BIOACTIVITY AND MICROBIAL CONTENT OF *LIPPIA MULTIFLORA* LEAVES, A HERBAL TEA FROM GHANA

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DECLARATION

I, the undersigned, declare that the entirety of the work contained in this thesis is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

The consumption of herbal teas is an increasing phenomenon among tea consumers globally. However, herbal teas that are not pre-treated to reduce their microbial load are a health risk to consumers, in spite of their potential health-promoting properties. The aim of this study was to develop a steam pasteurisation treatment to reduce the microbial load on *Lippia multiflora* Moldenke (Verbanaceae) tea leaves, a herbal tea from Ghana, identify the bacteria present, and to evaluate the effect of the steam treatment on the bioactive constituent of the leaves.

An HPLC method was developed and optimised for the identification and quantification of verbascoside, the major antioxidant compound of *L. multiflora* herbal infusion. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used to confirm the presence of the compound in the infusion. Ascorbic acid was used as a stabilising agent during the quantification process to prevent the degradation of verbascoside. The hot water infusion of *L. multiflora* was compared to those of *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush) on the basis of their soluble solids and total polyphenol contents, as well as on their antioxidant activities.

In addition to verbascoside, another compound with the same parent and fragment ions as verbascoside was present in the infusion. A 100 ml infusion of *L. multiflora* had significantly ($P < 0.05$) higher soluble solids and total polyphenol contents, and antioxidant activities than those of rooibos and honeybush. The rooibos infusion showed significantly ($P < 0.05$) higher soluble solids and total polyphenol contents as well as antioxidant activities than honeybush. On the basis of soluble solids, rooibos showed a significantly ($P < 0.05$) higher total polyphenol content and a lower ferric-reducing activity than *L. multiflora*. Both teas, however, did not differ significantly with respect to the DPPH antioxidant activity.

The effect of steam pasteurisation on the microbial load of *L. multiflora* herbal tea leaves was evaluated. Five samples of the tea were steam pasteurised at 99.8°C for 2.5 min and five samples were unpasteurised. Microbial enumeration was conducted in duplicate on potato dextrose agar (PDA), plate count agar

(PCA), violet red bile agar (VRBA), yeast peptone dextrose agar (YPDA), and de Man Rogosa Sharpe agar (MRS). Morphologically distinct colonies were isolated, sub-cultured and their Gram reaction recorded. These bacteria were identified to the species level using 16S ribosomal DNA (rDNA) sequence data.

Most of the bacteria identified belonged to the genus *Bacillus*. One species each from the genera *Pantoea* and *Kocuria* were also identified, but only the *Bacillus* species survived the steam treatment. Coliform bacteria detected prior to pasteurisation were not detected after steam treatment. Steam pasteurisation reduced the microbial load from 10^4 to 10^2 cfu.g^{-1}. The effects of the steam pasteurisation on the soluble solid, total polyphenol, and the active compound contents of *L. multiflora,* as well as the antioxidant activities were studied. Pasteurisation did not significantly ($P > 0.05$) change the soluble solids, total polyphenol and active compound contents, or the antioxidant activity.

Steam pasteurisation is potentially an effective method to treat *L. multiflora* herbal teas prior to consumption. However, the steam treatment should complement good agricultural and hygienic practices rather than replace them as some bacteria can survive this treatment. The identification and quantification of verbascoside in *L. multiflora* infusion, as well as the relatively higher antioxidant contents compared to rooibos and honeybush should provide the basis for future studies on the therapeutic application of this herbal tea. Also, verbascoside could potentially form the basis for future quality control of *L. multiflora*.

UITTREKSEL

Daar is 'n wêreldwye toename in die verbruik van kruietee. Kruietee wat egter nie vooraf-behandelings ontvang om die mikrobiese lading te verlaag nie kan, ten spyte van moontlike gesondheidsvoordele, ook 'n potensiële gesondheidsrisiko vir verbruikers inhou. Die doel van hierdie studie was om 'n stoompasteurisasie-behandeling te ontwikkel wat die mikrobiese lading op *Lippia multiflora* teeblare, 'n kruietee van Ghana, te verlaag. Verder is die teenwoordige bakterieë geïdentifiseer en die effek van 'n stoombehandeling op die bio-aktiewe komponente in die teeblare is ook geëvalueer.

'n Hoë-druk vloeistof-chromatografie metode is ontwikkel en ge-optimiseer vir die identifikasie en kwantifisering van verbaskosied, 'n hoof antioksidant komponent in *L. multiflora* kruie aftreksels. Vloeistof chromatografie, gekoppel aan in-lyn massa spektroskopie is ook gebruik om die teenwoordigheid van die komponent in die aftreksel te bevestig. Tydens die kwantifiseringsproses is askorbiensuur as 'n stabiliseringsagent gebruik om die degradasie van verbaskosied te voorkom. Die warm water aftreksel van *L. multiflora* is vergelyk met die van *Aspalathus linearis* (rooibos) en *Cyclopia* spp. (heuningbos) in terme van hul opgeloste vastestof- en totale polifenol inhoude, asook hul antioksidant aktiwiteite.

'n Ander komponent buiten verbaskosied, maar met dieselfde ouer en fragment ione, was ook in die aftreksel teenwoordig. 'n 100 ml *L. multiflora* aftreksel het beduidend ($P < 0.05$) meer opgeloste vastestowwe, totale polifenole en antioksidant aktiwiteit getoon as rooibos en heuningbos. Rooibos het weer beduidend ($P < 0.05$) meer opgeloste vastestowwe, totale polifenole, en antioksidant aktiwiteit as heuningbos. In terme van opgeloste vastestowwe het rooibos 'n beduidende ($P < 0.05$) hoër totale polifenol inhoud en laer ferriet-reduserende aktiwiteit as *L. multiflora*. Beide tee het egter nie beduidend verskil ten opsigte van hul antioksidant aktiwiteit nie.

Die effek van stoompasteurisasie op die mikrobiese lading van *L. multiflora* kruieteeblare is geëvalueer. Vyf teemonsters is gestoompasteuriseer by 99.8°C vir 2.5 min en 5 verdere monsters is nie gepasteuriseer nie. Mikrobe-tellings is in

duplikaat op potato dextrose agar (PDA), plate count agar (PCA), violet red bile agar (VRBA), yeast peptone dextrose agar (YPDA), en de Man Rogosa Sharpe agar (MRS) gedoen. Morfologies onderskeibare kolonies is geïsoleer, her-gekweek en hul Gram status genotuleer. Hierdie bakterieë is daarna tot op spesie-vlak geïdentifiseer deur 16S ribosomale DNS (rDNS) volgorde bepalings.

Die meerderheid van die geïdentifiseerde bakterieë behoort tot die genus *Bacillus* en een spesie elk van die genera *Pantoea* en *Kocuria* is ook geïdentifiseer. Slegs *Bacillus* spesies het egter die stoompasteurisasie behandeling oorleef. Kolivorme bakterieë wat voor pasteurisasie waargeneem is was afwesig na die stoom behandeling. Stoompasteurisasie het ook die mikrobiese lading van 10^4 na 10^2 kve.g^{-1} verminder. Die effek van stoompasteurisasie op die opgeloste vastestowwe, totale polifenole en die aktiewe-komponent inhoud van *L. multiflora*, asook die antioksidant aktiwiteit is bestudeer. Pasteurisasie het die opgeloste vastestowwe, totale polifenole, aktiewe komponente en die antioksidant aktiwiteit nie-beduidend ($P > 0.05$) verander.

Stoompasteurisasie kan potensieël 'n effektiewe metode wees vir die behandeling van *L. multiflora* kruietee voor verbruik. Die stoombehandeling moet egter saam met goeie landbou- en higiëniese praktyke gebruik word eerder as om dit te vervang aangesien sommige bakterieë hierdie stoombehandeling kan oorleef. Die identifikasie en kwantifisering van verbaskosied in *L. multiflora* aftreksels, asook die hoër antioksidant inhoud vergeleke met rooibos en heuningbos verskaf moontlikhede vir verder navorsing in die terapeutiese aanwending van hierdie kruietee. Verbaskosied kan ook moontlik die basis vorm vir toekomstige kwaliteitskontrole van *L. multiflora*.

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DEDICATION

To the enduring memory of my mother, the instructive example of my father, and the support of my family.

Most of all, this is to the love of my love, my wife Elfreda Naa Lomoteley Arthur who sacrificed self and personal ambition, so we could jointly attain what we could call a 'family goal'. Elfreda, I dedicate this thesis to your love. I love you more than you can imagine.

CONTENTS

The language and style used in this thesis are in accordance with the requirements of the International Journal of Food Science and Technology. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

During the past 10 years, the use of herbal teas has increased globally (Sheehan, 2002; Wright *et al.*, 2007; Van Wyk, 2008) and they are reportedly competing in the market with standard or 'true' teas (Anon, 2003; Zegler, 2007). In the United States, a larger percentage of consumers are purchasing and exploring the use of herbal products (Halberstein, 2005), and in Germany, herbal teas are frequently used in routine hospital care (Wilson *et al.*, 2004). Both the Chinese and the Japanese, who constitute a large segment of the herbal tea market, consider these teas as effective in the maintenance of health (Béliveau & Gingras, 2004).

Due to the surge in consumer interests (Postlewaite, 1998), many beverage companies have added herbal teas to their production lines and are advertising their health benefits (Sharon, 1991; Wong, 1998). Stickel *et al.* (2009) observed that consumers' growing interest in disease prophylaxis, nutrition and the quest to improve health and well-being underpin the increased use of herbal teas. The presence of antioxidant compounds in herbal teas has been linked to their health-promoting properties (Wong 1998; Popp, 2004). Subsequently, these compounds have become the focus of several research efforts, some of which are targeting the identification of plant species with novel bioactive constituents (Kang *et al.*, 2003; Lee *et al.,* 2003; Cano & Volpato*,* 2004; Sağlam *et al.,* 2007).

High microbial loads present on food products are a significant health risk to consumers. As primary agricultural products, herbal tea materials are easily contaminated by soil and environmental microorganisms. Moreover, in the regular production process of drying, cutting, packing, storing and distribution, they are usually not subjected to control measures that could reduce microbial contamination and growth (Martins *et al.,* 2001). High levels of microbial contamination has been reported in herbal teas and the consumption of some of these products has also been implicated in adverse effects reported by consumers (Satorres *et al.*, 1999; Martins *et al.,* 2001; Rizzo *et al.,* 2004; Wilson *et al.*, 2004; Nestmann *et al.*, 2006; Bianco *et al.*, 2008). This has raised public health concerns over their quality and safety (Street *et al.*, 2008).

Lippia multiflora is a herbal tea which is currently being introduced to the international market. It belongs to the Verbenaceae plant family (Pascual *et al.,* 2001), which comprises of many plants known for their antitumour, antiinflammatory and antioxidant properties (Kunle *et al.*, 2003; Viljoen *et al.*, 2005; Oliveira *et al.*, 2006). The genus *Lippia* consists of more than 200 species of herbs, shrubs and small trees, several of which occur in sub-tropical Africa, as well as South and Central America where they are traditionally used by infusing the leaves and aerial parts, for consumption as tea (Pascual *et al.*, 2001; Juliani *et al.*, 2006). *Lippia multiflora* which occurs in Ghana, Senegal and Cote d'Ivoire, is a perennial, aromatic shrub, of which the infusion is used as a herbal tea commonly known as 'Bush Tea' or the 'Tea of Gambia' (Abena *et al.,* 2003; Avlessi *et al.,* 2005; Juliani *et al.*, 2006). This tea is used as a sudorific, a febrifuge, a laxative and for the treatment of colic (Kunle *et al.*, 2003). Except for the communities where natural stands of the tea occur, and where the leaves are traditionally used as a herbal infusion, no commercial application of the tea has been reported. Recently, however, a development organisation, the Agribusiness in Sustainable Natural African Plant Products (ASNAPP) (www.asnapp.org) has been leading efforts to commercialise the tea.

For herbal teas such as *L. multiflora* to sustain the current interest by consumers, it is important to first identify their microbial contamination level as an indicator of safety and quality (Jarvis *et al.*, 2007), and to develop methods to control the microbial contamination of the final product. Concurrently, it is imperative that methods employed to control microbial populations, do not adversely affect the bioactive compounds and their medicinal properties for which consumers use these products. Therefore, the aim of this study was to identify the microbial load and species present on *L. multiflora* herbal tea and investigate the effect of steam sterilisation on the microbes. Furthermore, the major antioxidant compound was identified in the hot water infusion of the tea leaves and an HPLC method developed for its quantification. The effect of steam on the activity of the infusion and the major antioxidant compound was also studied. A final aim of this study was to benchmark *L. multiflora* herbal tea in terms of its antioxidant activity and total polyphenol content against those of rooibos and honeybush, two South

African herbal teas that have achieved commercial success (Joubert *et al.*, 2008) in order to provide a possible commercial pathway for *L. multiflora*.

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CHAPTER 2

LITERATURE REVIEW

A.   Background

Worldwide, tea is reputed to be the most consumed beverage apart from water (Santana-Rios *et al.*, 2001; Malinowska *et al.*, 2008). In the United Kingdom, tea is an obsession of the British that has become one of the defining idiosyncrasies of the national character (Anon, 2007). With sales totaling 100 000 tons, tea drinking is embedded in the cultural fabric of the British (Anon, 2007). Furthermore, the Chinese have, for nearly 2 000 years, used herbal preparations often taken as tea for all ailments ranging from simple warts to diseases as life-threatening as cancer (Wong, 1998).

Tea has been described as the oldest medicine (Dufresne & Farnworth, 2000). The Japanese, who average 7 cups of tea per day, have an extremely low incidence of heart disease (Vinson & Dabbagh, 1998). Weisberg (2001) reported that the consumption of four cups of black tea a day over an eight-week period by 50 patients with either a history of surgery to open blocked arteries or at least one coronary artery with greater than 70% blockage, greatly improved their condition. Patients suffering from various diseases including Parkinson's disease and Alzheimer's disease, cancer, hypercholesterolemia, atherosclerosis, ischemic damage, and inflammatory diseases have been known to show marked improvements in their conditions after drinking green tea (Landi, 2007). A long-term study indicated a significant lower risk of dying from coronary heart disease and a lower incidence of strokes when people consumed tea (Dufresne & Farnworth, 2000).

There currently exists a growing interest in herbal teas worldwide, and the prediction is that the industry will continue to expand as long as consumers continue to be health conscious (Dufresne & Farnworth, 2000; Sheehan, 2002; Schweizer, 2006). Tea sales will benefit from the current growth trend in natural products due to its health attributes (Postlewaite, 1998; Khan & Mukhtar, 2007). As the demand for healthy beverages continues to rise, manufacturers are looking to

botanical extracts which have medicinal or nutritional qualities and also provide herbal flavours and aromas to meet market demands (Wilson, 1999; Datamonitor, 2005a, 2005b). In some markets, including Western Europe and Japan, herbal notes and medicinal tastes are part of the perceived efficacy of drinks marketed as functional products (Foote, 2006).

B.   True teas

Berry (2005) estimated that about 3 000 varieties exist of what is referred to as 'true' tea, which is prepared from the evergreen plant *Camellia sinensis*. Four of these varieties are commonly known, and are classified on the basis of how the leaves are processed (Santana-Rios *et al.*, 2001). They include green, oolong black, and white tea (Figure 1).

Black tea is made from fresh leaves that are withered indoors in open-air shelves without any physical breaking of the leaf structure. After withering, the leaves are rolled, exposing the enzymes in the leaves to initiate a chemical oxidation process. The leaves are then exposed to high temperatures to stop the oxidation (Berry, 2005) (Figure 1). The leaves used for green tea are not subjected to this process, but rather steamed or otherwise heated immediately after picking to prevent any oxidation (Figure 1). Oolong tea is partially oxidised to a level between green and black tea (Vinson & Dabbagh, 1998; Berry 2005). While black, green and oolong teas are subjected to elaborate processing regimes, white tea typically undergoes a simple three-stage processing to produce its distinct characteristics (Berry, 2005). In the processing of white tea, only special leaves, i.e. newly grown buds and young leaves, are selected that contain high concentrations of catechins (Santana-Rios *et al.*, 2001; Malinowska *et al.*, 2008).
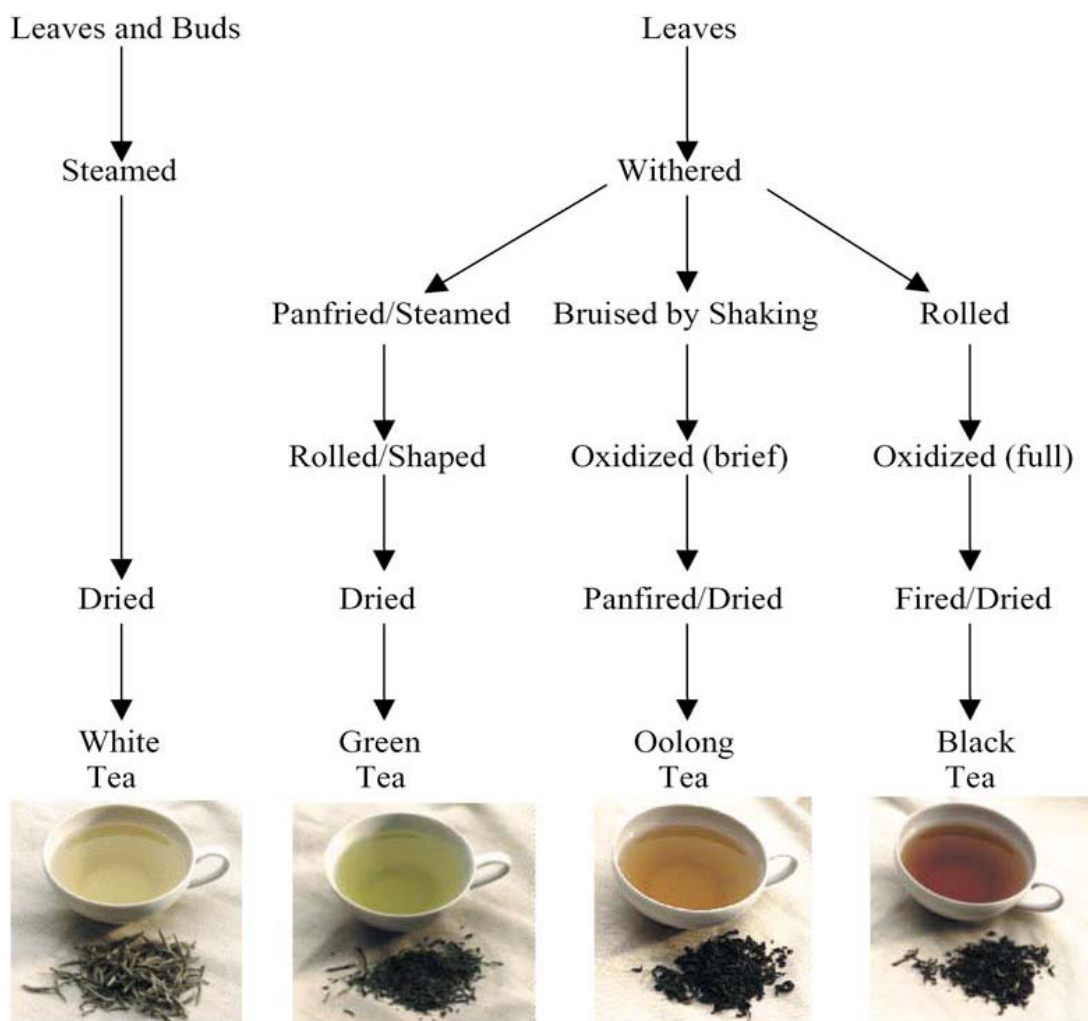
Figure 1. Tea manufacturing processes, showing oxidation steps that increase as tea is converted from white → green → oolong → black (Santana-Rios *et al.*, 2001).

All the different tea types have varying amounts of compounds with beneficial effects (Hou *et al.*, 2009). The major compounds of interest are the flavonols and their oxidation products. Black teas have a profile of flavonoids mainly of the flavanol class. They have high quantities of dimeric theaflavins and polymeric thearubigins; compounds which are virtually exclusive to black tea (Peterson *et al.*, 2004; De Mejia *et al.*, 2009). In processing black teas, the more severe stages of bruising, crushing and breaking allow polyphenol oxidases in the leaves to generate theaflavins, thearubigins and other more complex polyphenols from the endogenous catechins (Santana-Rios, 2001). Theaflavins, thearubigins, catechins, and caffeine are responsible for black tea quality (De Mejia *et al.*, 2009).

Theaflavins and thearubigins account for between 2 - 6% and 15 - 20%, respectively, of the dry weight of black tea solids (Lambert & Yang, 2003; Yang *et al.*, 2007) and antioxidant properties of the theaflavins, by the inhibition of transcription factors in mice microphages, have previously been reported (Łuczaj & Skrzydlewska, 2005). Thearubigins are much less understood (Khan & Muktar, 2007). Amongst the tea types, black tea contains the highest level of caffeine (Cheng, 2006). Caffeine has been associated with reduced tumorigenesis in animal models and other metabolic benefits (Khan and Muktar, 2007), in spite of mixed reports of its effects (Wei *et al.*, 2009). Black tea has also been found to be more efficacious than dexamethasone, a well known antiinflamatory drug against *Trypanosoma brucei* infection in mice, showing its therapeutic potential (Karori *et al.*, 2008).

Green tea contains approximately 30 % (m/m) of catechins in the tea leaves (Weisburger, 1997; Rapaka & Coates, 2006; Zaveri, 2006). Catechins such as (−)epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate (EGCG), (+)catechin, and (+)gallocatechin are known for their therapeutic properties. The most important of these catechins are the epicatechins of which EGCG is the most abundant (Rapaka & Coates, 2006). Epicatechin, epigallocatechin and epigallocatechin-3-gallate contribute significantly to the antioxidant activity of green tea (Rapaka & Coates, 2006; Landi, 2007). The tea leaves also contain approximately 50 mg per cup of tea or 40–50 % of the caffeine content of coffee (Weisburger, 1997) as well as varying amounts of tannins (De Mejia *et al.*, 2009; Wei *et al.*, 2009).

White tea represents a rare and highly-priced tea category less consumed around the world compared to black or green tea. Hence there are fewer compositional studies on this tea and their benefit to human health (Rusak *et al.*, 2008). However, antimutagenic activity of white tea has been reported (Santana-Rios *et al.*, 2001). In contrast to other teas, the higher content of buds in white tea is linked to its higher antimutagens content (Santana-Rios *et al.*, 2001).

In the search for similar health-promoting teas, research on herbal teas has increased over the last two decades. Those of commercial significance are often marketed as caffeine-free, with less or without tannins (Le Roux *et al.*, 2008).

C.   Herbal teas

Aside 'true' teas, consumers' demand for products of natural origin with health-giving properties is fuelling a rapidly growing herbal tea market across the globe. Presently, herbal teas are taking market share from 'true' teas (Anon, 2003), and particularly those with ingredients that purportedly have cancer-fighting properties and can help consumers live longer (Sheehan, 2002). Many beverage companies have consequently added herbal tea lines and are advertising the advantages of their intake, from increase in energy to calmness of the mind (Sharon, 1991; Wong, 1998).

