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Effects of high temperatures on leaf anatomy and phytochemistry in *Lippia javanica* (Verbenaceae)

By

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Declaration

I, Edith J. Singini, hereby declare that this dissertation is my original work submitted for the degree of Master of Science to the University of the Witwatersrand, Johannesburg. It has never been submitted to any other University for any other degree or examination.

The described experimental work in this Masters project was carried out at the University of Witwatersrand, Johannesburg, under the supervision of Dr. Ida Risenga (School of Animal, Plant and Environmental Sciences) and Prof. Luke Chimuka (School of Chemistry).

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Abstract

Lippia javanica (Burm.F.) Spreng is a medicinal plant used in various rural communities as a cheaper, safer and more desirable alternative for treating various ailments. In Southern African countries such as South Africa, Botswana and Zimbabwe, *L. javanica* is sold in local markets and informal sectors. The shrub contains phytochemicals that are responsible for a wide range of medicinal properties, which are profoundly affected by environmental stresses. High temperatures significantly affect plant growth and production, and it was predicted that South Africa would experience increased heat due to climate change. Therefore, the aim of this study was to assess the potential impact of high temperatures (47/37ºC-day/night simulation) on the leaf morphology, histology, essential oil yield and composition, and the total phenolic and flavonoid content in *L. javanica*. Mature plants of *L. javanica* were exposed to 47/37ºC episodically for 48, 96 and 144 hours in a climate test incubator, while control samples were kept at 25/20ºC in the greenhouse. The histological assessment was conducted using the glutaraldehyde-osminium method, while essential oils were analysed using the Gas Chromatography–Mass Spectrometry (GC-MS) and the total phenolic and flavonoid content were estimated using the UV-visible spectrophotometer. Control samples showed 144.75 mg GAE/g (phenolics) and 81.17 mg QE/g (flavonoids). The exposure to high temperatures resulted in an increase in total phenolics from 191.33 mg GAE/g (48 hours) up to 441.94 mg GAE/g (144 hours) ($F_{(7,40)} = 1670$; $P < 0.001$). Similarly, the total flavonoid content increased from 115.91 mg QE/g (48 hours) up to 268.66 mg QE/g (144 hours). The essential oil percentage yield increased significantly from 48 hours (3.2%) to 144 hours (6.3%) compared with the control (1.2%) ($F_{(3, 20)} = 16.31$; $P < 0.0001$), and the grouped essential oil components also showed an increasing trend. The histological assessment showed that the leaf midrib and blade had thickened. Trichome height ($F_{(7, 162)} = 32.09$, $P < 0.001$) and diameter ($F_{(7, 161)} = 3.56$, $P < 0.01$) also increased significantly from 48 hours to 144 hours, which possibly correlates with the increased phytochemicals. The present data suggest that *L. javanica* has possible mechanisms to adapt under high temperatures, which was further indicated by the growth of new shoots when treated samples were returned to the greenhouse (25/20ºC). *L. javanica*'s response to high temperatures may positively impact its medicinal properties and, consequently, the range of ailments that it treats.

Key words: Ailments, Cellular structures, Essential oils, Flavonoids, Histology, Medicinal plants, Morphology, Phenolics.

Abbreviations and Symbols

L. javanica	-	*Lippia javanica*
TPC	-	Total Phenolic Content
TFC	-	Total Flavonoid Content
LC	-	Least Concern
IUCN	-	International Union for Conservation of Nature
GC-MS	-	Gas Chromatography – Mass Spectrometry
GAE	-	Gallic Acid Equivalence
QE	-	Quercetin Equivalence
CSIR	-	Council for Scientific and Industrial Research
IHN-L	-	Indigenous Healing Network List
WHO	-	World Health Organisation
UV	-	Ultraviolet Radiation
ANOVA	-	Analysis of Variance
TIC	-	Total Ion Chromatogram
$AlCl_3$	-	Aluminium Chloride
$NaNO_3$	-	Sodium Nitrate
MeOH	-	Methanol
mm	-	millimetres
cm	-	centimeters
%	-	Percent
ppm	-	parts per million
mg	-	milligram
µm	-	micrometers
rpm	-	revolution per minute

Dissertation structure

The first chapter comprises a general introduction contextualizing the study and stating the aims, objectives and hypotheses of the study. It also includes the rationale and problem statement, along with research questions. The introduction highlights the use of medicinal plants globally and the development of natural chemotherapeutics and drugs from plants. It also stated the importance of integrating medicinal plants with orthodox medication. The second chapter is a detailed literature review on *Lippia javanica*, which gives background information on the study species, its geographic distribution, bioactive compounds secreted and economic value herewith. The third and fourth chapters are stand-alone chapters to be submitted as scientific papers. These two chapters have a degree of repetition, which is limited to the methodology section.

