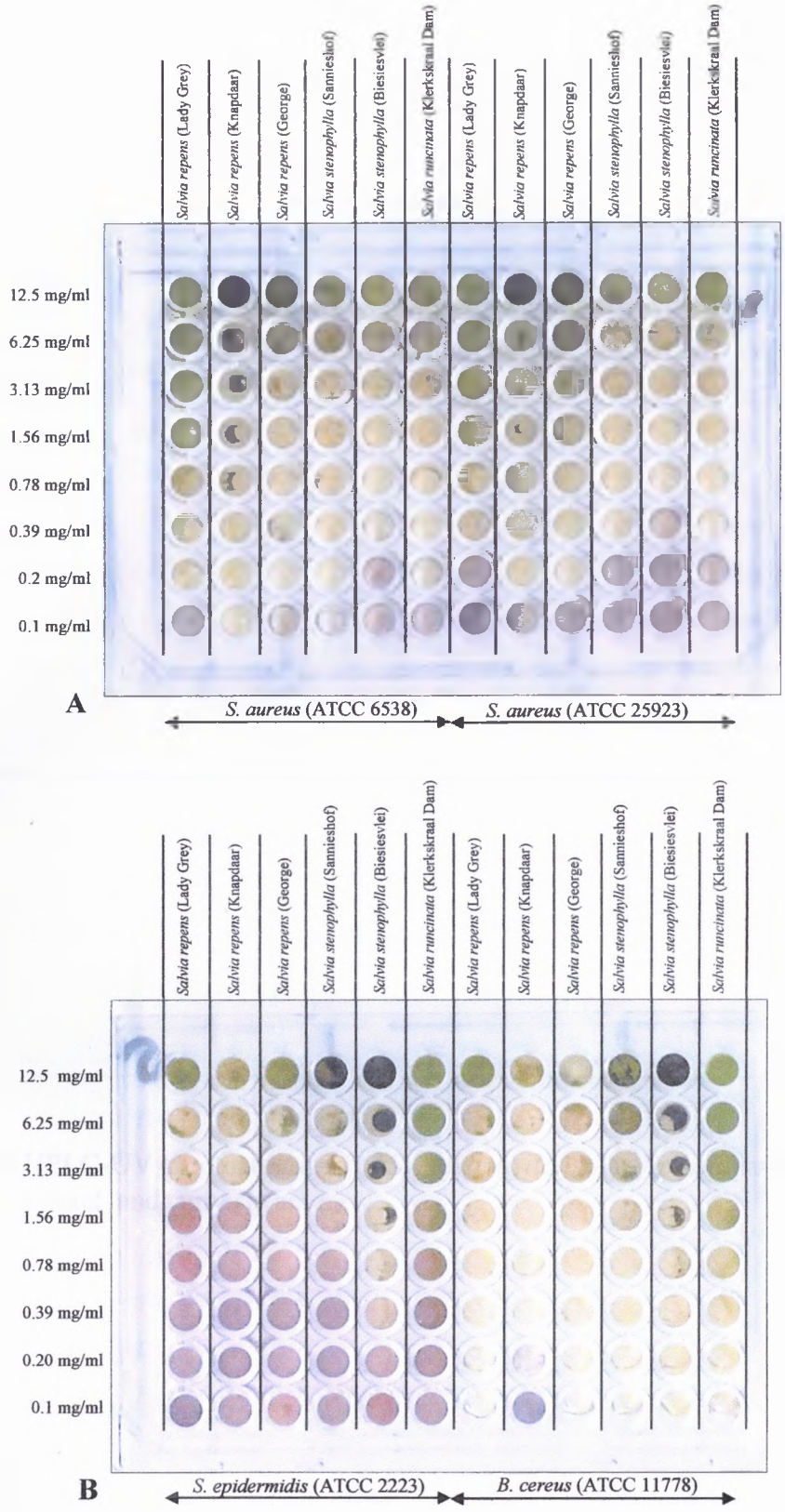


Figure 54 A & B. Microtiter plates showing the MIC values for *S. stenophylla, S. repens* and *S. runcinata.*