Herbal teas can be made from any plant, and from any part of the plant, including the roots, flowers, seeds, berries or bark, depending on the solubility of the active compounds (Apak *et al.* 2006). A herbal tea is prepared by covering the particular plant material with boiling water and allowing it to steep. Campanella *et al.* (2003) found that an infusion time of 5 min with hot water is optimal for extracting herbal tea antioxidants, after which the antioxidants either precipitate or form micelles decreasing both the antioxidant capacity and polyphenol content of the infusion. Teas like mate (*Ilex paraguariensis*), rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* species) are reported to have multiple biological effects, with proven antioxidant activity (Ivanova *et al.*, 2005).

Various parts of the world have popularised herbal teas believed to exert known beneficial effects. Rooibos and honeybush are popular South African teas (Joubert *et al.,* 2008). Herbal teas including Greek mountain tea, eucalyptus, linden, sage, chamomile, mint and dictamnus are widely consumed in the Mediterranean region (Atoui *et al.*, 2005). In Turkey, medicinal herbals used for teas include scarlet pimpernel, everlasting (immortelle), buckthorn, fumitory, plantain and mallow. Chamomile is widely consumed in Argentina and Portugal (Martins *et al.*, 2001; Satorres *et al.*, 1999). The popular herb, mate, is commonly consumed in South American countries such as Brazil, Paraguay and Uruguay with consumption growing around world markets (Filip, 2000; Bravo *et al.*, 2007; Furgeri *et al.*, 2009; Giulian *et al.*, 2009). Other herbal teas also common on the tea market include French lavender, shrubby germander, yarrow, puncture vine, coriander, sweet basil, lemon balm, coltsfoot, thyme, and marshmallow (Apak *et al.,* 2006).

Additionally, stinging nettle, linden, blackberry, fennel and sage used in tea bags for the preparation of infusions have also been reported (Apak *et al.*, 2006).

D.   Rooibos and honeybush

Two South African herbal teas that have transitioned from local consumption to international commercial application are rooibos and honeybush teas (Joubert *et al.*, 2008). Rooibos (*Aspalathus linearis*) tea is a popular tea enjoyed for its taste and aroma, as well as its health-promoting properties (Van de Merwe *et al.*, 2006; Joubert *et al.*, 2008; Marnewick *et al.*, 2009). International consumption of rooibos has been escalating since 1955. It grew from 524 tons in that year to 10 600 tons in 2003, with exports constituting 6 400 tons (Joubert & Schulz, 2006).

Traditionally, rooibos has been used in South Africa to alleviate infant colic, allergies, asthma and dermatological disorders (Joubert *et al.*, 2008). Young green twigs are harvested with a sickle and transported in bundles for oxidation during which water is added to the green tea material (Cheney & Scholtz, 1963). The wet material is bruised, placed in heaps in the open air and allowed to undergo oxidation at temperatures between 24 and 38°C. The leaves are dried afterwards for 1- 2 days before bagging (Cheney & Scholtz, 1963; Du Plessis & Roos, 1986). Usually, the leaves are brewed in hot water and the liquor is consumed hot or cold (Jaganyi & Wheeler, 2003; Joubert *et al.*, 2008). The use of rooibos as a herbal alternative to conventional tea dates back to the last century when it was consumed as a strong, hot brew with milk and sugar added (Joubert *et al.*, 2008).

The practice of incorporating rooibos extracts in topical cosmetic formulations has become a trend in the cosmetic industry (Joubert & Schulz, 2006). However, much of the research attention on rooibos tea is due to its antioxidant properties (Jaganyi & Wheeler, 2003). The unfermented rooibos tea contains approximately 15 g.kg^{-1} and 77 g.kg^{-1} of aspalathin (a dihydrochalcone and *C*-glycosidic compound) on dry matter and soluble solids bases respectively (Joubert, 1996). This compound has been found to possess bioactive properties including antioxidant (Von Gadow *et al.*, 1997; Krafczyk *et al.*, 2009), antimutagenic (Van de Merwe *et al.,* 2006; Snijman *et al.*, 2007) and antitumour activities (Marnewick *et al.*, 2005) as well as antidiabetic effects (Kawano *et al.,*

2009). Although the tea contains many flavonoids, aspalathin (Figure 2) is exclusive to rooibos (Koeppen & Roux, 1966).
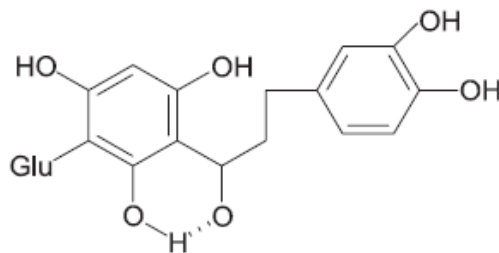
Figure 2. Chemical structure of aspalathin (Koeppen & Roux, 1966).

Another indigenous South African herbal tea that has achieved commercial success is honeybush tea. Produced from different *Cyclopia* spp., the tea has transitioned from limited and localised use to commercial cultivation to meet export market demands (Van der Merwe *et al.*, 2006). The species of commercial importance are predominantly *C. intermedia, C. subternata and C. genistoides* (Le Roux *et al.*, 2008). Traditional honeybush, as a herbal tea, requires that the plant material is subjected to high temperature oxidation to release its characteristic honey-like flavour and dark-brown leaf colour (Joubert *et al.*, 2008). The infusion has been used in South Africa as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis (Joubert *et al.*, 2008). Honeybush infusions are gaining popularity due to the characteristic honey-like flavour, low tannin content, absence of caffeine and potential health effects related to their antimutagenic and antioxidant properties (Joubert *et al.*, 2008).

The modern use of honeybush has in many ways followed the trend of rooibos, and is often enjoyed as an infusion prepared from a mixture with rooibos tea (Joubert *et al.*, 2008). Honeybush tea blends very well with other indigenous plants and fruits. Buchu leaves (*Agathosma betulina*), African potato (*Hypoxis hemerocallidae*) corms and dried marula (*Sclerocarya birrea*) among others, are mixed with the tea to impart additional taste and health benefits (Joubert *et al.*, 2008). Honeybush tea contains mangiferin (Figure 3), as the major monomeric polyphenolic compound (Joubert *et al.*, 2008).
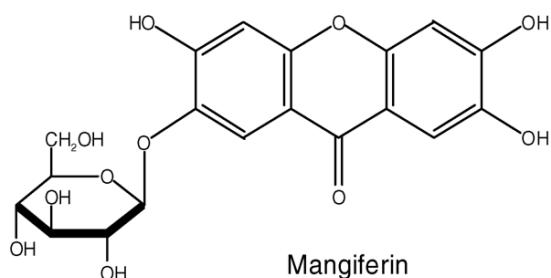
Figure 3. Chemical structure of mangiferin (Pinto *et al.*, 2005).

Mangiferin, a *C*-glycosyl xanthone has been shown to possess various properties (Pinto *et al.*, 2005), including radioprotection against [60]CO gamma radiation (Jagetia & Baliga, 2005), cytoprotection and antigenotoxic potential against cadium chloride toxicity in HepG2 cells (Rao *et al.,* 2009) in addition to its chemopreventive effects against benzo(*a*)pyrene-induced lung carcinogenesis in experimental animals (Rajendran *et al.,* 2008). Additionally, Wauthoz *et al.* (2007) reported many pharmacological activities, including antioxidant, antiallergic antitumour, immunomodulatory, antiinflammatory, antidiabetic, lipolytic, antibone resorption, monoamine oxidase-inhibiting, antimicrobial and antiparasitic properties for mangiferin

E.   Importance of natural antioxidants

The popularity of herbal teas has directed research towards the identification and isolation of new compounds with antioxidant potential and plant constituents that show free radical scavenging activity (Kang *et al.*, 2003; Lee *et al.,* 2003; Sağlam *et al.,* 2007). A large number of studies have been conducted on the traditional pharmacopoeia of indigenous peoples and rural communities within the tropics in order to understand their pharmacology and unveil potentially useful compounds (Cano & Volpato*,* 2004). In developed countries, public interest in the health benefits of phytoceuticals for reducing and inhibiting chronic diseases and ageing has stimulated the nutritional supplement industry to develop functional foods and herbal supplements containing these ingredients (Zhang, 2004). Developments include the formulation of natural antioxidants from plants such as rosemary, sage

and oregano for food, cosmetic and other applications (Arabshahi-Delouee & Urooj, 2007).

Antioxidants have gained nutritional importance due to their ability to quench the harmful effects of reactive free radical species that are generated as part of normal cellular activities (Sun *et al.,* 2007). Free radicals such as reactive oxygen species (ROS) can cause DNA damage, cancer, cardiovascular disease and ageing (Kang *et al.*, 2003; Sun *et al.*, 2007). Experimental evidence has suggested that there are six major ROS which can cause oxidative damage in the human body. These species are superoxide anion radical ($O_2^{.-}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^.$), hydroxyl radical ($^.OH$), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$) (Huang *et al.,* 2005).

In normal cellular systems, elaborate mechanisms exist for the detoxification of some free radical compounds (Lee *et al.,* 2003) by the conversion of ROS or reactive nitrogen species (RNS) to harmless compounds (Huang *et al.*, 2005). For instance, superoxide anion is converted to oxygen and hydrogen peroxide by superoxide dismutase, or reacts with nitric oxide ($NO^.$) to form peroxynitrite. Hydrogen peroxide (H_2O_2) can also be converted to oxygen and water by catalase (Lee *et al.,* 2003; Huang *et al.,* 2005), while glutathione peroxidase destroys toxic peroxides (Lee *et al.,* 2003).

No enzymatic action is known to scavenge peroxyl radical, peroxynitrite, hydroxyl radical and singlet oxygen. This makes the use of non-enzymatic antioxidants and other phytochemicals a valuable option to scavenge these free radicals in order to prevent their harmful effects to living systems (Huang *et al.*, 2005). Non-enzymatic antioxidants can be made *in vivo* or obtained from the human diet (Lee *et al.,* 2003).

The ability of many natural substances such as carotenoids, tocopherols, and polyphenols to act as dietary antioxidants have been reported (Chaillou & Nazareno, 2006; Alarcón *et al.*, 2008). Alarcón *et al.* (2008) reported that herbal infusions can be taken as an effective complement to the antioxidant intake of the human diet. While many compound groups in a given plant material could possess varying levels of antioxidant activities, polyphenols have been singled out as the most important sources of plant antioxidants (Katsube *et al.,* 2004; Bonanni *et al.,*

2007; Helmja *et al.*, 2007). This compound group is widely diverse with extensive distribution in plants (Robards *et al.*, 1999).

Trouillas *et al.* (2003) analysed sixteen water extracts of different plants used as herbal teas in France for their antioxidant, antiinflammatory and antiproliferative properties. It was found that antioxidant activities correlated with the amount of polyphenolic compounds present in the extracts. The phenolic compounds were again suggested as being responsible for the antiinflamatory effects observed, which were exerted through the inhibition of arachidonic acid metabolism. The observed antiproliferative effects were associated with the antioxidant and antiinflamatory properties of the herbal products.

Tiwari and Tripathi (2007) studied the free radical scavenging and metal chelation properties of *Vitex negundo* (Verbanaceae), an aromatic shrub used in India to manage pain, inflammation, and related diseases. They concluded that the leaves of *V. negundo* contain a number of antioxidant compounds that can effectively scavenge various ROS or free radicals under *in vitro* conditions. They also confirmed mild metal chelation, which would result in preventative antioxidant ability (Huang *et al.*, 2005).

F.   Methods for assaying total antioxidant capacity

Many analytical methods have been developed to determine the antioxidant activity in plant materials (Moreno-Sánchez, 2002; Schlesier *et al.,* 2002; Pérez-Jimenez *et al.*, 2008). Although *in vitro* results give a good idea of the protective effects of the antioxidants in such plants (Schlesier *et al.,* 2002), significant differences still exist between *in vivo* and *in vitro* results (Serrano *et al.,* 2007). Whenever *in vivo* studies compare antioxidants, qualitative rather than quantitative comparisons are made (Bartasiute *et al.*, 2007). Moreover, a significant part of the antioxidants contained in plant foods are not analysed in most antioxidant capacity assays, because the extraction process for most assays is not complete (Serrano *et al.*, 2007). Extraction processes using only hot water may fail to completely extract all available compounds possessing antioxidant activity. However, hot water extraction becomes the most appropriate method if one is interested in assessing the antioxidant activity of a given cup of tea. Where a full antioxidant profile is

required to measure the nutraceutical value of a plant, an organic solvent, alone or mixed with water, may be required.

Prior *et al.* (2005) noted a distinct challenge in the assay of antioxidant capacity within biological systems. This is because minimally, four general sources of antioxidants are known to occur in living systems. These are compounds and molecules derived from enzymes (superoxide dismutase, glutathione peroxidase and catalase), from large molecules (albumin, ceruloplasmin, ferritin and other proteins), from small molecules (ascorbic acid, glutathione, uric acid, tocopherol, catotenoids and polyphenols); and from hormones (estrogen, angiotensin and melatonin). The fact that there are multiple sources of free radicals and oxidants and also the differing chemical and physical characteristics of both oxidants and antioxidants appear to heighten the challenge of effectively assessing antioxidant capacities. Moreover, depending on the reaction conditions, antioxidants respond in different manners to different radical or oxidant sources (Prior *et al.*, 2005).

For these reasons, the first International Congress on antioxidant methods was convened in June 2004 to deliberate on the analytical issues related to the antioxidant capacity in foods, botanicals, nutraceuticals, and other dietary supplements (Prior *et al.*, 2005). It became clear that no one assay would truly reflect the total antioxidant capacity of a particular sample and that while there was not going to be a standard relationship between methods, the use of a single or multiple assays could help compare antioxidants from various food products (Prior *et al.*, 2005). In general, the differences in methods often arise by varying the oxidising compounds and the spectroscopic or chromatographic methodology used for monitoring the reaction progress (Chaillou & Nazareno 2006). For routine purposes, however, the most widely used methods are the ferric reducing/antioxidant power (FRAP), 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) or Trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the oxygen radical absorbance capacity (ORAC) methods (Pérez-Jimenez *et al.,* 2008). Traditional methods use stable coloured free radicals, such as a DPPH or an ABTS radical, due to their intensive absorbance in the visible region (Helmja *et al.*, 2007). Additionally, the stability of the DPPH radical allows for easy handling and manipulation during the assay (Frum *et al.*, 2007).

In selecting antioxidant assays, Prior *et al.* (2005) suggested that the mechanism of reaction and its relation to what might occur in the target application is a primary indicator of the suitability of the assay. For a typical antioxidant action, an assay based on a hydrogen atom transfer (HAT) mechanism is preferred to a single electron transfer (SET) reaction mechanism because of the role of the peroxyl ion as the predominant free radical found in lipid oxidation foods and biological systems (Prior *et al.*, 2005). Thus, the biological relevance, endpoint determination, and the method of quantification are indicative factors to be considered (Prior *et al.*, 2005). Table 1 shows a comparison of methods for assessing antioxidant capacity based on simplicity of the assay, instrumentation required, biological relevance, mechanism, endpoint, quantification method, and whether or not the assay is adaptable to measure lipophilic and hydrophilic antioxidants (Prior *et al.*, 2005). Of the methods shown in Table 1, many laboratories prefer to use DPPH due to the stability of the DPPH radical compared to the difficulty in working with actual free radical species which are highly unstable (Prior *et al.*, 2005; Frum *et al.*, 2007; Bartasiute *et al.,* 2007).

Table 1. Comparison of antioxidant assay methods (Prior *et al*., 2005).

Assay name	Simplicity	Instrumentation	Biological relevance	Mechanism	Endpoint	Quantification	Lipophilic and Hydrophilic AUC
ORAC	+ +	+	+++	HAT	Fixed time	AUC	+++
TRAP	---	-- specified	+++	HAT	Lag phase	IC_{50} Lag time	--
FRAP	+++	+++	--	SET	Time varies	ΔOD fixed time	---
CUPRAC	+++	+++		SET	Time	ΔOD fixed time	---
TEAC	+	+	-	SET	Time	ΔOD fixed time	+++
DPPH	+	+	-	SET	IC_{50}	ΔOD fixed time	-
TOSC	-	-	++	HAT	IC_{50}	AUC	---
LDL oxidation	-	+++	+++	HAT	Lag phase	Lag time	---
PHOTOCHEM	+	-- specified	++	unspecified	Fixed time	Lag time or AUC	+++

+, ++, +++ = desirable to highly desired characteristic, -, --, --- = less desirable to highly undesirable. The lipophilic assay is quantified by using the area under curve (AUC) measured over a defined time, and the hydrophilic assay is quantified based upon the lag phase. SET= single electron transfer, HAT= hydrogen atom transfer, ORAC = oxygen radical absorbance capacity , TRAP = total radical-trapping antioxidant parameter, FRAP = ferric reducing antioxidant power, CUPRAC = copper reduction assay, TEAC = trolox equivalent antioxidant capacity, DPPH = 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, TOSC = total oxidant scavenging capacity, LDL Oxidation = Low-density lipoprotein oxidation assay, PHOTOCHEM = photochemiluminescence assay, IC_{50} = half maximal inhibitory concentration, ΔOD = change in optical density.

G.   *Lippia* species

The genus *Lippia* belongs to the plant family Verbenaceae, a family widely distributed in the tropics (Abena *et al.*, 2003). Comprising of several genera such as *Alloysia*, *Verbena*, and *Lantana* (Hennebelle *et al.,* 2008), this family is known for its antitumour, antiinflammatory and antioxidant properties (Kunle *et al.*, 2003; Oliveira *et al.*, 2006; Viljoen *et al.*, 2005). Some species, including *Verbena officinalis* and *Lantana camara* have extensively been used in traditional folk medicine and nutrition (Oliveira *et al.,* 2006; Hennebelle *et al.,* 2008). Increasing research interest in the family Verbenaceae (Oliveira, 2006; Viljoen, 2005; Ghisalberti, 2000) is due to their health and nutritional importance and recently in their potential application for neutraceutical and pharmaceutical formulations (Pascual *et al.,* 2001; Hennebelle *et al.,* 2008). Other traditional applications of plants of the genus *Lippia* include their use as seasonings, as well as for analgesic, and antimalarial properties, and the treatment of respiratory disorders (Abena *et al.*, 2003).

Kunle *et al.* (2003) reported over 200 *Lippia* species that have previously been characterised. They suggested that the high number of species within the genus was likely due to the taxanomic inclusion of other genera sharing common properties. This was previously observed by Grayer & De Kok (1998) when they identified several species of the Labiatae family initially classified as Verbenaceae.

Several *Lippia* species occur in sub-tropical Africa, as well as South and Central America. *Lippia javanica* is reported to occur as a wild plant in South Africa (Viljoen *et al.*, 2005), while *Lippia micromera, L. alba, L. orignanoides* and *L. microphylla* are known to populate South and Central America (Pascual *et al.,* 2001; Abena *et al.,* 2003; Dos Santos *et al.,* 2004). Wherever they occur, species of *Lippia* appear to be widely used by the local populations (Valentin *et al.*, 1995; Pascual *et al.,* 2001). Some of their many medicinal applications such as analgesic, antimicrobial and sedative properties are summarised in Table 2.

Table 2. Traditional uses of selected *Lippia* species (Abena *et al.,* 2003).

Species	Traditional uses
L. alba	Analgesic, sedative, culinary seasoning
L. javanica	Analgesic, antispasmodic
L. micromera	Culinary seasoning, diuretic, respiratory disorders
L. microphylla	Respiratory disorders
L. multiflora	Antihypertensive, hepatic disease, antimalarial
L. nodiflora	Antimicrobial, diuretic, antimalarial
L. origanoides	Culinary seasoning, gastrointestinal disorders

Lippia multiflora is a stout, woody, aromatic perennial shrub. The leaves are simple, large, oblong-lanceolate in shape and green in colour and are thick in texture, with dentate margins and 7-8 pairs of lateral veins and a ridged stem. The leaf base is asymmetric with reticulate venation and an acuminate apex (Kunle *et al.*, 2003). In undisturbed sites, the plant can grow to a height of between 2.7 and 3.9 m. It may be found as a single stand, but mostly occur in colonies of 0.5 to 1.6 m apart. Figure 4 shows natural stands (A) and cultivated *L. multiflora* plants (B) in Ghana. *Lippia multiflora* is used in Africa as an infusion commonly known as 'Tea of Gambia' (Abena *et al.,* 2003). As a herbal tea, it has been consumed since ancient times without any reported adverse effects, therefore, it is considered safe for human consumption (Juliani *et al.*, 2006). This tea is widely used as a sudorific, a febrifuge, a laxative and for the treatment of colic. It is a common children's remedy for fever and constipation and for common colds and chest complaints (Kunle *et al.*, 2003). In most cases, the leaves or aerial parts and flowers are used. They are often prepared as a decoction and administered orally (Kunle *et al.*, 2003).

The presence of *L. multiflora* has been reported in Ghana, Senegal, and Cote d'Ivoire (Avlessi *et al.,* 2005; Juliani *et al.*, 2006). In Ghana, *L. multiflora* is found in the forest savannah, transitional zones, and coastal savannah climates. Commercially viable quantities of *L. multiflora* are found in several localities including Dagomba via Drobonso, Sampa, Ve Golokwati, Homako via Ejura and

Buem Nsuta where the local communities refer to the plant as 'saanumum' or 'saareso nunum' (Acquaye *et al.*, 2001). It is common to see several hectares of uncultivated fields with approximately 60% of the vegetation consisting of natural stands of *L. multiflora* on the outskirts of these communities (Acquaye *et al.*, 2001). Figure 5 shows the various areas where *L. multiflora* grows in Ghana. The listed communities where the plant is found are located within the Brong Ahafo, Ashanti and Volta regions of the country.

A

B

Figure 4. Natural stands (A) and cultivated plants (B) of *Lippia multiflora* respectively in a Ghanaian community and at the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Figure 5. Sites where *L. multiflora* grows in Ghana.

The use of *L. multiflora* as a herbal infusion appears to have been confined only to those communities endowed with natural stands of the plant (Kunle *et al.*, 2003; Avlessi *et al.,* 2005; Juliani *et al.*, 2006). The introduction of the tea to local markets has largely been minimal. Agribusiness in Sustainable Natural African Plant Products (ASNAPP), a development organisation in Africa, recently initiated the commercialisation of the plant on local, regional and international markets. ASNAPP supports local community groups to sustainably harvest the plants for sun-drying, milling and subsequent tea bagging. Harvesting is either by plucking the leaves from whole stems or coppicing the shoots and stripping off the leaves before sun-drying to attain a final moisture content of below 10%. For commercial quantities, coppicing is the preferred method. Sun-dried leaves are coarsely milled (ca 2 mm^2 in size), sieved with a 2 mm mesh size and packaged in standard tea bags each weighing approximately 2.5 g.

H.   Phytochemistry of *Lippia multiflora*

The essential oils from *L. multiflora* represent the class of compounds that impart the aroma and flavour to the tea (Juliani *et al.*, 2006). The oil is characterised by a small amount of sesquiterpenes represented by β-caryophyllene and *trans*-β-farnesene (Pascual *et al.,* 2001), and the monoterpenes, limonene, p-cymene, linalool, and camphor (Pascual *et al.,* 2001; Abena *et al.*, 2003; Kunle *et al.*, 2003). Most studies identify thymol, thymyacetate, p-cymene and carvacrol as key components of the oil (Oladimeji *et al.*, 2000; Bassole *et al.*, 2003; Kunle *et al.*, 2003).