The third chapter investigates the effects of high temperatures on the leaf morphology and histology of *Lippia javanica*. This chapter assesses the structural modifications of *L. javanica* after exposure to high temperatures with the aim of relating the results with the functionality of *L. javanica* under high temperatures. Thus the fourth chapter investigates the effects of high temperatures on the essential oil yield and composition, as well as the total phenolic and flavonoid content in *L. javanica*. This chapter provides the functionality aspect of *L. javanica* under elevated temperatures. The two chapters aim to elucidate the significant role that high temperatures have on the growth of medicinal plants and the production of bioactive compounds as a defence mechanism against environmental stress. The two chapters, therefore, provide evidence of the relationship between function and structure in response to environmental stresses.

CHAPTER 1

1.1 INTRODUCTION

1.1.1 General Background

For several years, plants have been considered an excellent source of natural ingredients for food, pharmaceuticals, and cosmetic industries (Wronka *et al*., 1995). They produce compounds that exhibit many medicinal properties such as; antioxidant, antimicrobial, anticancer, anti-inflammatory, antalgic and neuroprotection (Dzoyem and Eloff, 2015; Sulaiman and Balachandran, 2012). In most African countries, people rely on plants as their primary source of medicine for health care (Gurib-Fakim, 2006). Medicine based on indigenous plants are still used today worldwide, and as reported by the World Health Organisation (WHO), approximately 80% of the Earth's population depends on medicinal plants for health and wellbeing (Srivastava *et al*., 1996; Gurib-Fakim, 2006).

The dependence and demand for medicinal plants have led to the development of natural chemotherapeutics and drugs from plants (Hashempour *et al*., 2014). This development is due to the increase in metabolite diseases such as cancer, diabetes and cardiovascular infections (Gurib-Fakim, 2006). For instance, active compounds from plants are used as ingredients in the production of herbal health care formulations by pharmaceutical industries and herbal-based cosmetic products, along with herbal nutritional supplements (Wronka *et al*., 1995; Sulaiman and Balachandran, 2012). Thus, medicinal plants are of high economic importance in addition to their known medicinal and cultural uses. In the United Kingdom, for example, the use of herbal medicinal products has increased by 50% since 1995, where consumers spent approximately £65 million in 2000 (Van Wyk, 2008).

Medicinal plants contain active ingredients or chemical compounds that may treat diseases and maintain health (Houghton, 1995; Van Wyk, 2008). The medicinal properties of these plants lie in the phytochemicals or bioactive compounds such as flavonoids, phenolics, tannins, alkaloids and essential oils (Van Wyk, 2008). More medicinal plants are yet to be investigated and they could potentially be new sources of medicinal drugs (Fabricant and Farnsworth, 2001; Gurrib-Fakim, 2006). For instance, the production of morphine by E.

Merch in 1826 in Germany, marked the first commercialisation of drugs derived from plants (Dar *et al*., 2017). Herbal medications are still of use today and in some countries, medicinal plants are highly preferred than orthodox medication because of their high efficacy and affordability (Calixto, 2000; Gurib-Fakim, 2006). For example, to treat multi-drug-resistant malaria, an anti-malarial drug called Artemisinin, which is obtained from a plant called *Artemisia annua*, is used and it is mostly preferred because there is no inexpensive alternative for treating this ailment (Brisibe *et al*., 2008).

Although 50% of all drugs are derived from medicinal plants, more could be done to promote the inclusion of medicinal plants in producing conventional medication (Calixto, 2000). Scientific studies are constantly trying to bridge the gap between orthodox and traditional (plants) medicines because of their coupled effectiveness in treating illnesses (Fox, 2000). The bridge between these medicines could accommodate both poor and rich people as the prices become more affordable (Fox, 2000; Hall *et al*., 2000). Bridging the two types of medicines has spread worldwide and authorities in the United States, Germany, Canada, and India are lobbying for the combination of local herbs with orthodox medicines (Houghton, 1995; Fox, 2000). In Australia, the Indigenous Healing Network List (IHN-L) promotes educating lay people in indigenous methods of healing and they have established a centre for scientific research on medicinal plants (Hall *et al*., 2000). This shows that some countries are advancing the integration of orthodox and traditional (plants) medicines; however there are still a lot of countries that are yet to adopt this intervention (Houghton, 1995).