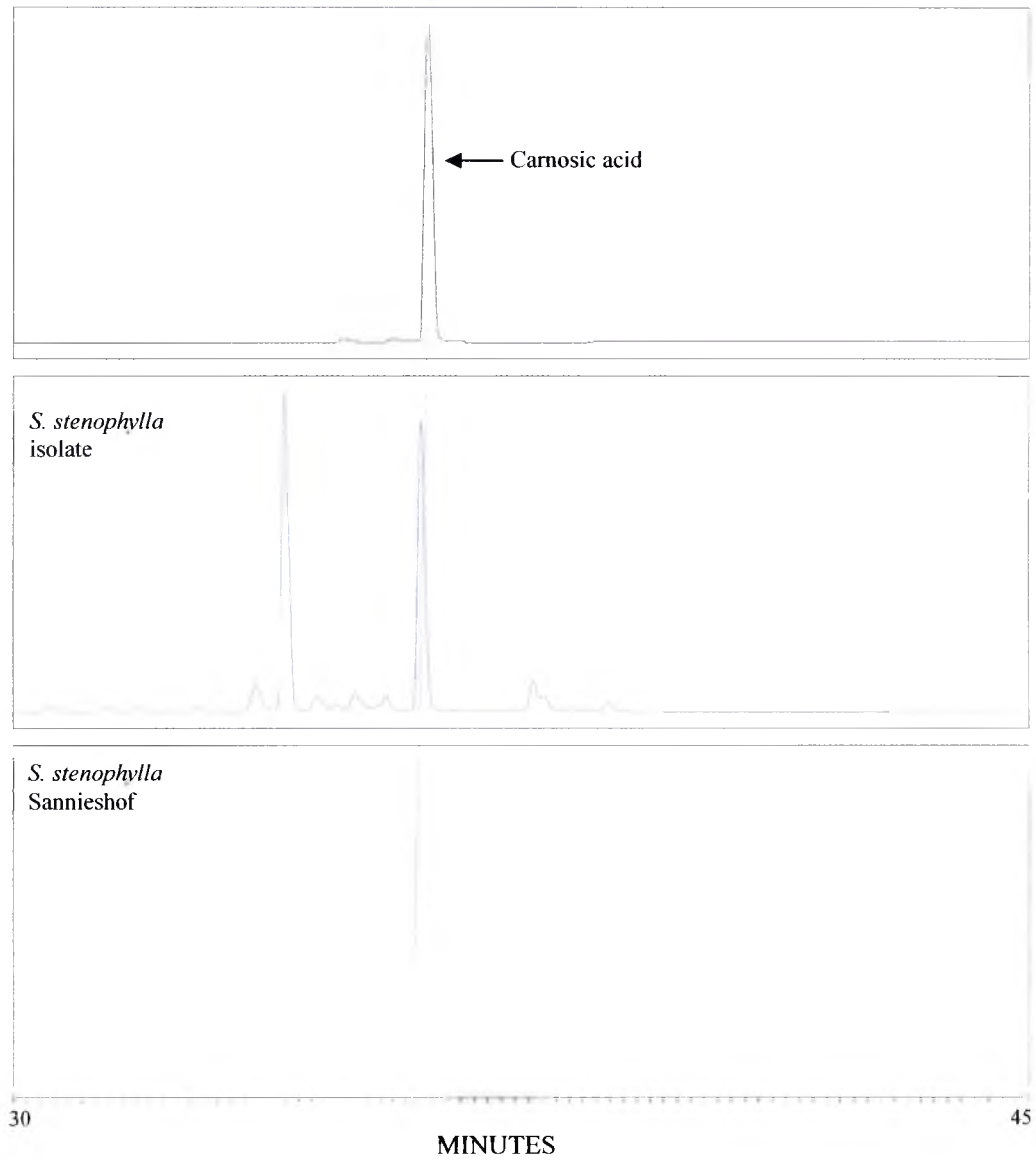


Figure 55. HPLC-UV chromatograms of *S. stenophylla* isolate in comparison with the crude extract and carnosic acid.

results confirm this finding in that the methanol extracts (Molteno and Sannieshof for *S. stenophylla*; George, Knapdaar and Lady Grey for *S. repens*) that had high concentrations (+++ and ++) of carnosic acid exhibited the highest antibacterial activity. The sample from Biesiesvlei did not have any carnosic acid but has bacterial activity almost as good as the Sannieshof and Molteno extracts which have carnosic acid. It is, however, rich in carnosol derivatives, which are also reported to have antibacterial properties (Table 14). As earlier reported in the text, carnosic acid is very unstable and quickly converts to other stable entities.