Bassole *et al.* (2003) identified three major components of the essential oil of *L. multiflora* collected from Burkina Faso. These were thymol (29.9%), p-cymene (26.2%) and thymylacetate (11.8%). Other minor components identified were γ-terpinene (4.5%), hexenyl valerate (4.5%), (Z)-isoeugenol (4.0%), α-phellandrene (3.0%), 2-methyl-3-buten-2-ol (2.7%) and myrcene (2.2%). Avlessi *et al.* (2005) also identified limonene, β-caryophyllene, p-cymene, camphor, linalool, α-pinene and thymol in *L. multiflora* samples collected from Benin. The different 'chemotypes' of *L. multiflora* (Oladimeji *et al.*, 2000; Pacual *et al.*, 2001; Bassole *et*

al., 2003) are largely due to differing geographical locations, as well as extraction and analytical methods. Table 3 provides the percentage composition of some compounds identified in the essential oil of *L. multiflora* obtained by conventional hydro-distillation.

Table 3. Chemical constituents of the essential oil of *Lippia multiflora* (Abena *et al.*, 2003).

| Constituent	% Composition of oil
α-Thujene	4.8
Myrcene	3.7
p-Cymene	41.1
Limonene	1.2
γ-Terpinene	2.4
Thymol	19.0
Carvacrol	5.2
Thymylacetate	14.2
β-Caryophyllene	4.0
Trans-β-farnesene	1.6

The biological activities of the essential oil of *L. multiflora* have been widely studied. Valentin *et al.* (1995) found that a hexane extract of *L. multiflora* essential oil exhibited antimicrobial activity against *Pseudomonas aeruginosa* and *Candida albicans.* This is consistent with the observation that thymol and its derivatives show strong antibacterial activities against Gram-negative bacteria and *Staphylococcus camorum* (Bassole *et al.,* 2003). *In vitro* studies have also found the essential oil of *L. multiflora* to inhibit the growth of the malaria parasite, *Plasmodium falciparum* at the trophozoite-schizont stage (Valentin *et al.*, 1995).

Research on the phenolic and pharmacological composition of *L. multiflora* is limited (Pascual *et al.*, 2001; Juliani *et al.*, 2006). Fractionation of a methanolic extract of *L. multiflora* was found to produce a fraction containing polyphenols, flavonoids and phenolic acids (Juliani *et al.*, 2006). The majority of flavonoids

which have been identified in the plant extract are flavones, frequently 6-hydroxylated flavones and methoxyflavones (Pascual *et al.,* 2003).

The major phenolic compound is a caffeic acid derivative called verbascoside (Pascual *et al.*, 2001; Juliani *et al.*, 2006). A number of plants containing verbascoside have been shown to exhibit different therapeutic properties. A Chinese herbal medicinal plant *Buddleja officinalis,* which has been proposed as a therapeutic strategy for the treatment of Parkinson's disease was found to contain verbascoside as a major bioactive compound (Sheng *et al.,* 2002). Backhouse *et al.* (2008) identified the compound in *Buddleja globasa*, a plant widely used in Chile for its pharmacological activities. This compound has also been found in *Ballota nigra,* present in most parts of the world with mild climatic conditions, and used in the treatment of stomach ache, nausea and vomiting (Vrchovská *et al.*, 2007). Frum *et al.* (2007) identified verbascoside in *Halleria lucida* (Scrophulariaceae), a popular medicinal plant found in the Olifants River Mountains in the Western Cape of South Africa. The Zulus in the Natal Province (South Africa) also use this plant in topical applications to relieve ear ache. Other plants sources of verbascoside include *Oxera crassifolia, O. balansae* and *O. pulchella,* and *Faradaya amicorum, F. lehuntei,* and *F. splendida*, of the Labiatae family (Grayer & De Kok, 1998).

The chemical structure (Figure 6) of verbascoside shows four important moieties, namely a dihydroxyphenylethanol, glucose, rhamnose and caffeic acid (Sheng *et al.,* 2002). The caffeic acid and dihydroxyphenylethanol moeities are largely responsible for its pharmacological activities (Juliani *et al.,* 2006).
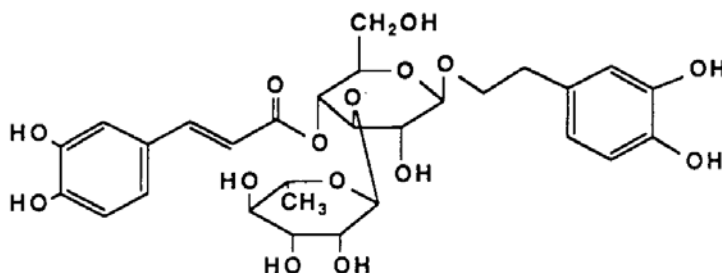
Figure 6. Chemical structure of verbascoside (Sheng *et al.*, 2002).

Verbascoside is reported to have a wide range of activities in biological systems, including neuroprotective effects (Sheng *et al.,* 2002; Zhao *et al.,* 2005), antimicrobial activity (Avila *et al.*, 1999), antiviral activity (Ghisalberti, 2000), as well as antioxidant activity (Juliani *et al.*, 2006; Frum *et al.,* 2007; Obied *et al.,* 2008). Herbert & Maffrand (1991) showed that verbascoside inhibits protein kinase C (PKC) activity, a cellular enzyme that is implicated in signal transduction, cellular proliferation and differentiation associated with tumour formation. To gain understanding into the site(s) on PKC to which verbascoside interacted to produce its inhibitory effect, these researchers assessed the effectiveness of verbascoside relative to another function within the regulatory subunit of PKC. This subunit is recognised by the presence of diacylglycerol and phorbol esters. They suggested that rather than acting on this regulatory subunit, verbascoside might be acting on the catalytic center of PKC to inhibit the activity of the protein kinase (Herbert & Maffrand, 1991).

Sheng *et al.* (2002) examined the inhibitory effect of verbascoside against neurotoxicity induced by 1-methyl-4-phenylpyridinium ion (MPP^+) in cultured PC12 cells. The PC12 cells were exposed to MPP^+ and cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. It was found that MPP^+ at a concentration of 200 µM killed 40–50 % of the cells within 48 h of treatment, but its cytotoxic effects were attenuated in the presence of verbascoside at concentrations of 0.1, 1 or 10 µg.ml^{-1}. Verbascoside at these concentrations exhibited cytoprotective effects in a dose-dependent manner (35%, 46% and 59% of neuroprotection, respectively) and the compound alone did not cause any apparent cytotoxicity (Sheng *et al.,* 2002). Although the cellular and molecular mechanism underlying this protective effect is not fully understood, its antioxidant capacity was suggested as a possible mechanism for the neuroprotective action (Sheng *et al.,* 2002).

Verbascoside was found to be present in large quantities in the nodules of tuberculosis-infected olive trees, raising questions of its potential role in the nodules. Olive tuberculosis caused by *Pseudomonas savastanoi* is a widespread disease in olive-growing areas where affected trees show little vigor, growth reduction and bitter taste of the fruits (Cayuela *et al.,* 2006). The relatively large

amount of verbascoside was attributed to its role in a defense mechanism by the olive tree against the tuberculosis attack (Cayuela *et al.,* 2006).

Verbascoside exhibited antiviral activity against respiratory syncytial virus (RSV) which is implicated in cough-related diseases (Ghisalberti, 2000). Verbascoside and ribavirin were compared for effectiveness in the treatment of RSV under *in vitro* conditions. Although ribavirin is an approved drug for the treatment of RSV infections in humans*,* verbascoside showed a better activity against RSV (Ghisalberti, 2000).

I.   Incidence of microbial contamination of herbal products and methods identified to control contamination

Herbal plant materials typically originate from rural communities and are handled by indigenous people with a lack of adequate infrastructure to ensure the safety of the products. These factors predispose the products to microbial contamination. As primary agricultural products, the risk of contamination becomes even more heightened due to the high probability of plant material coming into contact with the soil. Between the time of harvesting of raw herbal material and their final consumption, the potential for contamination may occur in any of the handling stages including processing, packaging, distribution, retail display, storage and use by the consumer (Gould 1996).

Satorres *et al.* (1999) analysed 100 herbal products sold in Argentina and identified *Clostridium botulinum* spores in *Lippia turbinata, Pimpinella anisu, Alternanthera pungens,* and *Senna acutifolia.* Bianco *et al.* (2008) found that 7.5% of 200 samples of chamomile tea (*Matricaria chamomilla*) in Argentina were contaminated by botulinum spores. Martins *et al.* (2001) also isolated *C. perfringes* spores from the same type of tea in Portugal. *Clostridium botulinum* is the cause of the dreaded botulism, and infant botulism is a major public health concern. Infant botulism is currently the most common form of botulism in Argentina and the United States (Martins *et al.,* 2001). The illness is caused by the botulinum neurotoxin produced by toxigenic clostridia that colonise the large intestine of infants less than 1 year old. The case of *C. botulinum* spore contamination of

herbal teas demonstrates the high risk of using primary agricultural products as food without adequate postharvest control.

Herbal products are also predisposed to aflatoxin contamination, especially in high-moisture and high-temperature storage environments (Craufurd *et al.,* 2006). Under such conditions, contamination can occur even after adequate drying of the plant material. Aflatoxins are mycotoxins produced by species of the genus *Aspergillus.* They are a group of highly toxic, mutagenic and carcinogenic polyketide compounds which present a great public health concern (Baiyewu *et al.,* 2007). When 152 herbal plants from Argentina were analysed, 27% of the plant samples were contaminated with *Aspergillus flavus* (Rizzo *et al.,* 2004). Out of the 152 samples, 52% were contaminated with various other species of the genus *Aspergillus. Aspergillus carbonarius, A. awamorii, A. sclerotium, A. japonicas,* or *A. niger* were present in 89% of the samples studied. Out of 40 strains of *A. flavus* and *A. parasiticus* isolated, 50% produced aflatoxins (Rizzo *et al.*, 2004).

Stickel *et al.* (2009) described two incidents of severe hepatic injury subsequent to the intake of Herbalife® products contaminated with *Bacillus subtilis.* The Herbalife® brand comprises of several herbal products taken for their health-promoting and disease-curing properties. Standard microbiology screening of four samples of Herbalife® products showed growth of Gram-positive rods after 48 h of incubation. Bacteria from three out of four samples were subsequently identified by sequencing the 16S ribosomal RNA gene as belonging to the genus *Bacillus.* These isolates were further identified as *Bacillus subtilis* by gyrB gene sequencing (Stickel *et al.*, 2009). Du Plessis & Roos (1986) recovered *Salmonella* species and high numbers of coliform bacteria including *E. coli* from processed rooibos tea. Table 4 shows the level of contamination in some herbal tea products as reported in the literature.

The health-promoting benefits of herbal teas and their commercial success would be negatively influenced by their microbial contamination levels if the incidences of contamination are not addressed through the use of proper control measures. While consumers assume hot water brewing could attenuate microbial contamination, brewing herbal teas in hot water may present a false sense of safety when one relies on the potentially high temperature of the brewing water

alone (Wilson *et al.,* 2004). Raw herbal tea may be highly contaminated with microorganisms even after brewing at temperatures of 90°C (Wilson *et al.,* 2004). Bacterial spores of the Bacillaceae family are resistant to thermal treatment usually applied in the preparation infusions. Thermal shock may even be counter-productive by stimulating spore germination (Martins *et al.,* 2001; Donia, 2008). Therefore, more elaborate processing regimes are required to effectively address contamination of herbal teas.

Table 4. Incidence of microbial contamination (cfu.g^{-1}) in selected plants used for herbal tea.

Product	Scientific name	Plate count	Yeast /moulds	Coliforms (+ *E. coli*)	Reference
Chamomile	*Maticaria chamomilla*	3.6×10^7	1.1×10^4	1.1×10^3	Donia, 2008
Camomile	*Maticaria chamomilla*	1.0×10^9	1.0×10^6	1.0×10^5	Kolb, 1999
Camomile	*Maticaria chamomilla*	3.2×10^5	NA	NA	Nemțanu *et al.*, 2008
Corn silk	*Zea mays*	NA	1.3×10^6	NA	Martins *et al.*, 2001
Herbal blend	-	5.0×10^6	1.3×10^4	NA	Halt & Klapec, 2005
Linden	*Tilia grandifolia*	NA	1.6×10^4	NA	Martins *et al.*, 2001
Peppermint	*Mentha piperita*	2.6×10^8	2.0×10^4	1.1×10^3	Donia, 2008
Rooibos	*Aspalathus linearis*	2.4×10^6	NA	1.7×10^5	Du Plessis & Roos, 1986
Thea	*Thea sinensis*	6.0×10^5	1.5×10	2.2×10^2	Donia, 2008

NA = Not analysed

Most microbial contamination of food stuffs can be prevented by using a range of techniques that inhibit microbial growth such as chilling, freezing, drying and curing (Gould, 1996). Other techniques include pasteurisation, sterilisation and irradiation for inactivating microorganisms. There are still others that act by restricting access of microorganisms to the food system. Restrictive techniques include aseptic processing and packaging (Gould, 1996). For each product however, it is important to identify suitable control techniques and to define control parameters taking into consideration prior handling and post-harvest practices that may predispose them to contamination. Generally, control techniques such as food irradiation, ozonation and steam sterilisation have proven very useful for a wide range of products (Crawford & Ruff, 1996; Kim *et al.*, 2008).

Food irradiation, also known as 'cold pasteurisation', remains a controversial technology as consumers are generally concerned over the negative publicity that forewarns of unpredictable radioactive side-effects (Crawford & Ruff, 1996). In this technology, radiation is directed to penetrate deep into products to generate desirable effects such as the reduction of microbial contaminants (Ivanov *et al.*, 2001; Cia *et al.*, 2007) or the liberation of fixed antioxidant compounds to potentially enhance their bioactivity (Kim *et al.,* 2008). In some cases, radiation treatment can result in chemical changes in irradiated herbal products. Jilan *et al.* (1995) observed radiolytic decomposition in 16 out of 40 herbs subjected to irradiation treatment at 10 kGy to control microbial contaminants. A moisture content of 9% that is acceptable for dried herbal products appears to worsen the negative effects of irradiation on herbal products (Fang & Wu, 1998). Despite being an effective tool for preventing or reducing microbial contamination, food irradiation is currently sparingly used (Crawford & Ruff, 1996).

The use of ozone to control microbial contamination in food products has been investigated (Allende *et al.,* 2006; Selma *et al.,* 2008; Najafi & Khodaparast, 2009) as ozone has a wide antimicrobial spectrum, making it an attractive option for the food industry (Tiwari *et al.*, 2009). The bactericidal, fungicidal and general disinfection and oxidising effects of ozone treatments are used worldwide to extend the shelf-life of many perishable products (Das, 2008). Some produce handlers and processors use ozone for water sanitation, cold room air treatment, and other

post-harvest applications (Najafi & Khodaparast, 2009). However, its application in countries such as the United States has not been widespread, in spite of the fact that in 1982 the Food and Drug Administration granted a 'generally-regarded-as-safe' (GRAS) status for its use in bottled water (Guzel-Seydim *et al.,* 2004) and subsequently in 2001 approved ozone as a direct addition to food (Tiwari *et al.*, 2009).

In their evaluation of the effect of ozone on microbial populations on date fruits in Iran, Najafi & Khodaparast (2009) demonstrated the ability of ozone to act as an antimicrobial agent against a variety of microbes. Total counts of mesophilic microorganisms, coliforms, *Staphylococcus aureus* and fungi were shown to be lower than those of untreated samples. In another study on dried figs, Öztekin *et al.* (2006) found that from an initial count of 1.46 log cfu.g^{-1} of coliform bacteria, treatment after 3 and 5 h with ozone at 1 ppm concentration, reduced coliform counts to 0.39 and 0.23 log cfu.g^{-1}, respectively. At 5 and 10 ppm levels, no coliform bacteria could be counted. An initial fungal count of 1.46 log cfu.g^{-1} decreased significantly as the ozone concentration increased. Similarly, Akbas & Ozdemir (2008) also reported 0.9 and 2.7 log reductions, respectively, in *E. coli* and *Bacillus cereus* populations on dried figs after treatment with 0.1 ppm of ozone. At 1.0 ppm, 3.5 log cfu.g^{-1} reduction was achieved for both bacteria. Although no significant changes ($P > 0.05$) occurred in the organoleptic properties of the dried figs (Akbas & Ozdemir 2008), the effects on the bioactive compounds were not reported and the time of 10 h taken to achieve these reductions would normally be too long in a commercial processing environment compared to other treatments. Moreover, detrimental changes in active ingredient content and physical quality attributes have been proven after ozone treatment. Tiwari *et al.* (2009) investigated the effect of ozone on the anthocyanin content and colour of blackberry juice. Ozone had a significant effect on the degradation of both the anthocynin content and the colour of the juice.

The application of ozone to control microbial contamination in herbal products is not widely reported. Zhao *et al.* (2009) listed this non-heating sterilisation technique as having been applied to only a few plant materials. In instances when it was applied to red ginseng powder, ozone was less effective

compared to gamma irradiation in controlling microbial contaminants (Byun *et al.*, 1998). It was shown that gamma irradiation at 7.5 kGy resulted in a reduction of viable aerobic bacteria, moulds and coliforms below detectable levels, while ozone treatment for 8 h up to 18 ppm did not sufficiently eliminate the microorganisms in the red ginseng powder (Byun *et al.*, 1998).

Steam sterilisation is also regarded as an important microbial control measure for a variety of food products. A number of methods specifying temperature-time regimes of steam treatments have been designed for specific products. Inglis & Lark (2007) developed and patented a method for herbs and spices (International Patent Application number WO 96/36237) whereby herbs, spices and other materials are cooled below 0°C, rapidly heated to a sterilisation temperature and re-cooled to approximately room temperature. The heating and re-cooling steps are performed at a pressure of less than 1 bar (100 kPa). Schweiggert *et al.* (2005) conducted sterilisation experiments in which they thermally treated a range of spices and plant materials leading to low counts of aerobic bacteria, aerobic spore-forming bacteria, *Escherichia coli*, coliforms, *Staphylococcus aureus, Bacillus cereus,* fungi, and sulphite reducing clostridia. *Salmonella* spp. or aflatoxins were not detected in any of the products after treatment.

Subjecting fresh coriander and lyophilised coriander powder to blanching temperatures of 90°C and above for between 5 and 10 min resulted in markedly low counts for aerobic spore-forming bacteria. Fungi were practically eliminated from all the samples of coriander tested (Schweiggert *et al.,* 2005), a product which is typically associated with high viable aerobic counts of more than 10^6 cfu.g^{-1} (McKee, 1995). The result of the coriander sterilisation experiment is summarized in Table 5. Waje *et al.* (2008) also achieved 3-log reduction in viable aerobic counts, 2-log reduction in yeasts and mould counts and 4-log reduction in total coliform bacteria after steam-treating ground black pepper at 100°C for 16 min. Du Plessis & Roos (1986) reported a decrease in viable aerobic counts from between 1.2 and 2.9 x 10^5 cfu.g^{-1} by 3 to 4 log reductions in rooibos tea subjected to steam treatment at 99.5^{o}C for 2 min. Coliform bacterial counts also decreased from 2.7 x 10^5 in untreated samples to <10 cfu.g^{-1} in steamed samples (Du Plessis & Roos,

1986). Interestingly, steam treatment ≥90 °C for 2 min has been found to increase the antioxidant activity of rooibos tea (Standley *et al.*, 2001).

Table 5. Microbial counts (cfu.g^{-1}) of fresh coriander (CO) plants and lyophilised coriander powders subjected to different steam treatments (Schweiggert *et al.,* 2005).

Process parameter	Aerobic count	Aerobic spore forming bacteria	Yeasts	Moulds
Coriander (during blossoming)	5.20 x10^7	1.12 x10^4	4.50 x10^6	–
Heating, mincing CO 1 (90°C, 5 min; steam)	1.08 x10^5	1.30 x10^3	–	–
CO 2 (90°C, 10 min; steam)	8.00 x10^4	2.20 x10^3	–	–
CO 3 (100°C, 1 min; steam)	1.76 x10^5	2.20 x10^3	–	–
CO 4 (100°C, 5 min; steam)	1.24 x10^5	1.10 x10^3	6.00 x10^2	–
CO 5 (100°C, 7 min; steam)	1.32 x10^5	3.00 x10^2	7.00 x10^2	–
CO 6 (100°C, 10 min; steam)	1.56 x10^5	4.00 x10^2	–	–
CO 7 (not heated)	6.80 x10^6	3.40 x10^3	1.20 x10^3	–
CO 8 (100°C, 7 min; steam)	1.80 x10^4	–	–	–
CO 9 (100°C, 7 min; water)	8.00 x10^3	–	–	–

–: below detection limits

Steam treatment appears to present the most practical method to ensure microbial load reduction on a product like *L. multiflora.* This process which involves heating the dried plant material at elevated temperatures, excludes the use of

chemicals and is, therefore, an approved method for products that are marketed 'organic' (Das, 2008), or 'natural' as in the case of *L. multiflora*. After comparing the results of chlorine and ultraviolet treatments with those of steam treatment, Du Plessis & Roos (1986) recommended the use steam for microbial decontamination in the processing of rooibos tea (Du Plesisis & Roos, 1986).

It is important to deploy a technique that is applicable at the community setting which farmers could easily adopt without the need for the acquisition of sophisticated equipment. Generally, steam sterilisation satisfies the various factors including cost, integrity and efficacy of the resulting product that are considered when selecting an appropriate method for microbial load reduction (Das, 2008). Because the majority of herbal products and a wide range of beverages are marketed for their antioxidant properties, sterilisation treatment are designed such that the antioxidant effects are not compromised (Das, 2008).

J.   Regulation of the microbial content of herbal infusions

At the international level, the World Health Organisation's guidelines for assessing quality of herbal medicines specifies a maximum viable aerobic bacterial count of 10^7 cfu.g^{-1} for herbal materials to which hot water is added before consumption (WHO, 2007). This guideline also requires that the level of yeast and mould, as well as other enterobacteria should not exceed 10^4 cfu.g^{-1} and that the *E. coli* content be kept below 10^2 cfu.g^{-1}. *Salmonella* should be absent in 25 g of the sample (WHO, 2007). Monographs of the US pharmacopoeia sets fungal limits for raw materials of natural origin to a maximum of 2 x 10^2 cfu.g^{-1} (Donia, 2008). However, the legal situation for herbal preparations varies from country to country, and in most developing countries there is hardly any legislative framework (WHO, 2001).

In the European Union (EU) where regulations exist, there is no consistency in the regulatory framework (Gulati & Ottaway, 2006). Furthermore, such regulations usually consider the entire range of herbal medicines with few specifics for herbal teas. The European pharmacopoeia sets a maximum of 10^5 cfu.g^{-1} for aerobic counts and 10^3 cfu.g^{-1} for yeasts and moulds, as well as enterobacteria

(Heinrich *et al.*, 2004 in Gurib-Fakim, 2006). This appears more stringent than the WHO requirement. However, members of the European Herbal Infusion Association (EHIA) drawn from Belgium, Britain, Germany, Italy, Netherlands and Spain have established microbial criteria specifically for herbal infusions. The EHIA requirement stipulates 10^7 cfu.g^{-1} for total plate count, 10^2 cfu.g^{-1} for *E. coli*, 10^5 cfu.g^{-1} for moulds and an absence of *Salmonella* in 25 g of the sample (Kolb, 1999), which is more consistent with the WHO requirement.