The adoption and use (accessibility and quantity) of medicinal plants, particularly in Sub-Saharan countries is being threatened by climate change (Hansen *et al*., 2001). Some medicinal plants tend to thrive in a wide range of temperatures, but low and high temperature extremes have adverse effects on the morphology and the production of secondary metabolites (Castro-Diez *et al*., 1997; Stinziano and Way, 2014). Temperatures are steadily increasing at an alarming rate due to global warming, defined as the increase in the earth's average temperatures (Kruger and Sekele, 2012; Change *et al*., 2007). These high temperatures have adverse effects on the quality and quantity of essential oils and other bioactive compounds, which are crucial components in the healing process of various ailments (Stinziano and Way, 2014). This implies that under high temperatures, specific medicinal properties in plants may be affected and consequently affect the potential to treat some ailments.

1.1.2 Rationale

Several studies have been conducted on the wide range of chemical extracts used in traditional healing (Calixto, 2000; Gurib-Fakim., 2006; Dar *et al*., 2017). Some studies focused on the bioactive compounds in *Lippia javanica* (Terblanche and Kornelius, 1996; Dlamini *et al*., 2006; Mujovo *et al*., 2008; Lukwa *et al*., 2009; Chagonda and chalchat., 2015; Mahlangeni *et al*., 2018), while others assessed the morphology and histology of the shrub to attain further insights on its medicinal attributes (Combrinck *et al*., 20017; Martinez-Nataren, *et al*., 2011; Asowata-Ayodele, 2015; Asowata-Ayodele *et al*., 2016). However, there have been no studies conducted on the effects of temperature stresses on the anatomy and secretion of bioactive compounds in *L. javanica*. Therefore, this study aimed to investigate the potential impact of high temperatures on the composition and yield of essential oils, the total phenolic and flavonoid content, as well as the leaf morphology and histology of *L. javanica*. Little is known about the responses of *L. javanica* to high temperature stresses; therefore, 47/37ºC (day/night simulation) was chosen as the maximum temperature for this testing design. The above mentioned temperatures were based on the average temperatures obtained in all the six South African provinces where *L. javanica* is currently distributed. High temperature stresses are plant-specific; therefore, this study focused mainly on *L. javanica* because it is extensively used for medicinal purposes and it is of high economic value (Stinziano and Way, 2014). This study will contribute to the understanding of how *L. javanica* responds to high temperatures, thus determining the best harvesting period of the plant for industries, local markets and traditional/herbal medicinal practitioners.

1.1.3 Problem Identification

High-temperature stresses are more frequent due to global warming and they trigger complex responses in plants (Lugina *et al*., 2005)). Increased temperatures have been evident over the last part of the 20th century (Kruger *et al*., 2012); thus, studies need to be conducted to determine the possible effect of these increased temperatures on the extracts of medicinal plants. No studies have assessed the impact of high temperatures on the anatomy and bioactive compounds in *Lippia javanica*; thus, this study aimed to determine the effects of high temperatures on the leaf histology, morphology, essential oil yield and compounds, and the total phenolic and flavonoid content in *L. javanica*. *L. javanica* is a medicinal plant widely

used by indigenous people and herbal/traditional practitioners; therefore, it is only through a greater understanding of the effects of high temperatures on *L. javanica* that we will be able to effectively detect the changes in the chemical composition and functionality of the medicinal properties of the shrub under stress. This information will help determine if the medicinal plant after exposure to environmental stress can treat the same range of ailments, and it will help indigenous people, traditional/herbal practitioners, and industries to determine the suitable harvesting time for maximum productivity and for treating specific diseases. This research will contribute knowledge on the responses of *L. javanica* to environmental stresses and, consequently, how their responses affect the medicinal properties.

This study aimed to answer the four research questions below:

- How is the secretion of phenolics and flavonoids in *Lippia javanica* affected by increased temperatures?
- What effect do high temperatures have on the essential oil components and essential oil yield in *Lippia javanica*?
- What effect do high temperatures have on the leaf histology and trichome density in *Lippia javanica*?
- How does the leaf morphology of *Lippia javanica* respond to the increased high temperatures?
- How well does *Lippia javanica* recover after being exposed to high temperatures?