3.6.2 Antioxidant activity

The radical scavenging properties were evaluated against the DPPH radical. Antioxidant compounds appeared as white spots against a purple background upon spraying the TLC plate with the DPPH radical (Figure 56). The R_f value (0.31) of some compounds matched that of rosmarinic acid (0.3), which was the standard. All the non-volatile extracts indicated the presence of rosmarinic acid, which is a common constituent of the Lamiaceae particularly the subfamily Nepetoideae (Janicsak *et al.* 1999; Petersen and Simmonds 2003). In *S. repens* samples (1–7), other antioxidant compounds were revealed with R_f values of 0.15 and 0.52. The compounds with R_f values 0.52 also occur probably at low concentration in *S. stenophylla*. Other compounds with R_f values of 0.92 and 0.87 could be detected in *S. stenophylla* and *S. runcinata* samples. Antioxidant properties have also been documented in many essential oils of the Lamiaceae. For instance, *Marjorana hortensis* (Moench) and rosemary exhibited high antioxidant activity than that of the standard α-tocopherol and butylated hydroxytoluene (BHT) using a modified thiobartituric acid reactive species (TBARS) method (Baratta *et al.* 1998). *R. officinalis* inhibit the non-enzymatic liposomal peroxidation (Sotelo-Felix 2002). The blocking of lipid peroxidation by *R. officinalis* may be due to the action of scavengers of Cl_3COO and OH, converting them into less toxic substances or acting as antioxidant (Sotelo-Felix 2002).

There are many other compounds that portrayed antioxidant properties on the TLC plate (Figure 35) for non-volatile compound and this was further confirmed by the HPLC/UV/MS results. The other four compounds (carnosol, carnosic acid, isocarnosol and oleanolic acid) that were identified by the HPLC/UV/MS have been reported to have antioxidant properties. Due to the complexity of the natural mixtures of non-volatile compounds, it is

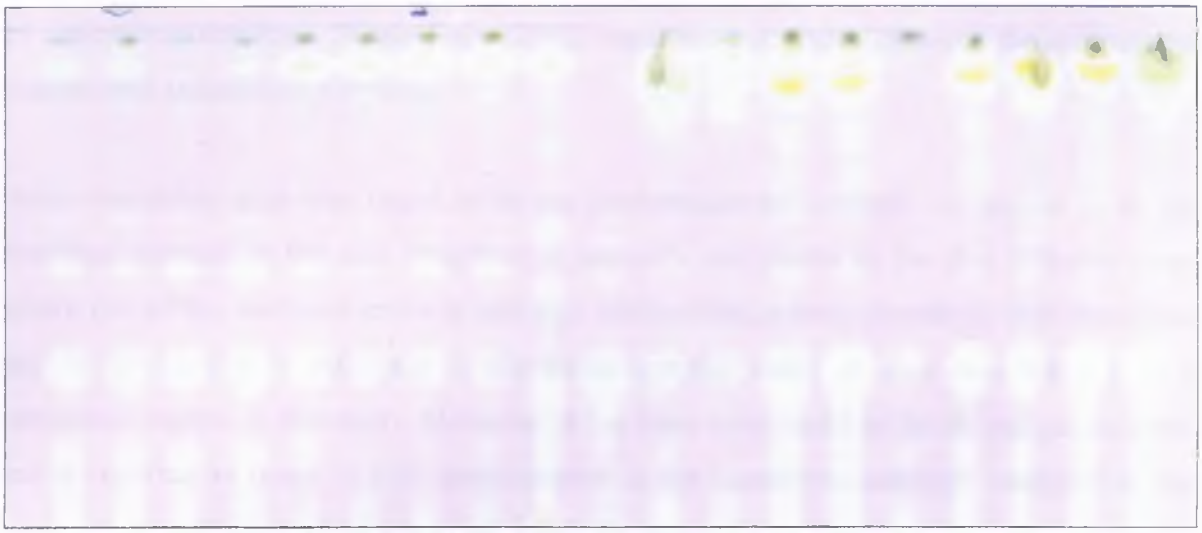


Figure 56. TLC plate of the methanol extracts of the *S. stenophylla* complex exhibiting the presence on rosmarinic acid and other antioxidant compounds. S stands for rosmarinic acid standard with $R_f = 0.3$.