Most African countries have no specific microbial guidelines for herbal teas largely because the industry is underdeveloped, informal and unregulated. The Ghana Standards Board (GSB) has no microbial specifications for herbal teas and as such none exists for *L. multiflora*. In South Africa where the herbal tea industry is more developed, the microbiological specification for bulk rooibos and honeybush teas sets the total viable counts to a maximum of 7.5×10^4 cfu.g^{-1} (Department of Health, 2008). In both teas, *Salmonella* should be absent in a sample of 25 g analysed and *E. coli* counts shall not exceed 20 cfu.g^{-1} for rooibos and shall be absent in 1 g of honeybush tea (Department of Health, 2008). The ASNAPP sanitary specification for natural products also sets a maximum of 10^5 cfu.g^{-1} for viable aerobic count, 10^5 cfu.g^{-1} for yeasts and moulds, 10^2 cfu.g^{-1} for *E. coli* and an absence of *Salmonella* in 25 g of *L. multiflora* tea leaves (ASNAPP-Rutgers, 2006). The South African specification and the ASNAPP in-house criterion are understandably stringent due to the high potential of microbial contamination associated with such farm based products.

K.   Conclusions

Herbal teas represent one of the fastest growing segments of the tea industry. The growth is primarily due to increased consumer awareness of the health-promoting properties of most herbal plants and the desire to seek alternative sources of health-care. Increasingly, the nutraceutical and the beverage industries will apply herbal ingredients in product formulation in the future to satisfy the increasing consumer demand.

Scientific evidence demonstrates that herbal products used as tea contain antioxidant compounds capable of promoting health and that the consumption of herbal teas has been associated with reduced risk of contracting cardiovascular diseases and cancer. Polyphenolic compounds which are widely sought after, constitute the most important sources of plant antioxidants. They are extensively distributed in various plants in nature. Some plants such as those of the Verbenaceae and Labiatae have engaged the attention of researchers due to the high levels of useful phytochemicals they possess.

Lippia multiflora (Verbenaceae) has been used in tropical Africa as a herbal tea and for the treatment of malaria. It has also been used as a sudorific, a febrifuge and for the treatment of colic. Due to the nature of handling herbal materials in rural communities where they are produced, the potential for contamination has been identified. In the case of *L. multiflora,* the need for developing a microbial load reduction process that has little or no effects on its health-promoting properties has become important as efforts to commercialise the tea increases. Equally significant is the need to understand the antioxidant potential of the tea which appears to underpin its health-promoting properties.

This study will address the development of a steam pasteurisation regime for *L. multiflora* herbal tea. The effect of this pasteurisation treatment on viable aerobic counts, yeast and moulds as well as on coliform bacteria will be assessed to provide an initial overview of the effectiveness of pasteurisation in reducing the microbial load. An HPLC method will also be developed to first identify and quantify the major antioxidant compound in the hot water infusion of *L. multiflora*, and to evaluate the effect of the pasteurisation treatment on the antioxidant activity of the infusion.

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CHAPTER 3

CHARACTERISATION AND BENCHMARKING OF *LIPPIA MULTIFLORA* HERBAL TEA WITH ROOIBOS AND HONEYBUSH TEAS

ABSTRACT

An HPLC method was developed and optimised for the identification and quantification of verbascoside, the major antioxidant compound of *Lippia multiflora* herbal infusion. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used to confirm the presence of the compound in the infusion. Ascorbic acid was used as a stabilising agent during the quantification process to prevent the degradation of verbascoside. The hot water infusion of *L. multiflora* was compared to those of *Aspalathus linearis* (rooibos) and *Cyclopia* sp. (honeybush), two South African herbal teas with commercial success, on the basis of their soluble solids and total polyphenol contents, as well as on their antioxidant activities. Antioxidant activities were assessed in terms of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$\cdot$) scavenging and ferric reducing activities. In addition to verbascoside, another compound with the same parent and fragment ions as verbascoside was present in the infusion. A 100 ml infusion of *L. multiflora* had significantly ($P < 0.05$) higher soluble solids and total polyphenol contents, and antioxidant activities than those of rooibos and honeybush. The rooibos infusion showed significantly ($P < 0.05$) higher soluble solids and total polyphenol contents, as well as antioxidant activities than honeybush. On the basis of soluble solids, rooibos showed a significantly ($P < 0.05$) higher total polyphenol content and a lower ferric-reducing activity than *L. multiflora*. Both teas, however, did not differ significantly with respect to the DPPH antioxidant activity. The identification and quantification of verbascoside in *L. multiflora* infusion, as well as the relatively higher antioxidant contents compared to rooibos and honeybush should provide the basis for future studies on the therapeutic application of this herbal tea. Also, verbascoside could potentially form the basis for future quality control of *L. multiflora*.

INTRODUCTION

The perceived health benefit that can be derived from the consumption of herbal teas is fueling a rapidly growing market for this category of teas (Sharon, 1991; Wong, 1998). Of particular interest in recent times are those herbal teas purported to have antioxidant constituents with cancer-fighting properties, and those that can help consumers live longer (Sheehan, 2002; Kang *et al.*, 2003). One such plant with the potential for growth is *Lippia multiflora.* It is a herbaceous plant which is widely distributed in West and Central Tropical Africa (Avlessi *et al.,* 2005). It belongs to the plant family Verbenaceae which typically colonise the tropics (Pascual *et al.*, 2001; Kunle *et al.*, 2003). Several species of the genus *Lippia* occur in South and Central America (Dos Santos *et al.,* 2004; Juliani *et al.,* 2006) where they are widely used for nutritional and pharmacological purposes by the local communities (Valentin *et al.*, 1995; Pascual *et al.,* 2001). They are particularly noted for their antitumour, antiinflammatory and antioxidant properties (Kunle *et al.*, 2003; Viljoen *et al.*, 2005; Oliveira *et al.*, 2006). Additionally, many species of the genus *Lippia* have been used as seasonings and for analgesic and antimalarial purposes, as well as for the treatment of respiratory disorders (Abena *et al.*, 2003).

The dried leaves of *L. multiflora* are infused to produce 'bush tea' or 'Gambian bush tea' (Lamaty *et al.*, 1990; Kunle *et al.,* 2003; Avlessi *et al.,* 2005). This tea is used as a sudorific, a febrifuge, a laxative and for the treatment of colic (Kunle *et al.,* 2003). It is a common children's remedy for fever and constipation and it is also useful for common colds and chest complaints (Kunle *et al.*, 2003). In French Guinea, a decoction of the leaves is used for fumigation, for bath and as a local hot application for ear troubles (Kunle *et al.*, 2003).

While the essential oils of *L. multiflora* have been studied, very little information is available in the literature on the polyphenolic composition of the infusion. Juliani *et al.* (2006) tentatively identified two major polyphenols, verbascoside and nuomoside in *L. multiflora*. Verbascoside (also called acteoside) is a caffeic acid derivative (Figure 1), known for its wide range of biological activities including neuroprotective effects (Sheng *et al.,* 2002; Zhao *et al.,* 2005), antimicrobial activity (Avila *et al.*, 1999), antiviral properties (Ghisalberti, 2000), and

antioxidant properties (Juliani *et al.*, 2006; Obied *et al.,* 2008). The strong iron-chelating activity of verbascoside contributes to its ability to protect plasmid DNA, *in vitro,* against hydroxyl radicals induced by Fenton type reactions (Zhao *et al.*, 2005). *In vitro* trials have also confirmed the antitumour activity of verbascoside as a result of its inhibitory effect on protein kinase C (PKC) (Herbert & Maffrand, 1991). These biological activities appear to support the potential application of *L. multiflora* for promoting the well-being of consumers.
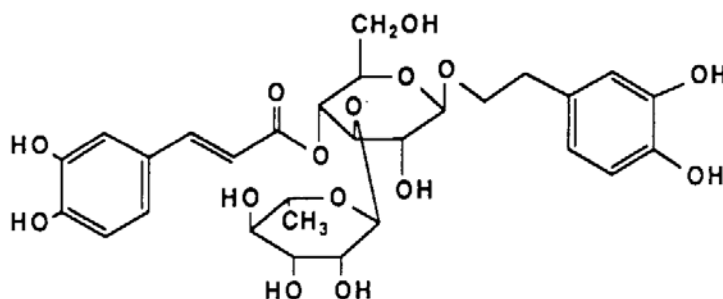
Figure 7. Chemical structure of verbascoside.

Although the long traditional use of *L. multiflora* herbal tea has been reported (Oladimeji *et al.,* 2000; Pascual *et al.*, 2001; Avlessi *et al.*, 2005; Juliani *et al.*, 2006), determination of its verbascoside content is yet to receive research attention, and comparative studies to place the health attributes of this tea into perspective in line with what presently exits on the herbal tea market remain undone. The two South African herbal teas, rooibos and honeybush, have received recognition on the global market for their health promoting properties, especially antioxidant properties (Joubert *et al.*, 2008a). Therefore, the present study was aimed at identifying and quantifying verbascoside and associated compounds in the hot water infusion of *L. multiflora* herbal tea. The antioxidant activity of the infusion was then benchmarked against those of rooibos and honeybush in an effort to carve a possible commercial pathway for *L. multiflora* which is a relatively new entrant to the commercial herbal tea market. An HPLC method was developed and optimised for the identification and quantification of verbascoside and its presence confirmed by liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques. Antioxidant activities were measured by the 2,2-diphenyl-1-

picrylhydrazyl (DPPH) and ferric reducing / antioxidant power (FRAP) assays. The three teas, *L. multifora*, rooibos and honeybush, were also assayed for their soluble solids and total polyphenol contents.

MATERIALS AND METHODS

Chemicals and reagents

Verbascoside (99.9% purity) was purchased from Extrasynthese (Genay, France; product code of 49945; lot number 08021501). The chemicals used for HPLC analysis were glacial acetic acid (Fluka, Buchs, Switzerland), HPLC grade acetonitrile 'Far UV' for gradient analysis (Merck, Darmstadt, Germany), dimethyl sulfoxide and ascorbic acid (Sigma Chemical Co., St. Louis, USA). Distilled water was de-ionised using a Modulab purifier (Continental Water Systems Corp., San Antonio, USA). The de-ionised water was further purified with a Milli-Q academic water purifier (Millipore, Bedford, USA) to obtain HPLC-grade water. A 0.1% formic acid solution was prepared by pipetting 2 ml of a stock formic acid solution (>98%) into a 2 litre volumetric flask and made to the mark with HPLC-grade water. This was well-mixed and filtered using an all-glass filter apparatus and a Millipore HV 0.45 mm membrane filter. The reagents used for the quantification of the total polyphenol content of the infusions were Folin-Ciocalteu's phenol reagent (Merck), anhydrous sodium carbonate (Saarchem, Gauteng, South Africa), and gallic acid (Sigma Chemical Co.). Trolox, sodium acetate, TPTZ (2,4,6-tripyridy-*s*-triazine), hydrochloric acid (HCl) and iron (III) chloride ($FeCl_3$) (Sigma Chemical Co.) were used for assaying antioxidant activities.

***Lippia multiflora* samples**

Five tea samples of *L. multiflora* were obtained from Agribusiness in Sustainable Natural African Plant Products (ASNAPP), South Africa. The samples originated from wild plants harvested in 2006 over a one month period in a community in Ghana and sun-dried before milling and packaging into tea bags. Five different

brands of rooibos (*A. linearis*) were purchased at a supermarket in Stellenbosch, South Africa. Five organically-produced honeybush samples (all blends of *C. intermedia* and *C. subternata*) were obtained from Cape Natural Tea Products (CNTP), a commercial tea manufacturer in South Africa.

Preparation of tea infusions

Lippia multiflora infusions representing 'cup of tea strength' were prepared from the five samples in single extractions. For each sample, 2 g of plant material was weighed with a Mettler (AE 160) balance into a 200 ml glass beaker. Distilled water (150 ml), brought to boiling in an LG microwave (Model number MS-4025ME), was then added to the plant material and the mixture stirred for 5 min using a magnetic stirrer. After 5 min, the aqueous extract was strained into a 200 ml volumetric flask and allowed to cool to room temperature. The remaining droplets of infusion on the leaves were then washed down with distilled water into the volumetric flask to make up the volume. The infusion was filtered using a Whatman No.4 filter paper and aliquots (1.5 ml) of each infusion were measured into several micro-centrifuge vials and kept frozen at –20°C until further analysis.

Both honeybush and rooibos teas were similarly extracted. Extraction using the infusion method was used because infusion represents the most widely consumed form of herbal teas (Billa *et al.,* 2008).

Determination of soluble solids content

The soluble solids content of each of the infusions was gravimetrically determined in triplicate. A 10 ml aliquot of each infusion was measured into a clean and previously weighed nickel moisture dish. The combined weight of the moisture dish and the infusion was then recorded. Prior to placing the dish and infusion into a vacuum oven, the moisture dish was placed on a boiling waterbath to evaporate moisture to near dryness. The moisture dish was then placed in a laboratory oven at 100°C for 1 h to dry completely and the final weight was recorded. The

difference between the initial and final weight of the moisture dish was calculated and expressed mg.100ml^{-1} of infusion.

Method development

Equipment

The HPLC set-up consisted of an Agilent 1200 system (Agilent, Santa Clara, USA) equipped with a quaternary pump, an autosampler, an in-line degasser, a column thermostat, and a diode array detector. Chemstation software for LC 3D systems (Agilent) was used to control the system and record data.

Optimisation of HPLC conditions

Optimisation of the HPLC conditions involved evaluating six different columns, each separately paired with two organic phases for separating the constituent compounds in the *Lippia multiflora* infusion. The organic phases included methanol and acetonitrile. Formic acid (0.1% v/v) was used as the aqueous mobile phase to enhance resolution and eliminate peak tailing of the constituent compound. Columns evaluated were Zorbax Eclipse XDB–5 µm C18 (Agilent Technologies, Waldbronn, Germany); Gemini 5 µm C18 110 Å (Phenomenex, Santa Clara, USA); Synergi 4 µm MAX–RP 80 Å (Phenomenex); Synergi 4 µm POLAR–RP 80 Å (Phenomenex); Synergi 4 µm Fusion–RP 80 Å (Phenomenex) and Prodigy 5 µm Phenyl–3 100 Å (Phenomenex). The dimensions of all six columns were 150 x 4.6 mm each. A scouting gradient changing linearly from 10% to 80% organic phase in 30 min at a flow rate of 0.8 ml.min^{-1} was used. Separation was performed at 30°C. Absorbance was measured at four different wavelengths, i.e. 225, 288, 330 and 350 nm, to optimise the number of peaks. After selection of the column and organic solvent, the gradient was optimised to reduce the analysis time.

HPLC analysis of infusion

Quantification of verbascoside was performed by HPLC-DAD. Separation was performed at 30°C on an Agilent Gemini 5 µ C18 110 Å (150x 4.6 mm) column protected with an RP/C18 guard column (Vici-AG International, Schenkon,

Switzerland), using gradient elution (Table 1) with acetonitrile (solvent A) and 0.1% aqueous formic acid (solvent B) and a flow rate of 0.8 ml.min^{-1}. The injection volume was 10 µl and detection was at 330 nm. Verbascoside in the *L. multiflora* infusion was identified by comparing its retention time and UV-Vis spectrum with that of the verbascoside authentic standard. Further confirmation was made using tandem mass spectrometry techniques.

Table 6. Mobile phase gradient table.

Time (min)	Solvent A (%)	Solvent B (%)
0	20	80
5	20	80
9	22	78
10	22	78
15	23	76
18	60	40
20	20	80
30	20	80

Solvent A: acetonitrile; Solvent B: 0.1% aqueous formic Acid

Stability of verbascoside

The use of 10% ascorbic acid to stabilise verbascoside was investigated. Ascorbic acid was prepared by weighing approximately 2 g into a 20 ml volumetric flask, and distilled water added to dissolve and filled up to the mark. To 950 µl portion of the *L. multiflora* infusion, 50 µl of the ascorbic acid solution was added before HPLC injection. Stability over a 24 h period was investigated.

Standard calibration curve and quantification of verbascoside

A stock solution of verbascoside was prepared by dissolving 2.41 mg in 2.4 ml dimethyl sulfoxide (DMSO). The working solution consisted of 200 µl of the verbascoside stock solution, 50 µl of 10 % (w/v) ascorbic acid solution and 750 µl of DMSO. Aliquots of the working solution (100, 50, 20 and 5 µl) were diluted with 400, 450, 480 and 495 µl of DMSO, respectively, to obtain the standard series

used for calibration. Quantification was achieved by the comparison of the peak area of the verbascoside present in the *L. multiflora* infusion and that of the verbascoside standard. Compounds 1, 2, 3 and 4 were quantified in terms of verbascoside equivalents.

Liquid chromatography and tandem mass spectrometry (LC–MS/MS)

LC–MS/MS analysis was performed using a Waters API Quattro Macro apparatus (Waters, Milford, USA), employing electrospray ionisation in the negative ion mode. The set-up had the following parameters: nitrogen was used as the cone (50 l/h) and desolvation (450 l/h) gas; desolvation temperature, 380°C; source temperature, 120°C; capillary voltage, 3500 V; cone voltage, 25 V, and sample injection volume was 5 µl. All other parameters were the same as for the HPLC analysis. LC-MS/MS analysis was performed using collision energy of 20 arbitrary units. The identity of the verbascoside peak as depicted in the HPLC chromatogram was further confirmed by comparing its mass spectra and fragmentation patterns with that of the verbascoside standard.

Determination of total polyphenols

The total polyphenol content was determined according to a modified version of the method of Singleton & Rossi (1965). A 1:10 Folin-Ciocalteau reagent (diluted with de-ionised water) and a 7.5% (w/v) aqueous solution of sodium carbonate were used. A stock solution of gallic was prepared and diluted in deionised water to give a standard series of 10, 20, 40, 60, and 100 mg.l^{-1}. *Lippia multiflora* and rooibos infusions were prepared for analysis by diluting 600 µl to 5 ml with deionised water. A dilution of 600 µl to 1 ml with deionised water was used for honeybush. Aliquots (20 µl) of the infusions, standard and distilled water controls were pipetted into allocated wells of a 96-well microplate. To each of these, 100 µl of the Folin-Ciocalteau reagent was added using a Gilson multi-channel pipette. Within 8 min, 80 µl of the Na_2CO_3 solution was added to each of the reaction mixtures. Absorbance values were read on a BioTek SynergyHT microplate-reader equipped

with Gen5 Secure software. By using the standard series, the corresponding total polyphenolic content was calculated for each of the infusions and the results expressed in millgrams of gallic acid equivalents (GAE) per 100 ml infusion. All determinations were done in triplicate.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Free radical scavenging activity was determined using the method of Rangkadilok *et al.* (2007) with slight modification. The DPPH solution was prepared by dissolving 3.94 mg of the DPPH radical in 100 ml methanol. The solution was covered with aluminum foil and kept in the dark until use. The radical stock solution was prepared fresh daily. Honeybush infusion was used without dilution. Dilutions of 120 µl to 1 ml and 80 µl to 1 ml were used, respectively, for rooibos and *L. multiflora* infusions. By using a 96-deep well microplate, 25 µl of the standards, distilled water blanks and tea infusions were measured into their allocated wells and 255 µl of the DPPH added. The plate was sealed with a silicon mat to prevent evaporation of the methanol and incubated in a dark cupboard. After 2 h of incubation, 200 µl of the reaction mixture was measured into the corresponding wells on a flat-bottom microplate and the decrease in absorbance monitored at 515 nm using a BioTek SynergyHT microplate-reader equipped with Gen5 Secure software. All determinations were done in triplicate and results expressed as µmol Trolox.100 ml^{-1} cup of infusion.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power assay (FRAP) was performed, using Trolox as standard with a concentration range of 50–600 µM. The freshly prepared FRAP reagent contained 100 ml of 300 mM acetate buffer (pH 3.6), 10 ml of 10 mM TPTZ (2,4,6- tripyridy-*s*-triazine) in 40 mM HCl, and 10 ml of 20 mM FeCl$_3$. Honeybush infusion was used without dilution while 120 µl aliquots of rooibos and *L. multiflora* infusions were diluted to 1 ml deionised water before used. Aliquots (20 µl) of the blank (dH2O), standards and tea samples were added into their allocated wells in a 96-well microplate, followed by the addition of 180 µl of the FRAP reagent using a

Gilson multi-channel pipette. Increases in absorbance due to the formation of a coloured TPTZ-Fe^{2+} complex were monitored spectrophotometrically at 593 nm, after a reaction time of 4 min at 37°C. All determinations were done in triplicate and results expressed as µmol Trolox.100 ml^{-1} of infusion.

Statistical analysis

Analysis of variance was performed on all variables accessed using the GLM (General Linear Models) procedure of SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC, USA). Student's t-least significant difference was calculated at the 5% level to compare treatment means.

RESULTS AND DISCUSSIONS

Method development

Verbascoside, a caffeic acid derivative compound, has a maximum absorbance, $\lambda_{max,}$ at 330 nm (Grayer & De Kok, 1998). This wavelength is important when working with compounds related to verbascoside. On the other hand, 225 nm is normally used to detect many organic compounds, while flavanols and flavonones are detected at 280 nm and flavonols and flavones are detected at 350 nm (Personal communication, Dr. D. de Beer, Infruitec-Nietvoorbij, Stellenbosch).

The effectiveness of columns and mobile phases (acetonitrile and methanol as organic solvents) in separating the constituent compounds in the *L. multiflora* infusion is summarized in Tables 2-5. Generally a column-mobile phase unit, which results in a shorter retention time and produces a higher number of peaks with good peak symmetry (close to 1.0), represents an efficient separation unit. The highest peak number (11 peaks) was obtained with a Prodigy 5µm Phenyl–3 100Å column using methanol as the organic solvent at 225 nm (Table 2). However, verbascoside showed a very long retention time (more than 17 min).

At UV wavelengths of 225 nm and 330 nm (Tables 2 and 4), both the Zorbax Eclipse XDB–C18 and Gemini 5 µm C18 110Å columns produced 10

chromatographic peaks each with acetonitrile as the organic solvent. Considering peak symmetry at 330 nm, and the shorter retention time for verbascoside, both the Gemini 5 µm C18 110Å and the Zorbax Eclipse XDB–C18 columns performed better with acetonitrile where they produced shorter retention times than with methanol. The Gemini 5 µm C18 110Å column, however, performed better than the Zorbax Eclipse XDB–C18 column with acetonitrile since the peak symmetry of the former (0.88) was closer to 1.0 than the latter (0.78). This subsequently led to the selection of acetonitrile, Gemini 5 µm C18 110Å, and 330 nm for all subsequent HPLC chromatographic separation and identification.

The sample verbascoside peak purity for the selected method was checked by evaluating spectra from five time points of the peak. Visual inspection showed a high degree of similarity between the five spectra, indicating a high degree of purity. The selected column and organic solvent were used to optimise the gradient. The goal was to minimize the analysis time, while maintaining good resolution between peaks.

Identification of verbascoside in *L. multiflora* infusion

Figure 2 is a chromatogram of the *Lippia multiflora* infusion. Verbascoside in the infusion was tentatively identified by comparing the UV-vis spectrum and retention time obtained with the infusion with that of the verbascoside standard. The verbascoside in the infusion exhibited a high level of conformity to that of the verbascoside standard with respect to UV-vis absorption spectrum and retention time (Figure 3, Table 6).
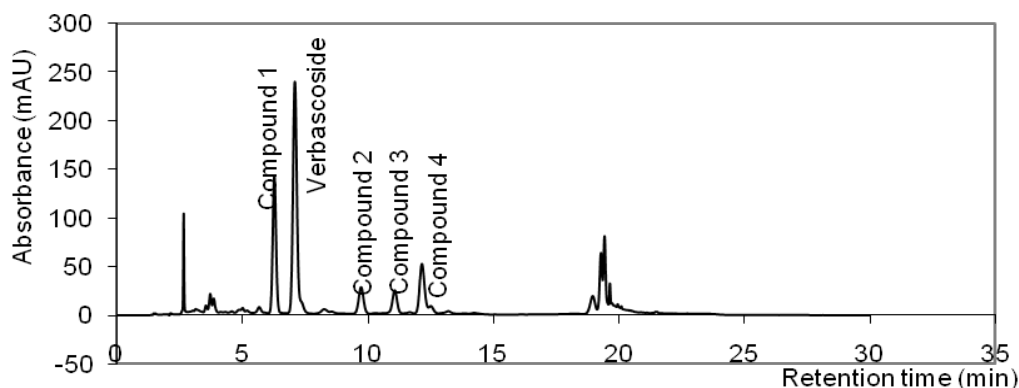
Figure 8. Chromatogram of the hot water infusion of *L. multiflora.*

Table 7. Column and mobile phase effectiveness at 225 nm.

Columns	MeCN Mobile Phase						MeOH Mobile Phase					
	ZB	GEM	SP	SM	SF	PP	ZB	GEM	SP	SM	SF	PP
RTst	9.12	10.01	10.02	9.71	10.05	9.96	16.45	17.29	18.54	17.33	17.24	17.26
RT	9.09	10.02	10.01	9.73	10.08	9.97	16.71	17.29	18.58	17.32	17.23	17.25
SymL	0.81	0.89	0.83	0.98	0.87	0.84	0.88	0.92	0.65	0.97	0.95	1.01
SymV	0.79	0.90	0.52	0.98	0.82	0.81	0.82	0.95	0.48	0.91	0.93	0.93
SymR	0.74	0.86	0.63	0.98	0.73	1.04	0.65	0.91	0.38	0.95	1.05	1.44
SymAV	0.85	0.97	0.55	0.99	0.77	0.89	0.94	0.85	0.51	1.08	0.91	1.05
# Peaks	10	10	7	9	8	10	10	9	5	9	10	11

ZB=Zorbax Eclipse XDB–C18; GEM=Gemini 5 µm C18 110 Å; SP=Synergi 4 µm POLAR – RP 80 Å; SM=Synergi 4 µm MAX–RP 80 Å; SF=Synergi 4 µm Fusion – RP 80 Å, and PP=Prodigy 5 µm Phenyl–3 100 Å. RTst=Retention time of standard verbascoside; RT=Retention time of *Lippia* infusion verbascoside; SymL=Symmetry of peaks to the left of *Lippia* verbascoside peak; SymV= Symmetry of *Lippia* infusion verbascoside; SymR=Symmetry of peaks to the right of *Lippia* verbascoside peak SymAV=Average peak symmetry of *Lippia* verbascoside.

Table 8. Column and mobile phase effectiveness at 288 nm.

Columns	MeCN Mobile Phase						MeOH Mobile Phase					
	ZB	GEM	SP	SM	SF	PP	ZB	GEM	SP	SM	SF	PP
RTst	9.12	10.01	10.02	9.71	10.05	9.96	16.45	17.29	18.54	17.33	17.24	17.26
RT	9.09	10.02	10.01	9.72	10.08	9.97	16.71	17.29	18.58	17.32	17.23	17.25
SymL	0.80	0.90	0.82	0.96	0.85	0.88	0.83	0.90	0.65	0.97	0.94	1.00
SymV	0.77	0.90	0.52	0.96	0.82	0.86	0.81	0.92	0.48	0.90	0.90	0.93
SymR	0.86	0.80	0.65	1.30	0.77	0.95	0.73	0.92	0.76	0.97	1.00	1.40
SymAV	0.77	1.00	0.67	1.09	0.96	0.89	0.92	0.81	0.59	1.12	0.88	0.99
# Peaks	6	8	5	7	7	7	7	6	6	6	7	8

ZB=Zorbax Eclipse XDB–C18; GEM=Gemini 5 µm C18 110 Å; SP=Synergi 4 µm POLAR – RP 80 Å; SM=Synergi 4 µm MAX–RP 80 Å; SF=Synergi 4 µm Fusion – RP 80 Å, and PP=Prodigy 5 µm Phenyl–3 100 Å. RTst=Retention time of standard verbascoside; RT=Retention time of *Lippia* infusion verbascoside; SymL=Symmetry of peaks to the left of *Lippia* verbascoside peak; SymV= Symmetry of *Lippia* infusion verbascoside; SymR=Symmetry of peaks to the right of *Lippia* verbascoside peak SymAV=Average peak symmetry of *Lippia* verbascoside.

Table 9. Column and mobile phase effectiveness at 330 nm.

| Columns	MeCN Mobile Phase						MeOH Mobile Phase					
	ZB	GEM	SP	SM	SF	PP	ZB	GEM	SP	SM	SF	PP
RTst	9.12	10.01	10.02	9.71	10.05	9.96	16.45	17.29	18.54	17.33	17.24	17.26
RT	9.09	10.02	10.01	9.72	10.08	9.97	16.71	17.29	18.59	17.32	17.23	17.25
SymL	0.80	0.90	0.82	0.96	0.85	0.88	0.83	0.89	0.65	0.94	0.91	0.98
SymV	0.78	0.88	0.52	0.94	0.82	0.86	0.79	0.92	0.47	0.90	0.90	0.93
SymR	0.81	0.80	0.66	1.22	0.77	0.97	0.66	0.92	0.78	0.97	1.00	1.41
SymAV	0.86	1.19	0.66	1.11	0.74	0.93	0.92	0.80	0.61	0.99	0.87	0.96
# Peaks	10	10	5	9	7	7	7	6	7	7	7	8

ZB=Zorbax Eclipse XDB–C18; GEM=Gemini 5 µm C18 110 Å; SP=Synergi 4 µm POLAR – RP 80 Å; SM=Synergi 4 µm MAX–RP 80 Å; SF=Synergi 4 µm Fusion – RP 80 Å, and PP=Prodigy 5 µm Phenyl–3 100 Å. RTst=Retention time of standard verbascoside; RT=Retention time of *Lippia* infusion verbascoside; SymL=Symmetry of peaks to the left of *Lippia* verbascoside peak; SymV= Symmetry of *Lippia* infusion verbascoside; SymR=Symmetry of peaks to the right of *Lippia* verbascoside peak SymAV=Average peak symmetry of *Lippia* verbascoside.

Table 10. Column and mobile phase effectiveness at 350 nm.

Columns	MeCN Mobile Phase						MeOH Mobile Phase					
	ZB	GEM	SP	SM	SF	PP	ZB	GEM	SP	SM	SF	PP
RTst	9.12	10.01	10.02	9.71	10.05	9.96	16.45	17.25	18.54	17.33	17.24	17.26
RT	9.09	10.02	10.01	9.72	10.08	9.97	16.71	17.29	18.59	17.32	17.23	17.25
SymL	0.80	0.91	0.83	0.97	0.84	0.88	0.82	0.90	0.66	0.95	0.92	0.98
SymV	0.78	0.90	0.52	0.96	0.83	0.87	0.79	0.93	0.47	0.90	0.90	0.93
SymR	0.76	0.80	0.64	0.99	0.74	1.02	0.63	0.93	0.34	0.98	1.12	1.54
SymAV	0.85	1.02	0.58	0.85	0.75	0.91	0.97	0.84	0.54	0.99	0.92	1.00
# Peaks	8	7	5	7	6	7	7	6	4	7	7	8

ZB=Zorbax Eclipse XDB–C18; GEM=Gemini 5 µm C18 110 Å; SP=Synergi 4 µm POLAR – RP 80 Å; SM=Synergi 4 µm MAX–RP 80 Å; SF=Synergi 4 µm Fusion – RP 80 Å, and PP=Prodigy 5 µm Phenyl–3 100 Å. RTst=Retention time of standard verbascoside; RT=Retention time of *Lippia* infusion verbascoside; SymL=Symmetry of peaks to the left of *Lippia* verbascoside peak; SymV= Symmetry of *Lippia* infusion verbascoside; SymR=Symmetry of peaks to the right of *Lippia* verbascoside peak SymAV=Average peak symmetry of *Lippia* verbascoside.

UV-vis spectra of constituent compounds in *L. multiflora* infusion

A

B

C

D

E

F
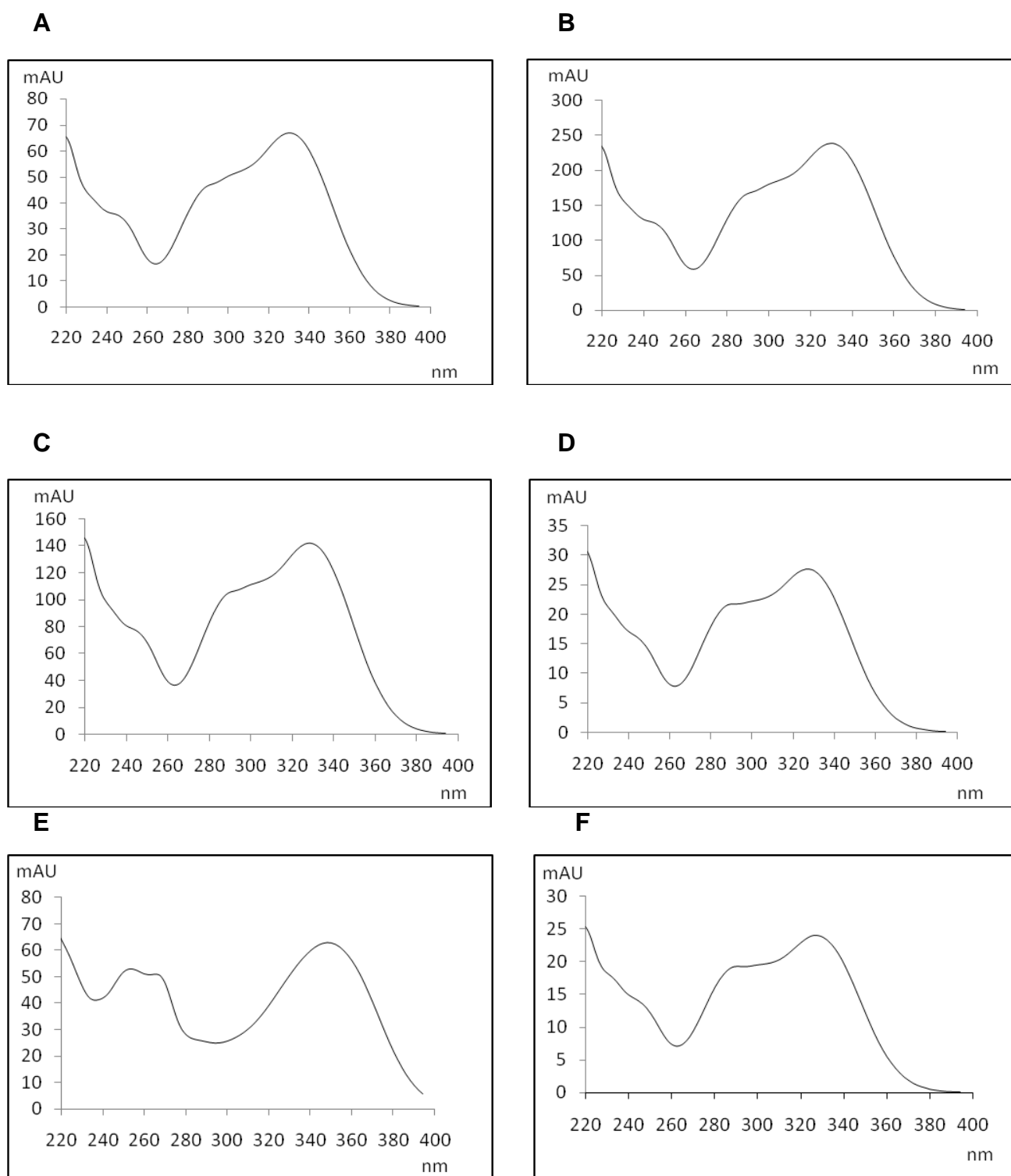
Figure 9. UV-vis spectra of the verbascoside standard (A) and verbascoside (B), compounds 1 (C), 2 (D), 3 (E) and 4 (F) from the hot water infusion of *L. multiflora*.

The other major compounds, except for compound 3, also exhibited spectra similar to verbascoside with absorption maxima between 320 and 340 nm. Apart from showing a distinct spectrum, compound 3 (E) had an absorption maximum at 348 nm, typical of a flavonoid compound such as a flavonol or a flavone.

LC- MS/MS analysis for confirmation of verbascoside in *L. multiflora* infusion

LC-MS[n] has become a versatile analytical technique for its ability to analyse compounds without the need for derivatization. It is capable of achieving simultaneous determination of non-volatile and thermally unstable compounds (Jong *et al.*, 2006) with specificity, sensitivity and speed (Xu *et al.,* 2007).

Generally, phenolic compounds give peaks of lower intensity in the negative than in the positive ionisation mode (Savarese *et al.*, 2007). However, in the present study, the analyses were conducted in the negative ionisation mode because of better ionisation for these compounds. Peak identification was carried out on the basis of *m/z* values of detected ions, and the retention time of chromatographic peaks. Table 6 shows the results of the mass spectrometric analysis of the authentic verbascoside standard and the *Lippia multiflora* infusion constituents.

Table 11. Results of MS/MS analysis of *L. multiflora* infusion constituents.

| No.	Compound	RT	Parent ion (m/z)	Major fragment ion (m/z)
1.	Verbascoside (std)	7.8	623	161
2.	Verbascoside (sample)	7.8	623	161
3.	Compound 1	7.0	609	161
4.	Compound 2	10.6	623	161
5.	Compound 3	11.6	461	285
6.	Compound 4	12.1	609	161

RT = retention time, m/z= mass to charge ration, std = standard

Both the verbascoside standard and the peak tentatively identified as verbascoside in the infusion produced a parent ion at m/z 623 and a major daughter ion at m/z 161 (Figures 4 and 5). The molecular mass of the parent ion corresponds to the de-protonated verbascoside, whereas the daughter ion corresponds to the caffeoyl moiety after the loss of water (Li *et al.*, 2008). This is consistent with the

observed fragmentation pattern of verbascoside given in the literature (Grayer & De Kok, 1998; Liu *et al.*, 1998; Savarese *et al.*, 2007). It should, however, be pointed out that the fragmentation pattern will depend on the experimental conditions such as type of ion source and mass analyser, as well as the collision energy. Savarese *et al.* (2007), also using negative ionisation, obtained the pseudo-molecular ion (m/z 623) with a few other fragments of lower intensity. Upon fragmentation, two ions with m/z 461 and 161, characteristic of verbascoside, were identified (Savarese *et al.*, 2007).
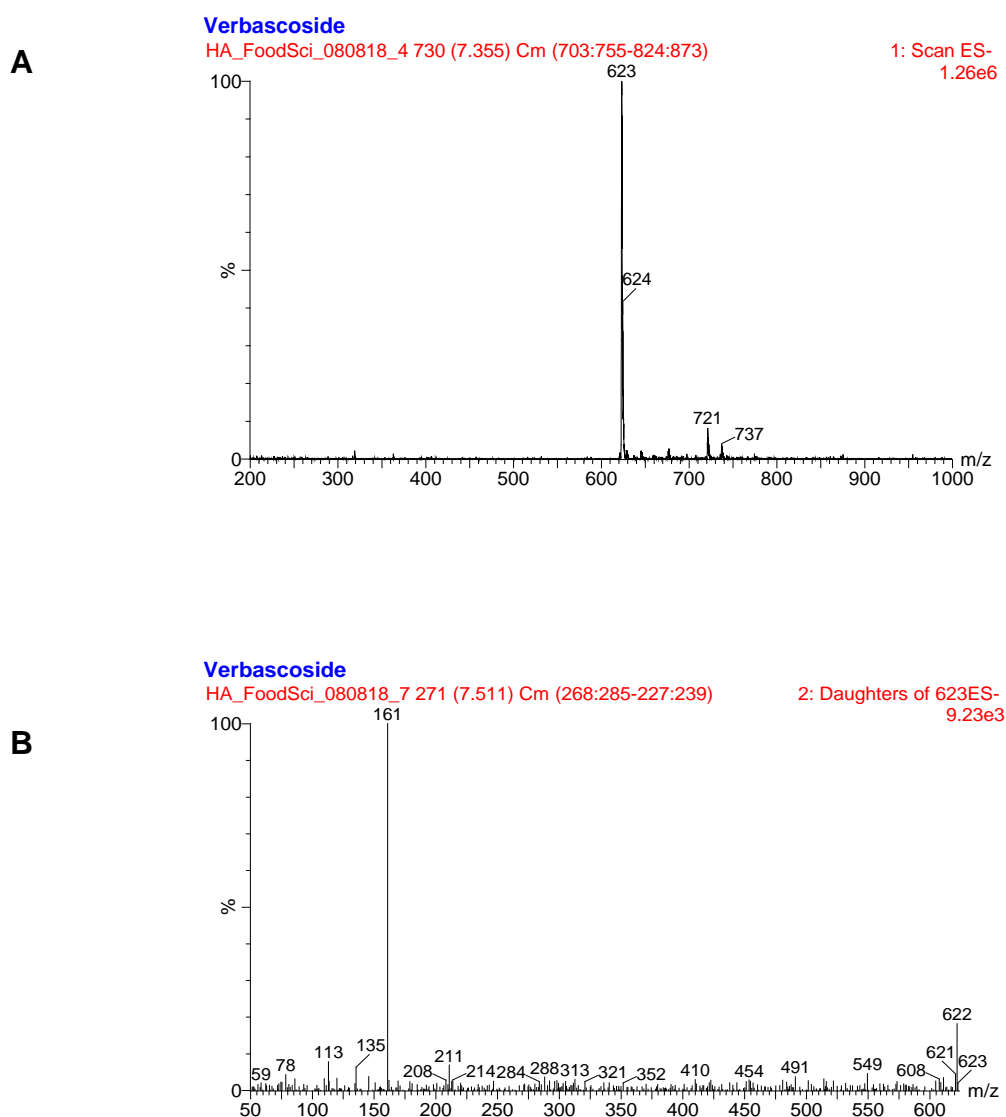
Figure 10. Mass spectrum (A) and MS/MS spectrum (B) of standard verbascoside with daughter ion m/z 161 from parent ion m/z 623.
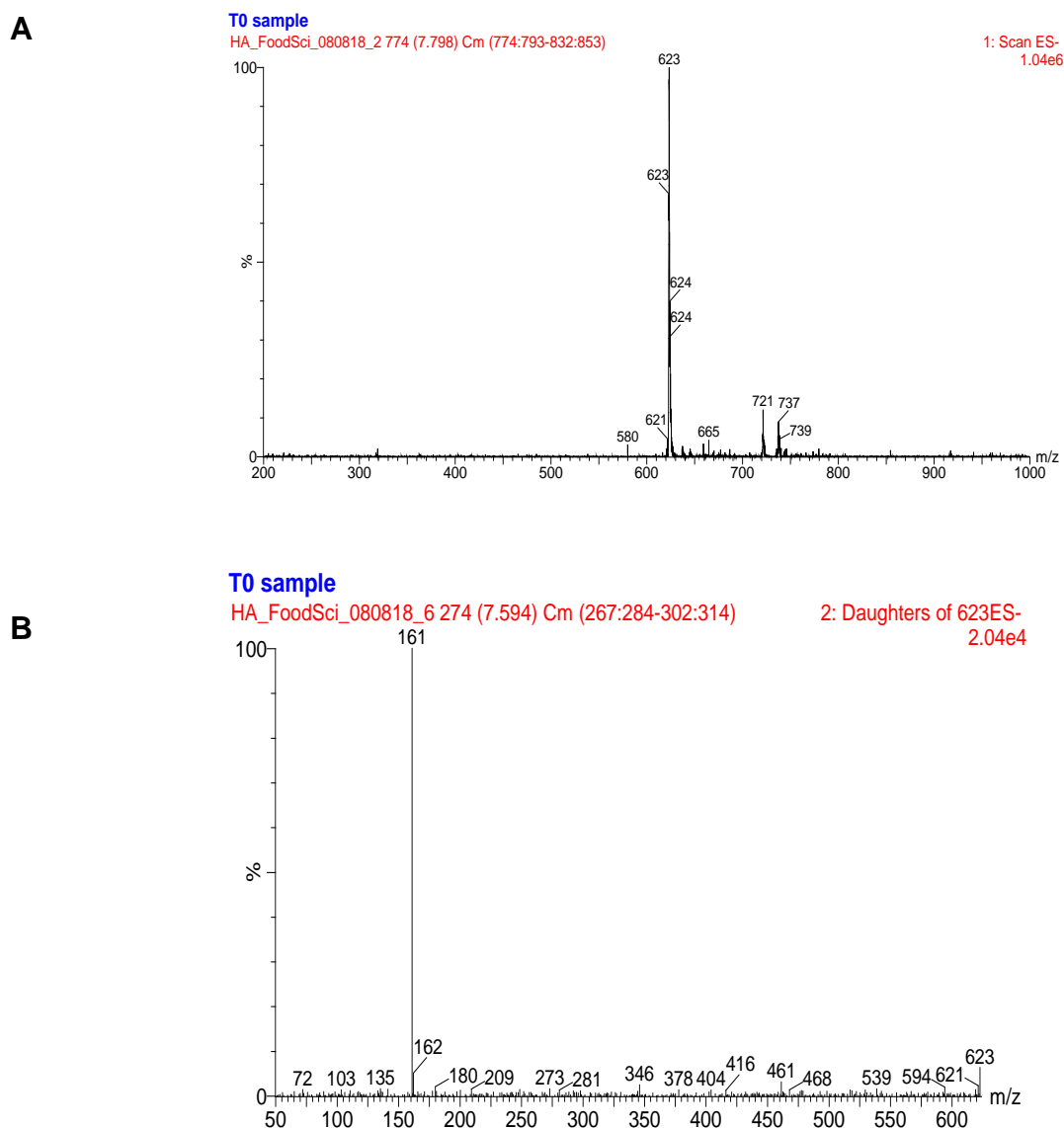
Figure 11. Mass spectrum of verbascoside ([M-H]$^-$ = m/z 623) (A); MS/MS spectrum of verbascoside (B) in *L. multiflora* infusion (parent ion m/z 623).