Key to the samples;

S. repens	*S. stenophylla*	*S. runcinata*
1. Lady Grey	6. Springfontein	13. Coligny
2. Knapdaar	7. Molteno	14. Klerkskraal Dam
3. George	8. Roossenekal	15. Lichtenburg
4. Dordrecht	9. Bethulie	16. Kroonstad
5. Indwe	11. Sannieshof	17. Kuruman
	12. Biesiesvlei	18. Delareyville
		19. Vryburg

rather difficult to characterize every compound and assess or compare their antioxidant activities. Each plant extract contains different compounds, which possess different amounts of antioxidant activities. Sotelo-Felix (2002) reports a correlation between the non-volatile content and antioxidant properties.

Since rosmarinic acid was found to be the predominant non-volatile compound in all the methanol extracts of the taxa examined, it supports our results in the disc diffusion assay where not all the methanol extracts had high antibacterial activity despite having rosmarinic acid in abundance, in reference to the bioautographic assay. It also disqualifies it as a taxonomic marker in this study. However, it has been very useful at family and genus levels and is reported to occur in high concentration in the Lamiaceae although restricted to the subfamily Nepetoideae (Janicsák *et al.* 1999; Pedersen 2000; Skoula *et al.* 2000). Petersen and Simmonds (2003), however, further states that rosmarinic acid cannot be used as a chemotaxonomic maker at family level also because it occurs widely in groups such as Boraginaceae, Apiaceae, Araliaceae, Cucurbitaceae, Hydrophyllaceae, Rubiaceae, Plantaginaceae, Sterculiaceae, Tiliaceae, Blechnaceae, hornworts, monocots like the sea grass family Zosteraceae, Potamogetonaceae, Anthocerotaceae and the Cannaceae.

3.6.3 Anti-inflammatory activity

Anti-inflammatory activity was assessed on cyclooxygenase-2 (COX-2), an enzyme that catalyses the conversion of arachidonic acid to prostaglandin. The taxa (*S. stenophylla*, *S. repens* and *S. runcinata*) that were screened for anti-inflammatory activity portrayed variation in inhibiting COX-2 from progressing the conversion of arachidonic acid to prostaglandin (Table 15). *S. stenophylla* collected from Biesiesvlei had the highest percentage of inhibition (73.4%), while the lowest percentage is shown by the extract collected from Sannieshof. *S. runcinata* from Vryburg had the highest percentage of inhibition at 60.48%, while the lowest is from Melville Koppies at 4.37%. Only two (Koster and Vryburg) out of the eleven *S. runcinata* extracts have anti-inflammatory activity above 50%. *S. repens* collected from Queenstown displays an inhibition of 60.21% (Table 15).

Table 15. Comparison of the percentages of inhibition of prostaglandin synthesis of the essential oils tested at 1% concentration.

Salvia essential oils	Locality	Inhibition (%)
S. stenophylla	Biesiesvlei	73.40
S. stenophylla	Dordrecht	21.83
S. stenophylla	Sannieshof	35.27
S. stenophylla	Smithfield	56.57
S. stenophylla	Steynsrus	51.72
S. repens	Dordrecht	60.21
S. runcinata	Bergville	35.95
S. runcinata	Coligny	40.68
S. runcinata	Delareyville	24.49
S. runcinata	Klerkskraal Dam	15.93
S. runcinata	Koster	53.61
S. runcinata	Kroonstad	23.84
S. runcinata	Kuruman	40.21
S. runcinata	Lichtenburg	19.36
S. runcinata	Melville Koppies	04.37
S. runcinata	Ventersdorp	24.53
S. runcinata	Vryburg	60.48