In Figure 6, the mass spectrum of an unidentified compound 1 (m/z 609) is presented. The parent ion m/z 609 appears to be a compound with similar mass to verbascoside that lost a CH_2 group (CH_2=14 and 623 − 14 = 609) (Li *et al.*, 2008). Further fragmentation of the parent ion m/z 609 also produced a daughter ion m/z 161 which is either a caffeoyl or glucose moiety which has lost a molecule of H_2O (Li *et al.*, 2008). Further investigation to elucidate the structure and identity of this compound is required.

A

B
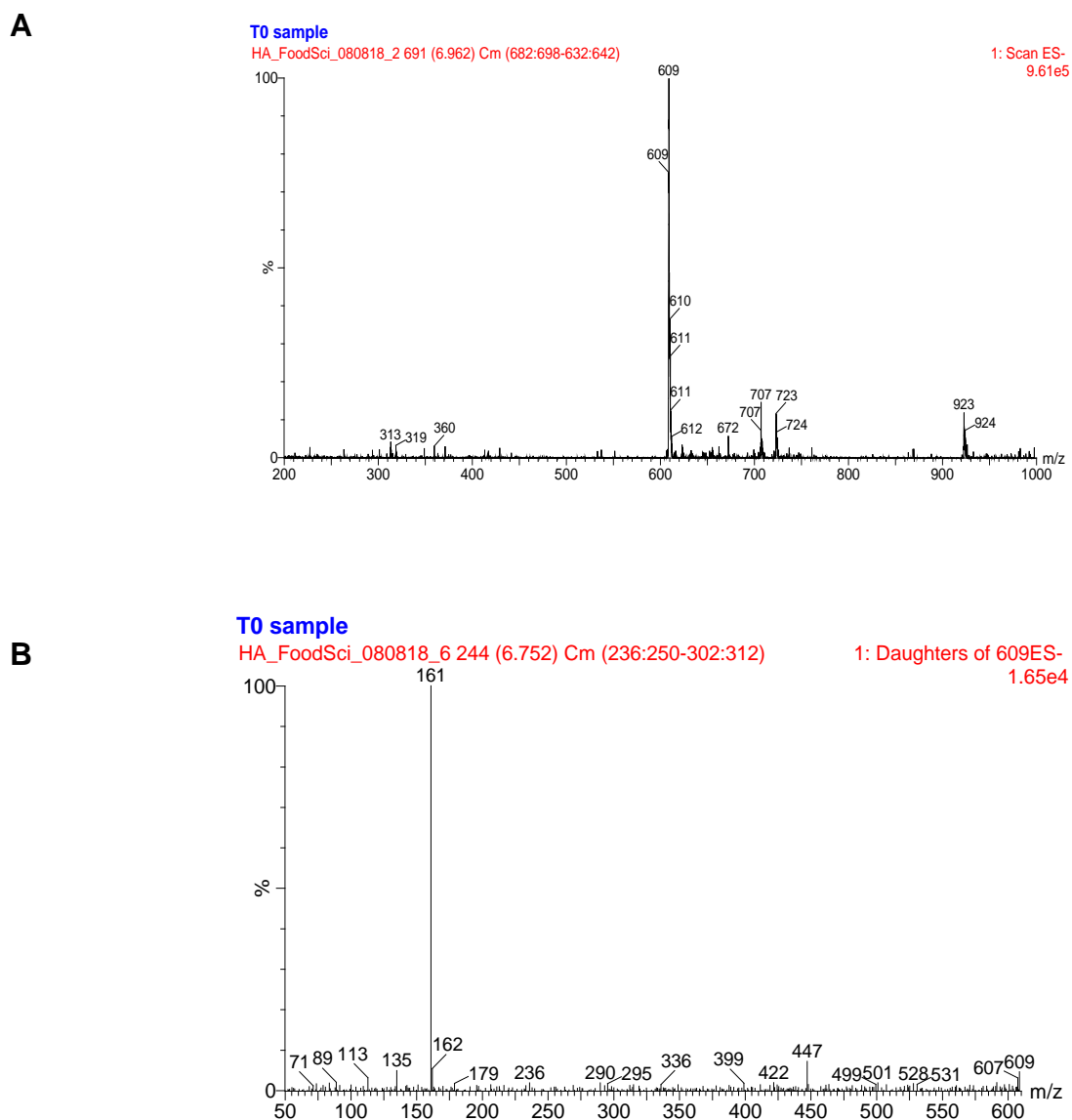
Figure 12. Mass spectrum of compound 1 ([M-H]$^-$ = m/z 609) (A); MS/MS spectrum of compound 1 (B) (parent ion m/z 609).

The unidentified compound 2 (m/z 623) (Figure 7) is a potential isomer of verbascoside as the parent ion of both compounds possess the same charge to mass ratios, and fragmentation yielded a typical verbascoside daughter ion at m/z 161 (Figures 7 and 4). This unidentified compound has a longer retention time compared to verbascoside. This would suggest a different spatial configuration which could cause the delay in retention time (Table 6). Isoverbascoside (isoacteoside) is retained longer on a C18 column with an acetic acid-acetonitrile gradient than verbascoside (Li *et al.*, 2008).
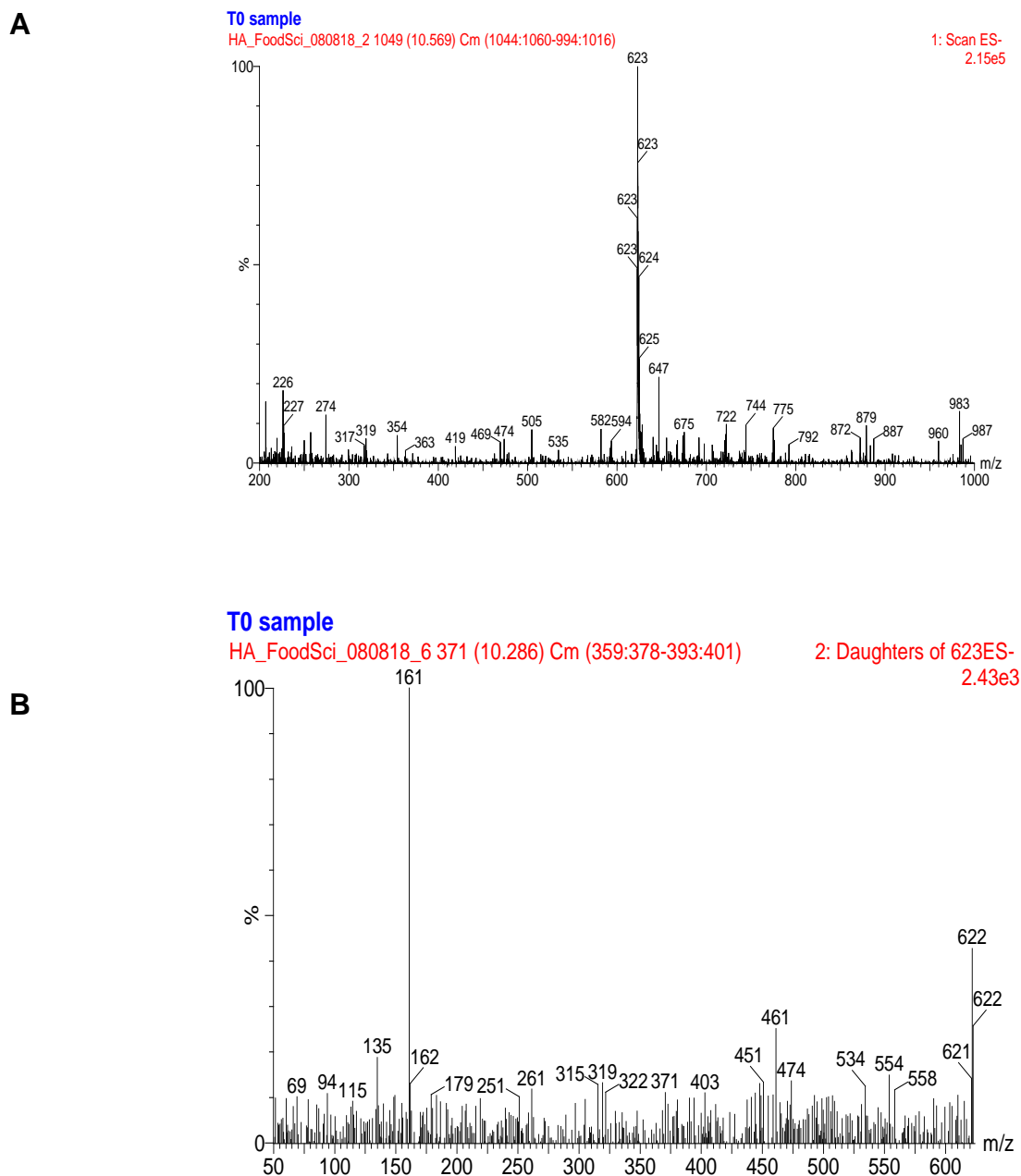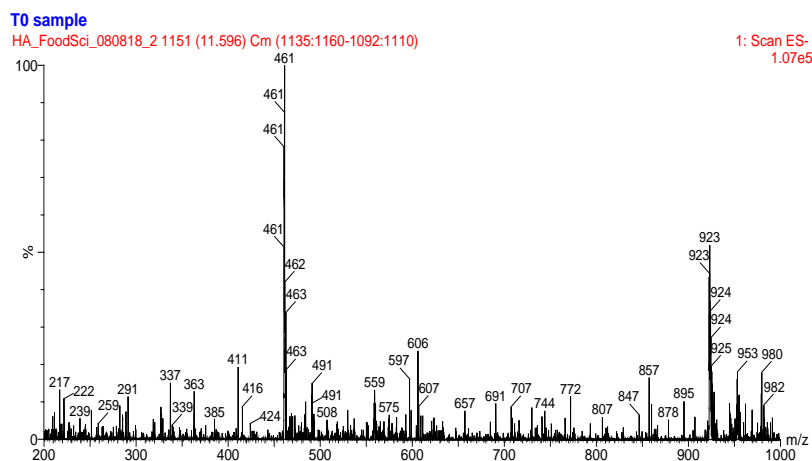
Figure 13. MS spectrum of compound 2 ([M-H]⁻ = m/z 623) (A);
MS/MS spectrum of compound 2 (B) (parent ion m/z 623).

In Figure 8A, two major ions, m/z 461 and 923, with the latter of lower intensity, were identified for compound 3. The ion at m/z 923 appears to be a dimer of the ion at m/z 461. The protonated monomer (molecular mass 462) would produce a depronated dimer at m/z 923 (Figure 8A). Compound 3, tentatively identified as a flavonoid based on its absorption spectrum with λ_{max} = 348 nm (Figure 3E), gave a daughter ion at m/z 285. Although the daughter ion at m/z 285, as well as the λ_{max} of 348 nm are characteristic of luteolin-7-diglucuronide, a flavonoid glucoside (Funes *et al.*,

2009; Meng *et al.*, 2009; Quirantes-Piné *et al.,* 2009), the charge to molecular mass ratio of luteolin-7-diglucuronide is m/z 637 (Quirantes-Piné *et al.,* 2009) compared to m/z 609 found in this study. The specific identity of compound 3, therefore, requires further investigation.
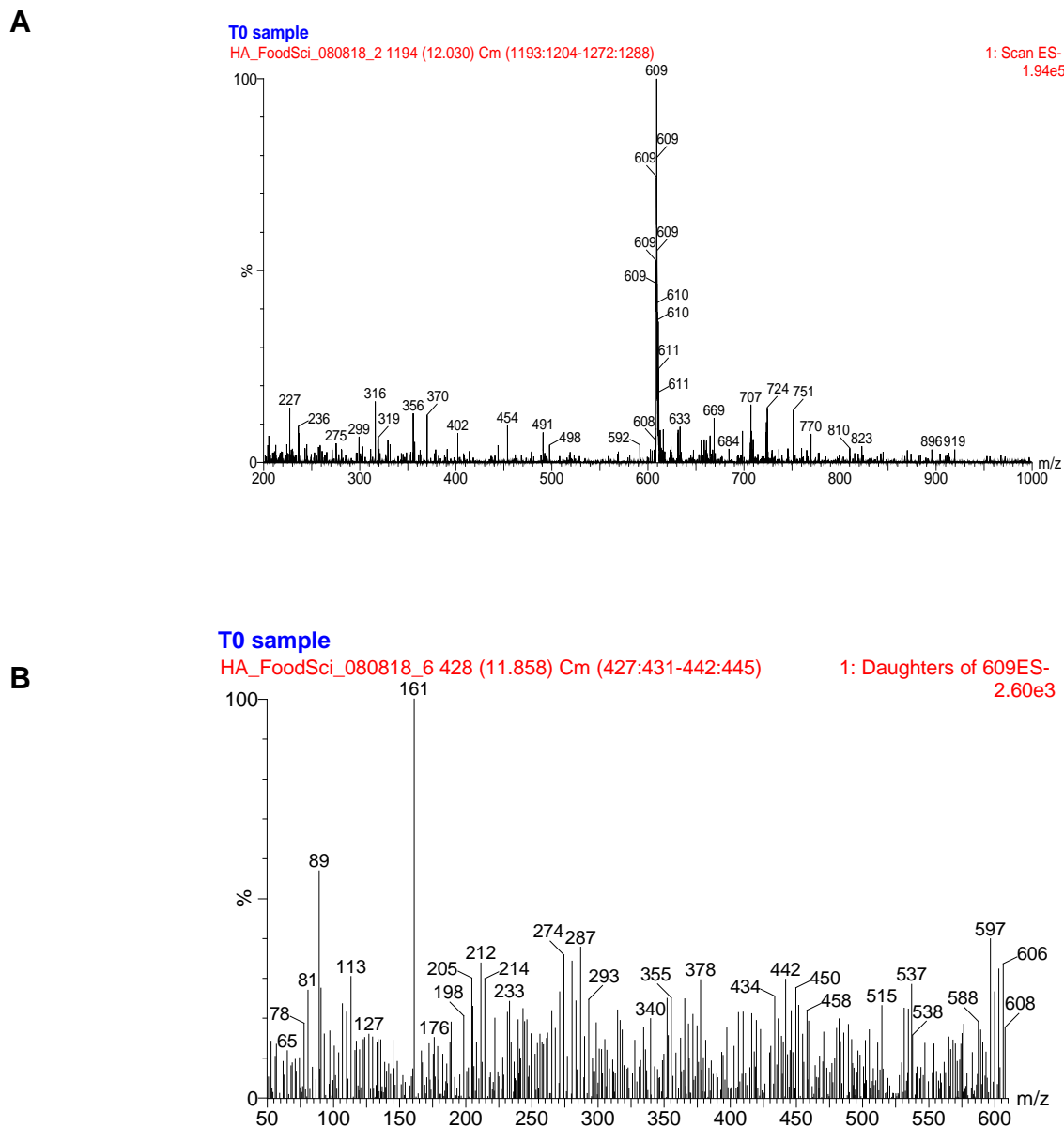
A

B
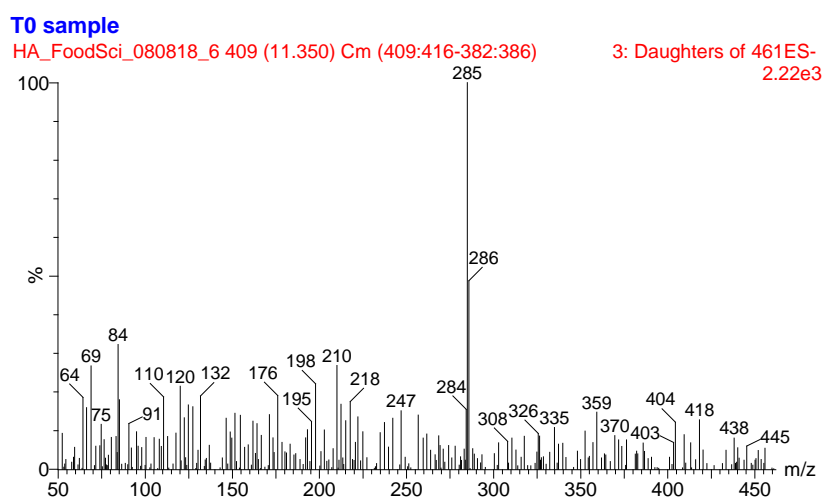
Figure 14. MS spectrum of compound 3 ([M-H]⁻ = m/z 461) (A); MS/MS spectrum of compound 3 (B) (parent ion m/z 461).

Unidentified compound 4 (Figure 9) had the same parent ion of m/z 609 as compound 1, and both produced the same major daughter ion of m/z 161. Isomeric compounds are indicated for 1 and 4 and further investigation is required.

A

T0 sample
HA_FoodSci_080818_2 1194 (12.030) Cm (1193:1204-1272:1288)   1: Scan ES-
1.94e5

B

T0 sample
HA_FoodSci_080818_6 428 (11.858) Cm (427:431-442:445)   1: Daughters of 609ES-
2.60e3

Figure 15. MS spectrum ([M-H]$^-$ = m/z 609) (A) and MS/MS spectrum (B) of compound 4 (parent ion m/z 609).

Stability of verbascoside

Verbascoside showed a marked degradation over a 24 h period from an initial 3074.68 mAU.s to 2657.63 mAU.s at 330 nm (Figure 10). Polyphenols with antioxidant capacity are themselves susceptible to oxidation (Bonanni *et al.*, 2007; Fukumoto & Mazza, 2000) and would normally require a stabilising antioxidant during their extraction and quantitative recovery (Robards *et al.*, 1999). Upon the addition of 10% ascorbic acid to the sample vial, however, stability prior to injection

was improved, but degradation still occurred at a lower rate (Figure 10). To further ameliorate the effect of degradation, the infusion and standard samples for HPLC identification and quantitative determinations were stored at -20^{0}C and analysed as soon as possible after defrosting.
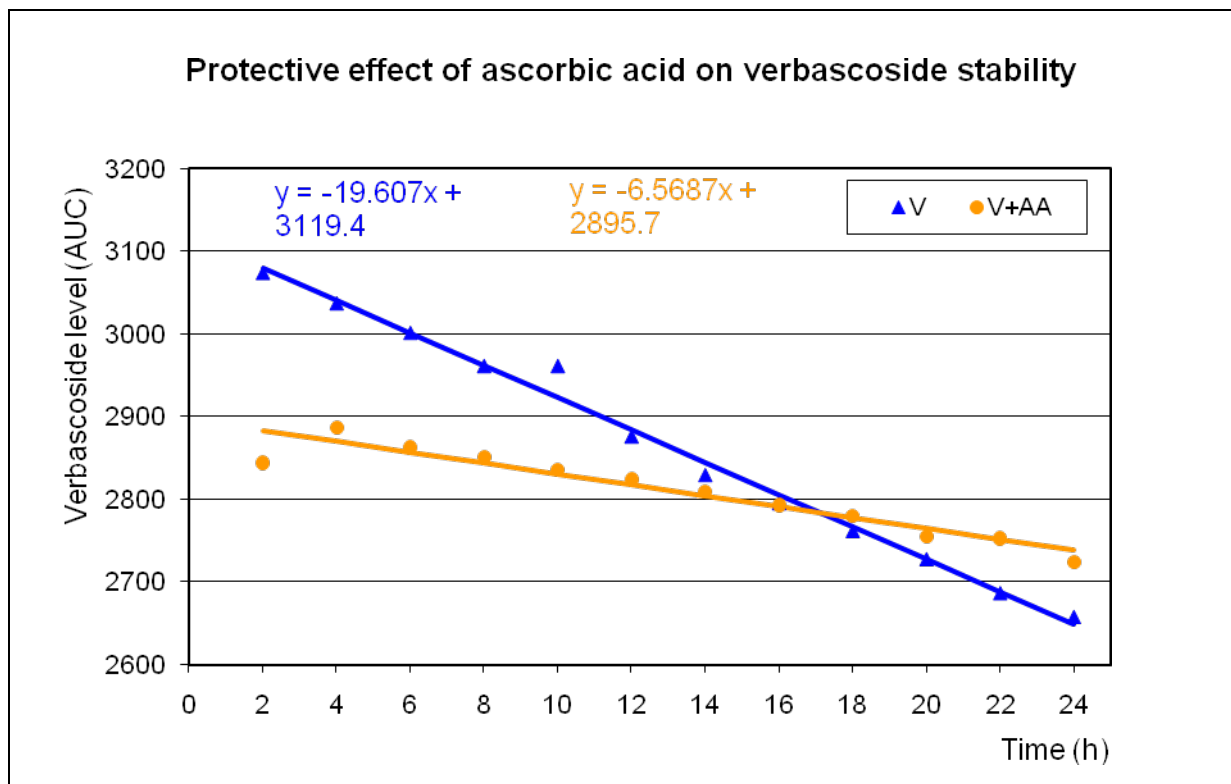


Figure 16. Stability of verbascoside (V) and the effect of ascorbic acid (AA) on its degradation.

Quantification of verbascoside and associated compounds

By using the calibration curve of the verbascoside standard (Figure 11), the amount of the compound in the infusion was calculated and the results summarised in Table 7. At a brewing strength equal to that of a 'cup of tea' (100 ml), the infusion of *L. multiflora*, contained 14.51 mg of verbascoside. The concentration of the unidentified compounds varied between 6.39 mg for compound 1 to 0.13 mg for compound 3. These quantitative data for verbascoside in *L. multiflora* could be exploited for the therapeutic application of this herbal tea and also form the basis for its future quality control.

Approximately, 40 mg of verbascoside has been found in 100 ml infusion of *Aloysia triphylla* (lemon verbena), also called *Lippia citriodora* (Verbenaceae), a plant native to South America (Carnat *et al.*, 1999). However, the extraction system consisted of 10 g of dried plant material infused for 15 min compared to the 2 g of *L. multiflora* infused for 5 min in this study. Vrchovská *et al.* (2007) also found approximately 10 mg of verbascoside in 1 g of a lyophilised infusion of dried *Ballota nigra* (Lamiaceae). The extraction process consisted of 5 g of the dried plant infused in 500 ml of boiling water for 15 min.
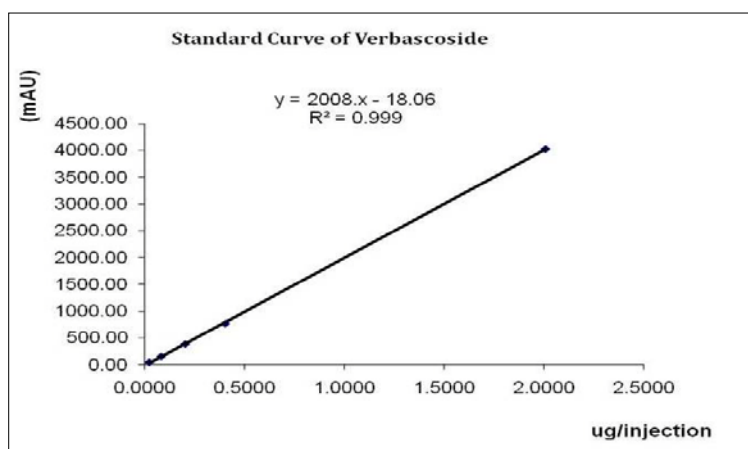
Figure 17. Calibration curve of standard verbascoside.

Table 12. Mean ± SD and range for the amount (mg VE[a]) of compound present in 100 ml of *L. multiflora* infusion ('cup of tea').

Compound	Mean ± SD	Range
Verbascoside	14.51 ± 0.58	13.62 – 15.02
Compound 1	6.39 ± 1.44	3.84 – 7.31
Compound 2	2.27 ± 0.22	2.01 – 2.46
Compound 3	0.13 ± 0.00	0.13 – 0.14
Compound 4	1.99 ± 0.23	1.70 – 2.27

[a]VE=verbascoside equivalent; SD=standard deviation; Means are the results of three determinations

Benchmarking of *Lippia multiflora* against rooibos and honeybush

Data obtained for the total polyphenol content and antioxidant capacity of *L. multiflora* tea were compared with those of rooibos and honeybush teas, two well-

known South African herbal teas that have made the transition from indigenous consumption to commercial patronage (Joubert *et al*, 2008a). The results are summarised in Tables 8 and 9.

Table 13. Soluble solids (SS) and total polyphenols (TP) contents, and antioxidant (DPPH and FRAP) activities of the tea infusions.

Tea	SS (mg)*	TP (mg GAE)*	DPPH (µmol Trolox)*	FRAP (µmol Trolox)*
LM	$188.53^a \pm 11.31$	$33.04^a \pm 1.39$	$368.73^a \pm 24.47$	$131.93^a \pm 7.92$
Rooibos	$124.13^b \pm 12.55$	$31.69^a \pm 3.84$	$256.08^b \pm 31.91$	$70.98^b \pm 10.13$
Honeybush	$122.87^c \pm 10.29$	$4.40^b \pm 0.43$	$21.31^c \pm 0.78$	$7.77^c \pm 0.52$
LSD	15.74	3.27	32.00	10.24

LM= *Lippia multiflora*, LSD= Least significance difference at 5% significance level.

Values with different superscripts in the same column differ significantly

* Results expressed on the basis of 100 ml infusion

Table 14. Total polyphenol (TP) content and antioxidant (DPPH and FRAP) activities of the tea soluble solids.

Tea	TP (g GAE.100 g^{-1})	DPPH (µmol Trolox.g^{-1})	FRAP (µmol Trolox.g^{-1})
LM	$17.55^b \pm 0.96$	$1957.46^a \pm 109.75$	$700.62^a \pm 39.97$
Rooibos	$25.51^a \pm 1.08$	$2060.27^a \pm 86.39$	$570.26^b \pm 33.45$
Honeybush	$3.58^c \pm 0.26$	$174.17^b \pm 12.17$	$63.44^c \pm 4.43$
LSD	1.17	111.54	41.61

LM= *Lippia multiflora*, LSD= Least significance difference at 5% significance level.

Values with different superscripts in the same column differ significantly

Lippia multiflora showed significantly higher ($P < 0.05$) values than rooibos and honeybush for all parameters measured on the basis of 'cup of tea' conversions (Table 8). The aspalathin-containing rooibos infusion exhibited significantly ($P < 0.05$) higher soluble solids and total polyphenol contents, and antioxidant activities than honeybush tea. An infusion prepared from *L. multiflora* showed almost twice as much antioxidant activity measured by the ferric reducing antioxidant power (FRAP) than rooibos infusion. This shows that *L. multiflora* was significantly ($P < 0.05$) stronger than rooibos and honeybush in reducing Fe (III) to Fe (II). Notably,

verbascoside, the active compound of *L. multiflora* has been identified as a potent Fe-complexing antioxidant (Zhao *et al.,* 2005; Mmatli *et al.*, 2007). Expressed on the basis of soluble solids, however, the DPPH antioxidant activities of *L. multiflora* and rooibos did not differ significantly ($P > 0.05$) (Table 9).

While the rooibos and honeybush teas had been subjected to processing, the *L. multiflora* tea had not, which could contribute to its distinctly higher soluble content and ferric reducing activity. The oxidation (fermentation) process to which rooibos is subjected to produce its sweet flavour and characteristic red-brown colour (Joubert & Schulz, 2006) could reduce the aspalathin content of processed rooibos by more than 90% (Joubert, 1996), and, therefore, its antioxidant activity. In some instances, processed iced teas tested negative for aspalathin and its associated bioactive compounds as a consequence of the severity and duration of the processing regime used (Joubert *et al.*, 2009).

Although honeybush infusions have typically lower polyphenol and antioxidant activities than rooibos, the unusually lower polyphenol content and antioxidant activity found in this study for honeybush are inconsistent with data reported by Joubert *et al.* (2008b). Factors such as the species of tea used, stem content, processing technique and the method and duration of preparation of the infusion could have accounted for the level of antioxidant activity of the infusion in this study. Marnewick *et al.* (2000) used an elaborate extraction system for both fermented and unfermented honeybush of the species *C. intermedia* that involved 30 min of hot water extraction and freeze-drying the extract before storage at –20℃. Also, the honeybush species used in the present study were blends of organically-produced *C. intermedia* and *C. subternata* which constitute two of the widely-used species by extract manufacturers (Joubert *et al.,* 2008b). The species used could, therefore, not account for the low activities exhibited. Differences in phenolic composition and, therefore, reactivity could explain this. It is also likely the low total polyphenol contents and antioxidant activity of the honeybush used in this study might be due to severe processing practices and potentially to the 'organic' production method used. Campanella *et al.* (2003) postulated 5 min of hot water extraction to be adequate for extracting antioxidant principles, although a lot more compounds with antioxidant activity may still remain un-extracted. However for consumption purposes 5 min is consistent with the normal time used by consumers of herbal teas.

CONCLUSIONS

An HPLC method has been developed for the identification of verbascoside in *L. multiflora* herbal tea. The presence of verbascoside in this herbal tea was confirmed using tandem mass spectrometry. Apart from verbascoside, other compounds were found in the tea that would require structural elucidation to confirm their identity.

Lippia tea was benchmarked against two well-known South African herbal teas, rooibos and honeybush on the bases of their polyphenol contents, and antioxidant capacities using the ferric reducing antioxidant power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The *L. multiflora* infusion gave significantly ($P < 0.05$) higher values than rooibos and honeybush with respect to soluble solids and total polyphenol contents as well as antioxidant activities as measured by the DPPH and FRAP assays. Both *L. multiflora* and rooibos infusions were significantly stronger than honeybush on all parameters measured namely, soluble solids and total polyphenol contents, and DPPH and FRAP antioxidant capacities.

The presence of verbascoside in *L. multiflora* infusion in addition to four other compounds of relatively high concentrations, as well as the significantly higher antioxidant activities in comparison to rooibos and honeybush is expected to provide the basis for future studies on the therapeutic application of this herbal tea. Verbascoside could potentially also form the basis for future quality control of *L. multiflora*.

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CHAPTER 4

EFFECT OF STEAM PASTEURISATION ON MICROBIAL AND QUALITY PARAMETERS OF *LIPPIA MULTIFLORA* LEAVES

ABSTRACT

The consumption of herbal teas is an increasing phenomenon among tea consumers globally. However, herbal teas that are not pre-treated to reduce their microbial load are a health risk to consumers. In this study, the effect of steam pasteurisation (99.8°C for 2.5 min) on the microbial load of *Lippia multiflora* herbal tea leaves was evaluated. Microbial enumeration was conducted in duplicate on potato dextrose agar (PDA), plate count agar (PCA), violet red bile agar (VRBA), yeast peptone dextrose agar (YPDA), and deMann-Rogosa-Sharp agar (MRS). Morphologically distinct colonies were isolated, sub-cultured and their Gram reaction recorded. These bacteria were identified to the species level using 16S ribosomal DNA (rDNA) sequence data. Finally, the effects of the period of the steam pasteurisation (0 – 150 s) on the soluble solids, total polyphenol, and verbascoside contents, as well as on the antioxidant activities of *L. multiflora* infusion were studied. Most of the bacteria identified belonged to the genus *Bacillus*. One species each from the genera *Pantoea* and *Kocuria* was also identified, but only the *Bacillus* species survived the steam pasteurisation. Coliform bacteria detected prior to pasteurisation were not detected after steam treatment. Steam pasteurisation reduced the microbial load from 10^4 to 10^2 cfu.g^{-1} and did not significantly ($P > 0.05$) change the soluble solids, total polyphenol and verbascoside contents, or the antioxidant activity. Steam pasteurisation is potentially an effective method to treat *L. multiflora* herbal teas prior to consumption. However, the steam treatment should complement good agricultural and hygienic practices rather than replace them as some bacteria can survive this treatment.

INTRODUCTION

Sales of herbal teas are increasing globally and are presently taking market shares from conventional teas (Anon, 2003; Malinowska, 2008). In 2005, traditional tea sales in the UK were decreasing yearly in terms of volume as consumers were increasingly consuming herbal teas (Datamonitor, 2005). The widespread popularity of herbal teas is due to their health attributes largely associated with antioxidant compounds (Weisberg, 2001; Béliveau & Gingras, 2004; Popp, 2004; Alarcón *et al.* 2007; Landi, 2007; Wright *et al.*, 2007). Antioxidant compounds show free radical scavenging activity and present various therapeutic bioactivities against chronic diseases (Kang *et al.*, 2003; Lee *et al.,* 2003; Huang *et al.*, 2005; Sağlam *et al.,* 2007). Their ability to modulate the harmful effects of reactive free radical species that are generated as part of normal cellular activities is the subject of intense scientific research.

Despite the significant role of herbal teas in improving nutrition and health, there have been reports of microbial contamination and adverse effects resulting from the consumption of these teas (Martins *et al.,* 2001; Ernst & Pittler, 2002; Nestmann *et al.*, 2006; Street *et al.*, 2008). Palma *et al.* (2003) reported a number of adverse effects linked to the consumption of herbal teas including neurological, cardiovascular and haematological hazards. Toxin-producing microbial contaminants often cause some of these adverse effects. Microbial contamination could occur when high bacteria counts such as 10^8 cfu.g^{-1} are found in raw tea products (Wilson *et al.*, 2004). Therefore, it is important to identify the microbial contaminants of herbal tea products as indicators of safety and quality (Master *et al.*, 2004; Schweiggert *et al.*, 2005; Jarvis *et al.*, 2007), and to develop methods of control.

Lippia multiflora is a herbal tea recently being introduced to the commercial tea market. It has been used in Africa as an infusion known as 'Tea of Gambia' (Abena *et al.,* 2003). This herbal tea is widely used as a sudorific, a febrifuge, a laxative and for the treatment of colic. The leaves or aerial parts and flowers are often prepared as a decoction and administered orally (Kunle *et al.*, 2003). The major antioxidant compound of *L. multiflora* is verbascoside (Juliani *et al.*, 2006; Pascual *et al.*, 2006), which has been shown to have wide biological activity, supporting its traditional use (Avila *et al.*, 1989; Sheng *et al.,* 2002; Zhao *et al.,* 2005; Obied *et al.,* 2008).

Although *L. multiflora* herbal tea has had a long history of traditional use (Abena *et al.,* 2003; Kunle *et al.*, 2003; Juliani *et al.*, 2006), recent efforts to commercialise the tea necessitated the need to ensure adequate microbial safety of the tea. Amongst the several microbial control methods described in the literature for plant material and food products (Gould, 1996), including irradiation (Crawford & Ruff, 1996) and ozonation (Allende *et al.,* 2006; Öztekin *et al.,* 2006; Selma *et al.,* 2008; Tiwari *et al.,* 2009; Najafi & Khodaparast, 2009), steam pasteurisation (Schweiggert *et al.,* 2005; Das, 2008) is preferred as a cost-effective method which is able to ensure the microbial safety and efficacy of the resulting product (Das, 2008).

The aim of this study was to enumerate the microbial species that occur on *L. multiflora* herbal tea leaves and to identify the bacteria present using 16S ribosomal DNA (rDNA) sequencing. A further aim was to investigate the effect of steam pasteurisation on the microbial population and potentially important quality parameters such as soluble solids, total polyphenols and verbascoside contents, as well as antioxidant activity of the infusion. This is the first attempt at investigating the possibility of steam pasteurisation to control microbial loads in support of the commercialisation of *L. multiflora* herbal tea.

MATERIALS AND METHODS

Chemicals and reagents

Verbascoside (99.9% purity) was purchased from Extrasynthese (Genay, France). The chemicals used for HPLC analysis were glacial acetic acid (Fluka, Buchs, Switzerland), HPLC grade acetonitrile 'Far UV' (Merck, Darmstadt, Germany) for gradient analysis, dimethyl sulfoxide and ascorbic acid (Sigma, St. Louis, USA). Distilled water was de-ionised using a Modulab purifier (Continental Water Systems Corp., San Antonio, USA). The de-ionised water was further purified with a Milli-Q academic water purifier (Millipore, Bedford, USA) to obtain HPLC-grade water. A 0.1% formic acid solution was prepared by pipetting 2 ml of a stock formic acid solution (>98% v/v) into a 2 L volumetric flask and made to the mark with HPLC-grade water. This solution was filtered using an all-glass filter apparatus and a

Millipore HV 0.45 mm membrane filter. The reagents used for the quantification of the total polyphenol content of the *L. multiflora* infusions were Folin-Ciocalteu's phenol reagent (Merck), anhydrous sodium carbonate (Saarchem, Gauteng, South Africa), and gallic acid (Sigma Chemical Co.). The reagents used for assaying antioxidant activities included Trolox (Sigma), sodium acetate (Sigma), TPTZ (2,4,6-tripyridy-*s*-triazine) (Sigma), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma), hydrochloric acid (HCl) (Sigma) and iron (III) chloride ($FeCl_3$) (Sigma).

Lippia multiflora tea

Lippia multiflora tea samples were randomly selected from the *Mpuntu* store of Agribusiness in Sustainable Natural African Plant Products (ASNAPP), Stellenbosch, South Africa. The samples originated from wild plants harvested in 2006 over a one month period in a community in Ghana and sun-dried before milling and packaging into tea bags. Ten tea samples (30 g each) were used for microbial analysis, five of which were pasteruised and five unpasteurised. Thirty tea samples (30 g each) were used to study the effect of steam pasteurisation on specific quality parameters of *L. multiflora* leaves.

Microbial enumeration

Five different microbial media were used for enumeration and isolation of the microbes present on the *L. multiflora* tea leaves. These were potato dextrose agar (PDA) for yeasts and moulds (Wen *et al.,* 2006) incubated at 25°C for 5 d, plate count agar (PCA) for viable aerobic counts (Wen *et al.*, 2006) incubated at 30°C for 48 h, violet red bile agar (VRBA) for coliform bacteria and *E. coli* (Bloch *et al.*, 1996) incubated at 37°C for 48 h, yeast peptone dextrose agar (YPDA) for yeasts (Mangia *et al.,* 2008) incubated at 25°C for 5 d, and the deMann-Rogosa-Sharp (MRS) agar for lactobacilli (Waite *et al.*, 2009) incubated at 30°C for 48 h. All the media were supplied by Merck.

Five tea samples were unpasteurised and 5 tea samples were steam-pasteurised at 99.8°C for 2.5 min. Serial dilutions (10^{-1} to 10^{-4}) of each tea sample were obtained by mixing 1 g tea leaves in 9 ml 0.85 (w/v) NaCl (Merck) followed by further dilutions. For each diluted sample, 1 ml was pour-plated in duplicate with all

the microbial media, incubated and colonies counted. Morphologically distinct colonies were randomly selected and sub-cultured by surface plating on the same media from which they had previously been isolated. Pure isolates were subjected to Gram staining and their morphology studied by microscopy.

Bacterial identification

Bacterial isolates were identified using DNA sequence data of a 1.5 kilobase fragment of the 16S rDNA. PCR amplification of this fragment was performed using the primers F8 (5'CAC GGA TCC AGA CTT TGA TYM TGG CTC AG 3') and R1512 (5'GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT 3') (Felske *et al.*, 1997). The PCR reaction mixture (50 µl total volume) contained 2 µl (400 nM) of each primer, 1 µl (5 U) *Taq* DNA polymerase (5 U.µl^{-1}, Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 5 µl 10 X buffer (with $MgCl_2$) (Super-Therm), 2 µl (0.4 mM) dNTPs (10 mM, AB gene, supplied by Southern Cross Biotechnologies), 2 µl 99% (v/v) dimethyl sulfoxide (DMSO) (Merck) and 2 µl DNA template. Thermal cycling parameters were as follows: initial denaturation at 92°C for 3 min; 35 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 30 s, elongation at 68°C for 60 s and final elongation at 72°C for 7 min (Felske *et al.*, 1997). PCR fragments were separated on a 1% (m/v) agarose gel (Merck), stained with ethidium bromide and visualised under UV light.

DNA sequencing of the successful PCR fragments was carried out at the Stellenbosch University DNA Sequencing Facility using ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, USA). Sequences data were compared to sequences in the National Center for Biotechnology Information (NCBI) database using the BLAST search option to determine the closest relatives (Altschul *et al.*, 1997).

Steam pasteurisation and quality parameters

The effect of pasteurisation on the soluble solids, total polyphenol, and verbascoside contents, as well as the antioxidant activities of the hot water infusion of the leaves was evaluated using 30 samples (30 g each) of the tea leaves from 6 pasteurisation time treatments (0, 30, 60, 90, 120 and 150 s) at 99.8°C replicated at random in 5

block replicates. An ARC-steam sterilising cabinet (ARC Infruitec-Nietvoorbij, Stellenbosch) was used for all pasteurisation treatments.

Lippia multiflora infusion

Lippia multiflora infusions were prepared by weighing 2 g of plant material with a Mettler (AE 160) balance into a 200 ml glass beaker. Boiling distilled water (150 ml) was added to the plant material and the mixture stirred for 5 min using a magnetic stirrer. After 5 min, the infusion was strained into a 200 ml volumetric flask and allowed to cool to room temperature. The remaining droplets of infusion on the leaves were then washed down with distilled water into the volumetric flask to make up the volume. The infusion was filtered using a Whatman No.4 filter paper and aliquots (1.5 ml) of each infusion were measured into micro-centrifuge vials and kept frozen at −20°C until further analysis.

Analysis of infusion

Soluble solids, total polyphenol, and HPLC analysis for the determination of verbascoside content, as well as antioxidant activities using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant power (FRAP) assays were performed on the infusion as described below.

Soluble solids content

The soluble solids content of each infusion was gravimetrically determined in triplicate. A 10 ml aliquot of the infusion was measured into a clean and previously weighed nickel moisture dish. The combined weight of the moisture dish and the infusion was then recorded. The moisture dish was placed on a boiling waterbath to evaporate moisture to near dryness, whereafter drying was completed in a laboratory oven at 100°C for 1 h and the final weight was recorded. The difference between the initial and final weight of the moisture dish was calculated and expressed as $mg.ml^{-1}$ of the infusion.

Total polyphenols

The total polyphenol content was determined using a modified Folin-Ciocalteau method as described by Singleton and Rossi (1965). A 7.5% (w/v) stock solution of sodium carbonate was prepared by weighing 75 g of Na_2CO_3 into a 1000 ml volumetric flask, dissolved and filled to the mark with distilled water. Gallic acid was used as a standard solution from which a dilution series of 10 mg.l^{-1}, 20 mg.l^{-1}, 40 mg.l^{-1}, 60 mg.l^{-1}, 80 mg.l^{-1}, and 100 mg.l^{-1} were prepared into 5 ml volumetric flasks. Tea extracts were prepared by measuring 600 µl into 5 ml volumetric flasks and filled to the mark. A 96-well microplate was prepared containing 20 µl each of tea extracts, standards and distilled water used as control. The Folin-Ciocalteau reagent (Merck) was 10 x diluted and 100 µl of the diluted reagent was added to each of the tea extracts, standards and water controls. Within 8 min, 80 µl of the Na_2CO_3 solution was added. Absorbance values were read from a BioTek SynergyHT microplate-reader equipped with Gen5 Secure software. By using the standard curve, the corresponding total polyphenolic content was calculated for each of the tea extracts.

Quantification of verbascoside

Quantification of verbascoside was performed by HPLC-DAD on an Agilent 1200 system (Agilent, Santa Clara, CA) consisting of a quaternary pump, an autosampler, an in-line degasser, a column thermostat, and a diode array detector. Chemstation software for LC 3D systems (Agilent) was used to control the system and record data. Separation was performed at 30°C on an Agilent Gemini 5 µm C18 110 Å column (150x 4.6 mm) protected with an RP/C18 guard column (Vici-AG International, Schenkon, Switzerland), using a gradient elution with acetonitrile (solvent A) and 0.1% aqueous formic acid (solvent B) at a flow rate of 0.8 ml.min^{-1}. The injection volume was 10 µl and detection was at 330 nm.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Free radical scavenging activity was determined using the method of Rangkadilok *et al.* (2007) with slight modification. The DPPH solution was prepared by dissolving 3.94 mg of the DPPH radical (Sigma) in 100 ml methanol (Merck). The solution was covered and kept in a dark cupboard until use. The radical stock solution was prepared daily. Using a 96-deep well microplate, 25 µl of the standards, distilled water controls and tea infusions were measured into their allocated wells and 255 µl

of the DPPH added. The plate was sealed with a silicon mat to prevent the evaporation of the methanol and incubated in a dark cupboard. After 2 h of incubation, 200 µl of the reaction mixture was measured into the corresponding wells on a flat-bottom microplate and the decrease in absorbance monitored at 515 nm using a BioTek SynergyHT microplate-reader equipped with Gen5 Secure software. All determinations were performed in triplicate and results expressed as µM trolox.mg^{-1} infusion.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power assay (FRAP) was performed, using Trolox as standard with a concentration range of 50–600 µM. The freshly prepared FRAP reagent contained 100 ml of 300 mM acetate buffer (pH 3.6), 10 ml of 10 mM 2,4,6-tripyridy-*s*-triazine (TPTZ) (Sigma) in 40 mM HCl, and 10 ml of 20 mM FeCl$_3$ (Sigma). Aliquots (20 µl) of the blank (dH2O), standards and tea samples were added into their allocated wells in a 96-well microplate, followed by the addition of 180 µl of the FRAP reagent and incubated at 37°C for 4 min. Increases in absorbance due to the formation of a coloured TPTZ-Fe^{2+} complex were monitored spectrophotometrically at 593 nm. The FRAP values of the extracts were expressed as µM trolox.mg^{-1} infusion. All determinations were performed in triplicate.

Statistical analysis

The data obtained for the quality parameters of the infusion were subjected to analysis of variance using the General Linear Models (GLM) procedure of SAS statistical software version 9.1 (SAS Institute Inc., Cary, USA). Student's t-least significant difference was calculated at the 5% level to compare treatment means. A probability level of 5% was considered significant. The microbial colony counts were expressed as mean ± standard deviation.

RESULTS AND DISCUSSIONS

Microbial content of *Lippia multiflora* leaves

The microbial content of *Lippia multiflora* tea leaves before and after pasteurisation is summarised in Table 1. The mean viable aerobic count on the untreated herbal tea leaves was 1.3 x 10^4 cfu.g^{-1} on the PCA growth medium. Steam pasteurisation, reduced this counts to 5.5 x 10^2 cfu.g^{-1}. However, the initial aerobic count was in conformity with the requirements of the WHO standards, the US pharmacopoeia, and the specifications of the European Herbal Infusion Association (EHIA), as well as other requirements cited in the literature (Shinagawa, 1990). In some cases viable aerobic plate counts of up to 10^7 are acceptable (Kolb, 1999), confirming that the levels of aerobic counts were within specifications.