The screening of anti-inflammatory properties indicates activity on all the essential oils. These properties are based on the inhibition of cyclooxygenases of which monoterpenes are reported to be responsible for (Perry *et al.* 2001; Petersen and Simmonds 2003). Anti-inflammatory properties have been attributed to the sesquiterpene (-)-α-bisabolol (http://www.dragoco. com/engl/pages/kos_w_irri.htm), while its isomer (+)-*epi*-α-bisabolol is reported to have cicatrizant properties (wound healing). This was shown in a study by Villegas *et al.* (2001) where *in vivo* cicatrizant activity of *Peperomia galioides* were attributed to α-bisabolol, (+)-*epi*-α-bisabolol and α-terpineol and their acyclic isomers, *trans*-nerolidol and *trans*-farnesol were inactive (Viljoen *et al.* 2002). Different results were obtained in this study as *S. stenophylla* from Biesiesvlei showed the highest percentage (73.4%) of cyclooxygenase inhibition and yet contains 1.77% of α-bisabolol compared to *S. stenophylla* from Smithfield and Dordrecht, which accumulated 17.37% and 46.5% α-

bisabolol respectively. *epi*-α-Bisabolol (2.07%) was found only in the essential oil of *S. runcinata* collected from Klerkskraal Dam, which also had the second highest level of α-bisabolol (41.05%), but had a low inhibition of 15.93%. Two *S. runcinata* essential oils from Koster and Vryburg do not contain any bisabolol compounds (isomers or epimers) but had cyclooxygenase inhibition of over 50%. Like many *Salvia* species, these results show considerable anti-inflammatory properties to support the use of *S. stenophylla* and *S. repens* in traditional medicine to treat inflammation. From these results we can attribute anti-inflammatory activity to volatile compounds occurring in the essential oil other than α-bisabolol, which may have suppressed activity due to the interaction with other compounds. Inflammation is a complex reaction, which is based on pathways that mainly involve two enzymes (lipooxygenase and cyclooxygenase). In this study we looked at one aspect of the complex biochemical cascade by assessing the inhibition of an isomer of cyclooxygenase.

Most of the anti-inflammatory activity of plants reported has been attributed to non-volatile compounds, which are widely distributed in nature (Perry *et al.* 2001). In this study, only essential oils were screened for cyclooxygenase inhibition following reports of the anti-inflammatory activities of α-bisabolol (an essential oil constituent). Methanol extracts would probably have exhibited anti-inflammatory activity above 90% seeing that all the extracts indicated the presence of rosmarinic acid, carnosic acid and carnosol, which are active anti-inflammatory compounds. In a study by Shale *et al.* (1999), the methanol and water extracts of *S. repens* collected from Lesotho exhibited inhibition of 83% and 47% respectively upon analysing the extracts using COX-2 assay. Anti-inflammatory constituents of *Salvia* species, so far identified, include flavonoids and terpenoids. Terpenoids such as oleanolic acid, ursolic acid and carnosol isolated from *S. officinalis* (Baricevic *et al.* 2001), aethiopinone and *o*-naphthoquinone isolated from *S. aethiopis* (Hernandez-perez *et al.* 1995) have shown remarkable anti-inflammatory activity. In another study by Benrezzouk *et al.* (2001), aethiopinone showed anti-inflammatory activity and *in vivo* inhibition of 5-lipooxygenase (5-LOX) after topical administration. 5-LOX inhibition may be beneficial in some inflammatory and respiratory diseases. The 5-LOX pathway is constitutively activated in the lungs of patients with idiopathic pulmonary fibrosis resulting in the overproduction of leukotrienes. These mediators of inflammation have an important role in the biology of asthma and administration of 5-LOX inhibitors or specific LTD 4 antagonists represents a new approach to the anti-inflammatory treatment of

asthmatic patients. Aethiopinone shows selectivity for 5-LOX relative to COX and is active after oral administration. COX exists in two isoforms, constituting COX-1, which is responsible for basal formation of prostaglandins in many tissues, including the stomach, kidney and platelet, which is induced by cytokines, growth factors, and mutagens that catalyse the synthesis of high levels of prostaglandins in the inflammatory response (Wallace and Chin 1997; Benrezzouk *et al.* 2001). Selective inhibitors of COX-2 inhibit prostaglandin synthesis at sites of inflammation but spares prostaglandin synthesis in the gastrointestinal tract and therefore, not induce ulcers like standard NSAIDs (Wallace and Chin 1997). There has been research directed towards the development of highly selective inhibitors of COX-2, and hence the assessment of essential oils on COX-2 (Wallace and Chin 1997).

4. CONCLUSIONS

- Essential oil chemistry is not significantly variable within a defined population but quantitative and qualitative variation has been documented among populations of the same taxon. Chemical data support the continued recognition of the three taxa as weak taxonomically separate entities within a larger species complex.
- The transplant experiment indicates that the observed chemical variation has a genetic basis and is not significantly influenced by environmental/ecological factors.
- Six essential oil chemotypes within the species have been identified;
    - Chemotype I – α-bisabolol. δ-3-carene and limonene rich oils.
    - Chemotype II – (*E*) nerolidol, γ-terpinene, δ-3-carene and *p*-cymene rich oils.
    - Chemotype III – β-phellandrene, β-caryophyllene and ledol rich oils.
    - Chemotype IV – α-bisabolol and (*E*) nerolidol-rich oils.
    - Chemotype V – (*E*) nerolidol-rich oils.
    - Chemotype VI – β-caryophyllene and α-pinene-rich oils.

- The qualitative essential oil data matrix produces a dendrogram, which is congruent with the *a priori* assignment of taxa in the study. With the exception of two OTU's (*S. stenophylla* of Biesiesvlei and *S. runcinata* of Lichtenburg) the clusters generally support the species delimitation of Codd (1985). The present study analysis supports the chemotaxonomic value of essential oils. However, the dendrogram of the quantitative data indicate high levels of chemical polymorphism and the possible existence of intermediate entities (perhaps hybrids) within the species complex
- The chemical analysis supports the suggestion that is introgression/hybridisation between *S. repens* and *S. runcinata* as proposed by Codd (1985). The *S. stenophylla* specimens collected at two localities in the Harts River catchment in the North-West Province may well represent a new taxon as they are morphologically similar and share a diagnostic morphological character (within the *S. stenophylla* complex) in the form of two-flowered verticils. Chemically, however, the specimens from these localities are significantly different.
- The cluster analysis performed on the qualitative essential oil data indicates a phenetic similarity between *S. repens* and *S. stenopylla*.

- AFLP data indicates a genetic similarity between *S. stenophylla* and *S. runcinata,* which is not congruent with the chemical evidence. A wider sampling may reveal more conclusive patterns.
- The study has identified high yielding clones of two commercially important essential oil constituents; (*E*)-nerolidol and α-bisabolol.
- Non-volatile extracts are not as variable among taxa/samples as the essential oil composition, therefore limiting their chemotaxonomic value. All taxa accumulate rosmarinic acid together with carnosic acid and derivatives thereof.
- The ambiguous nature of many points in the taxonomic key provided by Codd (1985) do not only reflect the presence of many intergrading forms, but has led to great confusion with regards to the geographic distribution of the three species comprising the *S. stenophylla* complex. On the basis of a limited survey of herbarium records and the fieldwork conducted during this study it is evident that the three species are often misidentified and Codd's distribution maps do not concur with the species delineations provided in his key (Codd 1985).
- There is no distinct pattern in the type and size of trichomes between plants of different species. Trichome density is variable and *S. stenophylla* has the highest density of peltate trichomes. This species also produced the highest yield of essential oil. This study supports the delimitation of *S. stenophylla* and *S. runcinata* on the basis of trichome density. Characters that define the specific limits of *S. stenophylla* include the density of peltate trichomes, which corresponds to the higher yield of essential oil and the two chemotypes. *S. repens*, on the other hand, is densely pubescent with eglandular trichomes and yields very little essential oil. *S. runcinata* is densely covered with capitate trichomes and does not yield as much essential oil as species with abundant peltate trichomes.
- The essential oils have negligible antimicrobial activity. Methanol extracts of all taxa showed good antibiotic properties with *S. stenophylla* having superior activity compared to *S. runcinata* and *S. repens*.
- The TLC overlay bioautographic assay indicated that the active fraction contained carnosic acid and derivatives thereof.
- In addition to rosmarinic acid, which is known for its antioxidant activity, all taxa produced a suite of phytochemicals with radical scavenging properties as seen in the

TLC-DPPH assay. The phytochemicals include carnosic acid, carnosol, isocarnosol and oleanolic acid as elucidated by HPLC/UV/MS.