The *L. multiflora* tea leaves that were unpasteurised showed the highest mean fungal count of 2.1 x 10^4 cfu.g^{-1} on the YPDA, and 1.6 x 10^4 cfu.g^{-1} on the PDA media (Table 1). Although these counts are generally lower than what has been reported in the literature for similar herbal products (Halt & Klapec, 2005; Schweiggert *et al.*, 2005), they are higher than the fungal requirements of 1.0 x 10^4 cfu.g^{-1} (WHO, 2007) and 2 x 10^2 cfu.g^{-1} (Donia, 2008) stipulated by the World Health Organisation and the US pharmacopoeia, respectively. Steam pasteurisation reduced the fungal counts to 3.3 x 10^2 cfu.g^{-1} and 5.5 x 10^2 cfu.g^{-1} on the PDA and YPDA media, respectively. These counts are within the fungal specifications of the WHO of 1.0 x 10^4 cfu.g^{-1} and the EHIA of 1.0 x 10^5 cfu.g^{-1}, but failed to meet the more stringent stipulations of the US pharmacopoeia.

The mean colony count on the untreated tea leaves was 5.9 x 10^3 cfu.g^{-1} on the MRS growth medium and was reduced to 7.3 x 10^2 cfu.g^{-1} upon steam pasteurisation. Although it is possible for other lactic acid bacteria of different genera to proliferate on the MRS medium (Jackson *et al.*, 2002), this medium has been used successfully to enumerate Gram-positive, non-spore-forming *Lactobacillus* species (Jang *et al.*, 2007; Waite, 2009). The WHO requirement for herbal materials and the South African regulation for the export of rooibos and honeybush have no specifications for lactobacilli limits.

Coliform counts were 4.0 x 10^2 cfu.g^{-1} on the VRBA growth medium and further examination by fluorogenic methods (González *et al.*, 2003) excluded the

presence of *E. coli*. Coliform bacteria of less than 10^2 cfu.g^{-1} are usually acceptable (Suwansonthichai & Rengpipat, 2003). Steam pasteurisation reduced the initial coliform load to undetectable levels. However, the initial presence of coliform bacteria is an apparent indication of a failure of hygienic and sanitary measures during the preparation of the tea (Avila *et al.*, 1989). Good hygienic and sanitary controls would have to be implemented to prevent future contamination with coliform bacteria.

Generally, steam pateurisation at 99.8°C for 2.5 min showed a substantial reduction of microbial counts on all the media used. Complete product pasteurisation was not achieved, but may not be desirable considering the potential negative impact of pasteurisation on the bioactive compounds of herbal teas (Joubert *et al.*, 2009).

Table 15. Colony counts (cfu.g^{-1}) obtained on different microbial media by culturing samples of the herbal tea.

Media	Untreated		Steam-pasteurised	
	Range	Mean ± SD	Range	Mean ± SD
PCA	6.1×10^3 –2.2×10^4	$(1.3 \pm 0.7) \times 10^4$	2.0×10^2 –8.5×10^2	$(5.5 \pm 2.7) \times 10^2$
PDA	7.9×10^3 –2.4×10^4	$(1.6 \pm 0.9) \times 10^4$	1.0×10^2 –4.0×10^2	$(3.3 \pm 1.5) \times 10^2$
YPDA	3.6×10^3 –3.8×10^4	$(2.1 \pm 1.3) \times 10^4$	2.0×10^2 –8.0×10^2	$(5.6 \pm 2.5) \times 10^2$
MRS	2.3×10^3 –1.5×10^4	$(5.9 \pm 6.2) \times 10^3$	3.5×10^2 –9.5×10^2	$(7.3 \pm 2.3) \times 10^2$
VRBA	ND –4.0×10^2	$(4.0 \pm 0.0) \times 10^2$	ND	ND

PCA = plate count agar; PDA = potato dextrose agar; YPDA = yeast peptone dextrose agar; MRS = malt extract agar; VRBA = violet red bile agar; ND = not detected; SD = standard deviation

Identification of bacterial isolates

The microorganisms isolated from both pasteurised and unpasteurised tea leaves were mostly Gram-positive, rod-shaped sporeformers. Isolates 00011, 00012, 15005, and 15009 had unexpectedly mixed Gram reactions. This was possibly due to the age of the microbial cells. However, isolates were further subjected to molecular identification to confirm their specific identity.

PCR products of 1.5 kb in size were successfully amplified utilising the universal primers, F8 and R1512, to amplify a part of the 16S rDNA. For all the isolates, a minimum of 800 bases were sequenced and used for comparison on the NCBI database, except for isolate 15002 where only 760 bp were used (Tables 2 and 3). Three *Bacillus pumilus* strains (NCBI Accession EU927407.1, 99% sequence similarity; NCBI Accession EU366363.1, 99% sequence similarity; and NCBI Accession FJ032017.2, 99% sequence similarity) were identified in the unpasteurised *L. multiflora* herbal tea leaves. *Bacillus vallismortis* (NCBI Accession FJ009394.1, 95% sequence similarity), *Kocuria marina* (NCBI Accession EU073966.1, 99% sequence similarity) and *Pantoea ananatis* (NCBI Accession DQ777968.1, 97% sequence similarity) were also identified in the unpasteurised tea leaves (Table 2).

Only four *Bacillus* species were identified in the steam-pasteurised tea leaves. These were *Bacillus licheniformis* (NCBI Accession EU231634.1, 99% sequence similarity), *B. pumulis* (NCBI Accession FJ032017.1, 99% sequence similarity), *B. subtilis* (NCBI Accession EU221673.1, 99% sequence similarity) and *B. megaterium* (NCBI Accession FJ009396.1, 99% sequence similarity) (Table 3), suggesting that only bacteria of the genus *Bacillus* tolerated the steam pasteurisation (Table 3). This corroborates earlier observations that bacterial spores belonging to the *Bacillaceae* family exhibit resistance to thermal treatment (Martins *et al.*, 2001; Donia, 2008).

Except for the *Bacillus pumilus* strain (NCBI Accession FJ032017.1, 99% sequence similarity) which was present in both the unpasterised tea leaves (as isolate 00015) and in the steam-pasteurised leaves (as isolate 15009), all the other *Bacillus* species were either identified only in the unpasteurised leaves or in the pasteurised samples. Therefore, while the thermal resistance (at 99.8°C for 2.5 min) of all the surviving *Bacillus* species can be inferred, this is particularly so for the *B. pumilus* strain (NCBI Accession FJ032017.1, 99% sequence similarity).

Table 16. Results obtained from comparing DNA sequence data of isolates from unpasteurised *L. multiflora* tea leaves to their close relatives in the NCBI database.

Isolate	Identification	NCBI Accession	No of bases sequenced	Sequence similarity (%)
00003	*Bacillus pumilus*	EU927407.1	823	99
00004	*Kocuria marina*	EU073966.1	810	99
00010	*Bacillus vallismortis*	FJ009394.1	809	95
00011	*Pantoea ananatis*	DQ777968.1	836	97
00014	*Bacillus pumilus*	EU366363.1	834	99
00015	*Bacillus pumilus*	FJ032017.1	805	99

Table 17. Results obtained from comparing DNA sequence data of isolates from steam-pasteurised (temperature 99.8°C for 2.5 min) *L. multiflora* tea leaves to their close relatives in the NCBI database.

Isolate	Identification	NCBI Accession	No of bases sequenced	Sequence similarity (%)
15002	*Bacillus licheniformis*	EU231634.1	760	99
15009	*Bacillus pumilus*	FJ032017.1	826	99
15010	*Bacillus subtilis*	EU221673.1	837	99
15011	*Bacillus megaterium*	FJ009396.1	847	99

Most species of the genus *Bacillus* are regarded as ubiquitous, endospore-forming Gram-positive bacteria which are non-pathogenic, with the exception of *B. cereus* and *B. anthracis* (Sabaté *et al.*, 2009). They are capable of forming endospores that are resistant to heat, dehydration, other physical and chemical stresses, and can grow under aerobic or anaerobic (facultatively) conditions (Shinagawa, 1990; Borsodi *et al.*, 2007). All four species that survived the steam pasteurisation in this study (Table 3) have been previously described as non-pathogenic. Some investigators have published the beneficial application of *B.*

subtilis as a probiotic microorganism (Endres *et al.*, 2009). Moreover, *B. subtilis* is capable of producing a number of toxins that act against disease-causing pathogens in humans and plants (Pan *et al.,* 2008). *Bacillus licheniformis* is an industrial microorganism with broad applications (Da Silva *et al.*, 2009), including the production of thermostable α-amylases (Hmidet *et al.*, 2008). Additionally, *B. megaterium* has attained a generally-regarded-as-safe (GRAS) status (Wang *et al.*, 2006) and *B. pumilus* has been isolated from Korean soyabean with antifungal activities against the aflatoxin-producing fungi, *Aspergillus flavus* and *A. parasiticus* (Cho *et al.*, 2009), where it acts as a biocontrol agent against the toxigenic fungi. Finally, *Bacillus pumilus* is a known plant-associated bacterium with protease activity and may hence participate in the decomposition of dead plants because of its ability to degrade protein, cellulose and pectin (Borsodi *et al.,* 2007).

In spite of the generally safe status of these *Bacillus* species, some of them have also been implicated in food poisoning cases where they have been shown to produce heat-stable toxins. Heat-stable, toxin-producing strains include *B. licheniformis* and *B. pumilus* (Neiminen *et al.*, 2007). The toxic agents produced are connected to the clinical symptoms of cows suffering from mastitis (Nieminen *et al.*, 2007). *Bacillus pumilus* is also sometimes isolated as the sole contaminant in cases of food poisoning (Suominen *et al.*, 2001). Therefore, the pathogenic potential of the specific strains isolated in this study will need further instigation.

Isolate 00011 is an enterobacterium strain related to *Pantoea ananatis* (NCBI Accession DQ777968.1, 97% sequence similarity) (Table 2). Several species of the genus *Pantoea* are known to be plant pathogens usually isolated from soil, fruit and vegetables (De Baere *et al.*, 2004; Goszczynska *et al.*, 2006; Brady *et al.*, 2008). Members of the genus *Pantoea* have been isolated from young *Eucalyptus* trees in Uganda, Argentina and Uruguay which showed a disease similar to bacterial blight (Brady *et al.*, 2008). These bacterial strains are reportedly linked to several human infections but have weakly understood ecology and host interactions (Brady *et al.*, 2008). Blood cultures of a patient with a history of cerebrovascular accidents and deep venous thrombosis tested positive for *P. ananatis* (De Baere *et al.*, 2004).

Isolate 00004 was identified as *Kocuria marina* (NCBI Accession EU073966.1, 99% sequence similarity). *Kocuria* species are members of the bacterial group referred to as Coryneform bacteria (Denis & Irlinger, 2008). Members of *Kocuria* are Gram-positive, aerobic, coccoid, non-encapsulated, non-halophilic

and non-endospore-forming (Kim *et al.*, 2004). *Kocuria marina* is a Gram-positive, aerobic, non-motile, halotolerant coccoid bacterium (Kim *et al.* 2004). It is not cited as a human pathogen.

The colony forming unit of a contaminating bacterium per gram (cfu.g^{-1}) in a given food matrix is used as an indicator of its safety in that food system. In this study, steam pasteurisation resulted in approximately 2-log reductions of fungal colony counts from 2.1×10^4 to 5.6×10^2 cfu.g^{-1}. This is considered acceptable in the literature (Romagnoli *et al.,* 2007; Donia, 2008), and meets the requirement of the World Health Organisation (WHO, 2007). While national standards differ on the limits of aerobic counts and in some cases 10^7 cfu.g^{-1} is considered within acceptable limits (Kolb, 1999), coliform bacteria usually do not exceed 100 cfu.g^{-1} (Suwansonthichai & Rengpipat, 2003). In this study, coliform bacteria that were initially detected in the untreated samples at 400 cfu.g^{-1} were not detected after pasteurisation.

Similar to other products, botanical materials do not necessarily have to be completely sterile. However, it is important that they are subjected to microbiological control to ensure that both the product and the consumer are not exposed to unacceptably high levels of contaminants (De la Rosa *et al,* 1995).

Effect of steam pasteurisation on antioxidant parameters of *L. multiflora* tea

After subjecting the tea leaves to a pasteurisation temperature of 99.8°C for different time periods ranging from 0 to 2.5 min, the soluble solids, total polyphenol and the verbascoside contents did not significantly change ($P > 0.05$) (Table 4). At time 0 s, SS was 1.89 mg.ml^{-1}, TP 17.55 g GAE.100^{-1}, and verbascoside content was 7.71 g.100 g^{-1}. These increased to 1.92 mg.ml^{-1}, 17.91 g GAE.100 g^{-1}, and 7.74 g.100 g^{-1}, respectively, at the end of pasteurisation. In all cases, however, there were no apparent increasing or decreasing trend with the increasing pasteurisation time, and the changes observed were not significant ($P > 0.05$). The increase in FRAP antioxidant from 700.62 µmol.g^{-1} at the start of pasteurisation to 751.02 µmol.g^{-1} at the end of the process was also not significant ($P > 0.05$).

The DPPH antioxidant activity did not change significantly ($P > 0.05$) after 2.5 min of steam pasteurisation. At time 90 s of steam treatment, however, the DPPH content appeared to have significantly reduced from its value at time 30 s, but the

trend of reduction was not sustained as the values at 60 s, 120 and 150 s did not significantly differ ($P > 0.05$) from the value at time 0 s. The highest antioxidant value (DPPH) occurred at time 30 s, but this did not show a reducing or increasing trend either. It is possible that the samples treated at 90 s were less homogenous with respect to the amount of actual tea leaves present to contribute towards the antioxidant value, as the antioxidant compound is not evenly distributed in plant leaves, twigs and other parts of the plant (Juliani *et al.*, 2006).

Table 18. Effect of steam pasteurisation period at 99.8°C on the soluble solids (SS), total polyphenol (TP) and verbascoside contents, as well as on the antioxidant activity of *L. multiflora* herbal tea leaves.

Time	SS (mg.ml^{-1})	TP (g.GAE.100 g^{-1})	Verbascoside (g.100 g^{-1} SS)	DPPH (µmol.g^{-1})	FRAP (µmol.g^{-1})
0	1.89^a ± 0.11	17.55^a ± 0.96	7.71^a ± 0.30	1957.46^{ab} ± 109.75	700.62^a ± 39.97
30	1.95^a ± 0.06	17.76^a ± 0.65	7.61^a ± 0.27	1974.96^a ± 61.90	722.62^a ± 41.40
60	1.87^a ± 0.08	17.65^a ± 0.53	7.74^a ± 0.26	1919.30^{ab} ± 41.15	703.02^a ± 66.45
90	1.85^a ± 0.11	17.85^a ± 0.91	7.70^a ± 0.45	1863.40^b ± 113.27	724.47^a ± 59.03
120	1.91^a ± 0.14	17.51^a ± 0.73	7.65^a ± 0.52	1935.36^{ab} ± 89.39	707.87^a ± 51.62
150	1.92^a ± 0.05	17.91^a ± 0.62	7.74^a ± 0.31	1944.56^{ab} ± 81.90	751.02^a ± 55.06
LSD	0.13	0.89	0.28	95.68	63.40

LSD = least significant difference; GAE = gallic acid equivalent; same superscript in the same column don't differ significantly

The effect of pasteurisation on the stability of antioxidant compounds appears to be a function of the thermal stability of the specific compound and varies from one product to another. Detrimental effects are often associated with those compounds with less thermal resistance. Antioxidant compounds such as furosine (Zanoni *et al.*, 2003), rutin (Sun *et al.,* 2007), oleuropein and verbascoside (Conde *et al.*, 2009) have shown high thermal resistance after steam pasteurisation with no significant decrease in their antioxidant activities. In the present study, verbascoside, being the main antioxidant compound of *L. multiflora* herbal tea (Juliani *et al.,* 2006; Pascual *et al.*, 2006) appeared to have exhibited a considerable thermal stability for which reason the antioxidant activities did not significantly change ($P > 0.05$).

CONCLUSION

Microbial contaminants on *L. multiflora* herbal leaves tea have been identified. These were largely rod-shaped, spore-forming, Gram-positive *Bacillus* species. Although generally regarded as harmless to humans, other studies have found toxigenic strains of the species identified in this study. Toxicity tests for the specific strains identified in this study are required to confirm the status of these specific strains. *Kocuria marina* and an *Enterobacteriaceae* bacterium related to *Pantoea ananatis* were also identified.

The microbial counts on the tea leaves were highest on YPDA at 2.1×10^4 cfu.g^{-1} and this was reduced to 5.6×10^2 cfu.g^{-1} after steam pasteurisation. This level of microbial counts is within the acceptable limits as stated by the specifications of the World Health Organisation for herbal infusions. Viable aerobic counts were lower than the maximum limits permitted by the WHO and coliform bacteria were not detected after pasteurisation.

Steam pasteurisation did not significantly alter the soluble solids, total polyphenol and verbascoside contents. Similarly the antioxidant activities as measured by the FRAP and DPPH assays did not change significantly ($P > 0.05$). Verbascoside, the major antioxidant compound of *L. multiflora* herbal tea appears to show considerable thermal stability for which reason the overall antioxidant activity of the tea did not significantly change ($P > 0.05$) with steam pasteurisation.

This study shows that *L. multiflora* herbal tea material can be pasteurised at 99.8°C for 2.5 min without significantly affecting the stated quality parameters of the tea. It is, however, recommended that pasteurisation treatment should complement the adherence to good agricultural and hygienic practices as some bacteria could survive this treatment.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

The consumption of tea, particularly herbal teas is increasing worldwide (Malinowska *et al.*, 2008). Countries such as the United Kingdom (Anon, 2007), China (Wong, 1998), Germany (Wilson *et al.*, 2004), South Africa (Joubert *et al.*, 2008; Van Wyk, 2008), Canada and the United States of America (Nestmann *et al.*, 2006; Schweizer, 2006) are seeing an increased consumption of herbal teas. Empirical evidence has suggested that herbal teas contain antioxidant compounds capable of promoting health (Weisberg, 2001; Béliveau & Gingras, 2004), and that their consumption has been associated with reduced risk of contracting cardiovascular diseases and cancer (Weisberg, 2001; Kang *et al.*, 2003; Reddy *et al.*, 2003).

Despite the intent to use these products for the purpose of providing nutritional and beneficial health effects, there have been recent high-profile reports of adverse effects attributed to their consumption (Nestmann *et al.*, 2006). Some herbal products have been found to contain unacceptably high levels of pathogenic microbial contaminants that may undermine their potential health benefits (Rizzo *et al.,* 2004; Bianco *et al.,* 2008; Stickel *et al.*, 2009). As primary agricultural products, herbal teas are naturally predisposed to microbial contamination, which may occur during the manufacturing process or during storage (Martins *et al.,* 2001; Ernst & Pittler, 2002). Therefore, the search for novel techniques that may destroy undesired microorganisms, but with less adverse effects on product quality has engaged the food industry in recent years (Master *et al.*, 2004).

Lippia multiflora herbal tea is in the process of entering the international market, necessitating the development of a treatment method to control possible microbial contamination. The initial viablet aerobic counts of about 1.3×10^4 cfu.g^{-1} on the tea leaves were much lower than results reported in the literature (Kolb, 1999; Satorres *et al.*, 1999; Martins *et al.*, 2001). This was further reduced to 5.5×10^2 cfu.g^{-1} on the PCA media after steam pasteurisation at 99.8°C for 2.5 min. The steam pasteurisation also resulted in the reduction of the microbial load on the YPDA media from 2.1×10^4 cfu.g^{-1} to 5.6×10^2 cfu.g^{-1}. These counts comply with the requirement of the World Health Organisation (WHO, 2007). Coliform bacteria which were initially present at 100 cfu.g^{-1} on the VRBA agar, but were undetectable after

pasteurisation, did not include *Escherichia coli* following further examination by fluorogenic methods as prescribed by González *et al.* (2003). The microbial counts after pasteurisation appear to suggest that this treatment led to approximately 2-log reductions of the microbial load on all the media used for enumeration.

This study also provided a profile of the microbial contaminants of *L. multiflora* herbal tea, which predominantly included rod-shaped, spore-forming, Gram-positive *Bacillus* species. Although generally regarded as harmless to humans and in some cases even beneficial (Dedej *et al.*, 2004; He *et al.*, 2006; Hmidet *et al.*, 2008; Pan *et al.*, 2008; Cho *et al.*, 2009; Da Silva *et al.*, 2009; Endres *et al.*, 2009; Sabaté *et al.*, 2009), other studies have found pathogenic strains of the *Bacillus* species identified in this study (Suominen *et al.*, 2001; Wang *et al.*, 2006; Neiminen *et al.*, 2007). While pathogenic tests for the specific strains identified in this study will help in understanding the risk that these microbes pose, it is important for the producers of the tea to observe good agricultural and hygienic practices during the production and processing stages.

Steam pasteurisation did not significantly alter the antioxidant activities of the tea as determined by the DPPH and FRAQP assays. Soluble solids, total polyphenol and verbascoside contents of the infusion were also not significantly affected by the pasteurisation treatment. This indicates the potential to successfully pasteurise the tea before it is sold to consumers.

When compared to commercial samples of rooibos and honeybush teas, two South African teas which have gained recognition on the international market (Joubert *et al.*, 2008), *L. multiflora* showed significantly higher ($P < 0.05$) antioxidant activities than these teas on the basis of 'cup of tea' strengths. This provides opportunities to position and market the tea on the international market.

An HPLC method was developed for the quantification of verbascoside, the major antioxidant compound of *L. multiflora* (Pascual *et al.*, 2001; Juliani *et al.*, 2006). The method could, in future, be employed in research on this bioactive compound of *L. multiflora* and for quality control purposes of its derived products.

Concluding remarks

This study showed the potential application of *L. multiflora* as a source of verbascoside, a compound that possesses useful bioactive properties. Further

research into harnessing this compound for nutraceutical purposes is recommended. There is the need to establish the variation in verbascoside content of *L. multiflora* plants as it will affect its antioxidant activities and other bioactivities associated with verbascoside. Future research should investigate the health-promoting properties of *L. multiflora* as other compounds in the plant could be important. A toxicological assessment should also receive attention. Positive outcomes will support the use of *L. multiflora* as a source of the compound and promote the large scale commercial use of this herbal tea. Such commercial application would, however, have to be based on a comparative study showing the feasibility, sustainability and the profitability of obtaining the compound relative to several other plants which have also been identified as sources of verbascoside. Furthermore, research is required to identify and characterise the four other compounds found in relatively high amounts in the infusion since they could, synergistically, be contributing to the observed antioxidant activities, and potentially other bioactivities. The identification and quantification of verbascoside in the *L. multiflora* infusion, as well as the relatively higher antioxidant contents compared to rooibos and honeybush should also provide the basis for future studies on the therapeutic application of this herbal tea. Verbascoside could potentially form the basis for future quality control of *L. multiflora*.

Finally, adequate microbial control of plants that are used in herbal infusions would address safety concerns and enhance their consumption. In some cases, contaminating microorganisms should be subjected to further toxicological analysis so that seemingly harmless microbes do not present health hazards to consumers.

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