- The essential oils displayed variable anti-inflammatory activity as assessed with the COX-2 radiometric assay. No association could be made between essential oil composition and anti-inflammatory activity.
- The collective results on biological activity provide an *in vitro* scientific basis supporting the use of these species in traditional herbal preparations.

5. RECOMMENDATIONS

- There is need for further morphological and biogeographical study to establish the level at which to recognise members of the *S. stenophylla* species complex. However, it should be realised that the species may not be perfectly demarcated, as evolution is an ongoing and dynamic process. The results obtained in the present study confirm the complexity of *S. stenophylla* and the related taxa.
- The molecular study using the AFLPs technique of the *S. stenophylla* species complex should be continued and furthered in order to verify the genetic variation that exists between the taxa and identify the possible multiple hybrids that may have arisen with time causing identification problems. Despite the fact that the number of taxa used in this study represents only a small sample, the potential resolving power of AFLP analysis is evident. Further taxon sampling and generation of additional markers using alternative primer combinations will allow improved assessment of the genetic variability across the geographical range of the complex. These data should then allow more accurate circumscription of species boundaries and a closely related out-group should be selected to enhance the delimitation at hierarchical level
- If this preliminary study is concurred by further evidence it may be necessary to either redefine *S. stenophylla, S. repens* and *S. runcinata* taxonomically as part of a specie complex or merge them under a single name representing a polymorphic/aggregate taxon.
- Verify the taxonomic status of a possible separate taxon at Biesiesvlei through further morphological, chemical and genetic analysis. Only then can it be formally named if it is indeed a new taxon.

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APPENDIX I

THE CHEMOTAXONOMY AND BIOLOGICAL ACTIVITY OF *SALVIA STENOPHYLLA* AND RELATED SPECIES.

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Salvia stenophylla Burch. (Lamiaceae) is endemic to southern Africa, occurring in Namibia, Botswana, and South Africa. It is closely related to *Salvia runcinata* L. f. and *Salvia repens* Burch. with which it forms a species complex. The taxa, however, had been delimited using floral characters only (corolla and calyx). The taxa represented in this species complex are well known in folk medicine of southern Africa. Plant extracts have been used in the treatment of urtricaria, body sores and stomach ailments. *Salvia stenophylla* is reported to contain α-bisabolol, a compound that has anti-inflammatory properties. Based on the traditional uses of these plants and the international use of α-bisabolol to develop active cosmetic products, establishing a scientific rationale for the traditional use is one of the objectives of this research. Antimicrobial and anti-inflammatory properties of the species complex will be documented. Taxonomic delimitation problems were resolved by chemical analysis.

Guided by information obtained from herbarium specimens, the three taxa were collected from natural populations. Bulk material from different populations of each species was collected and the aerial parts of the plants were hydrodistilled for 4 hrs to extract the essential oils (volatile compounds). Several chemical analyses like gas chromatography (GC), gas chromatography coupled to mass spectrophometry (GC/MS) and thin layer chromatography (TLC) were performed. Extraction of phenolic compounds was done by solvent extraction and then analyzed by TLC and high performance liquid chromatography (HPLC). Anti-inflammatory activity was done using the COX-2 assay and antimicrobial activity assays were performed using selected bacterial organisms, fungi and yeasts. The GC/MS results showed that the three taxa display different chemical profiles. The compound in highest concentration is the sesquiterpenoid α-bisabolol (~20%) for *S. stenophylla*, α-bisabolol (~35%) and ledol (24%) for *S. repens* and (*E*)-nerolidol (~42%)

for *S. runcinata*. Some *S. runcinata* populations however, had an average of 39% of α-bisabolol in high concentration, while some plants accumulated 26% of guaiol The essential oils exhibited very little anti-inflammatory and anti-bacterial activity while the methanol extracts exhibited remarkable activity in *S. stenophylla*, variable activity in *S. repens* and very minimal activity in *S. runcinata*. This clearly separates the taxa at chemical and biological level.