1.1.4 Aim

To assess the potential impact of high temperatures (47/37ºC - day/night simulation) on the leaf morphology, histology, essential oil yield and composition, and the total flavonoid and phenolic content in *Lippia javanica*.

1.1.5 Objectives

- To assess the effects of high temperatures (47/37ºC) on the leaf morphology and histology of *Lippia javanica* over 48, 96 and 144 hours.
- To screen the effects of high temperatures (47/37ºC) on the essential oil composition, yield (quantity) and percentage of major essential oil compounds in *Lippia javanica* over 48, 96 and 144 hours.

- To investigate the total phenolic and flavonoid content when *Lippia javanica* is exposed to high temperatures (47/37ºC) over 48, 96 and 144 hours.
- To assess the recovery of *Lippia javanica* at optimum conditions (25/25ºC) in the greenhouse after being exposed to high temperatures (47/37ºC).

1.1.5 Hypotheses

a) Under high temperatures, *Lippia javanica*'s leaves will change colour from green to brown and turgidity will gradually decline, causing all the leaves to wilt, curl and roll.
b) The cellular structure of *Lippia javanica* under high temperatures will experience changes such as; increased thickness of cell wall, swelling of trichomes and tightly packed mesophyll cells, which then decrease the intercellular air spaces.
c) The production of essential oils and the total flavonoid and phenolic content will increase under high temperatures. The yield, number of components and percentage of essential oil compounds will increase under high temperatures.

CHAPTER 2

2.1 LITERATURE REVIEW

2.2.1 Botanical overview and distribution of *Lippia javanica*

The plant family, Verbenaceae, encompasses 36 genera and a total of 1034 species (Xaba and SANBI, 2010). *Lippia* is one of the genera in the family and it comprises approximately 240 species of small trees, shrubs and herbs (Terblanche and Kornelius, 1996). It is extensively spread in the tropics of America, India and Africa (Viljoen *et al*., 2005; Chagonda and Chalchat, 2015). *Lippia*'s wide distribution and use in traditional medicine has led to several studies being conducted on the pharmacological and phytochemical activities (Chagonda and Chalchat, 2015).

Lippia javanica (Burm.F.) Spreng is commonly known as "Fever tea" or "Lemon bush" (English), "Koorsbossie" (Afrikaans), "Umsuzwane" (isiZulu) and "Musukudu" (Tswana) (Van Wyk, 2008; Retief, 2006; Xaba and SANBI, 2010; Dzoyem and Eloff, 2015). It is one of the four *Lippia* species that is indigenous to South Africa (Van Wyk, 2008; Viljoen *et al*., 2005). The other indigenous *Lippia* species with an overlapping distribution in South Africa are; *L. scaberrima, L. rehmannii* and *L. wilmsii*. *L. javanica* is an erect, aromatic woody shrub with a lemon-minty smell when crushed and it is said to be the most aromatic amongst South African indigenous shrubs (Watt and Breyer-Brandwijk, 1962; Van Wyk, 2008). The strong aroma protects the plant from browsing animals, thus improving the plant's survival rate (Mujovo *et al*., 2008; Xaba and SANBI, 2010).

L. javanica is drought resistant, grows easily in different soil types and grows relatively fast in sunny environments compared with cold areas (Palgrave *et al*., 2003; Mahlangeni *et al*., 2018). The shrub is mostly dominant in disturbed areas such as woodlands, grasslands and bushvelds (Mahlangeni *et al*., 2018). In particular, it tends to thrive in hostile environments with very little maintenance and it is known to propagate at altitudes as high as 2000m above sea level (Palgrave *et al*., 2003; Mahlangeni *et al*., 2018).

The shrub grows up to five metres in height (Olivier *et al*., 2010) and has dense cream-white flowers that cluster in dense circles of approximately 1 cm in diameter which blossom between February and May (Fig. 1 C) (Oyourou *et al*., 2013). The shrub bears small dry fruits that contain small brown seeds that are positioned right below the flowers after fertilization (Fig. 1 A) (Van Wyk, 2008; Olivier *et al*., 2010). The leaves are hairy, simple, and opposite and they are found in circles of three (Fig. 1 B) (Van Wyk, 2008; Oyourou *et al*., 2013). These leaves are rough with a sharp apex where the base tapers, running down the petiole (Fig. 1) (Olivier *et al*., 2010). The veins of the leaves are highly noticeable, and the margins are saw-like (Palgrave *et al*., 2003).

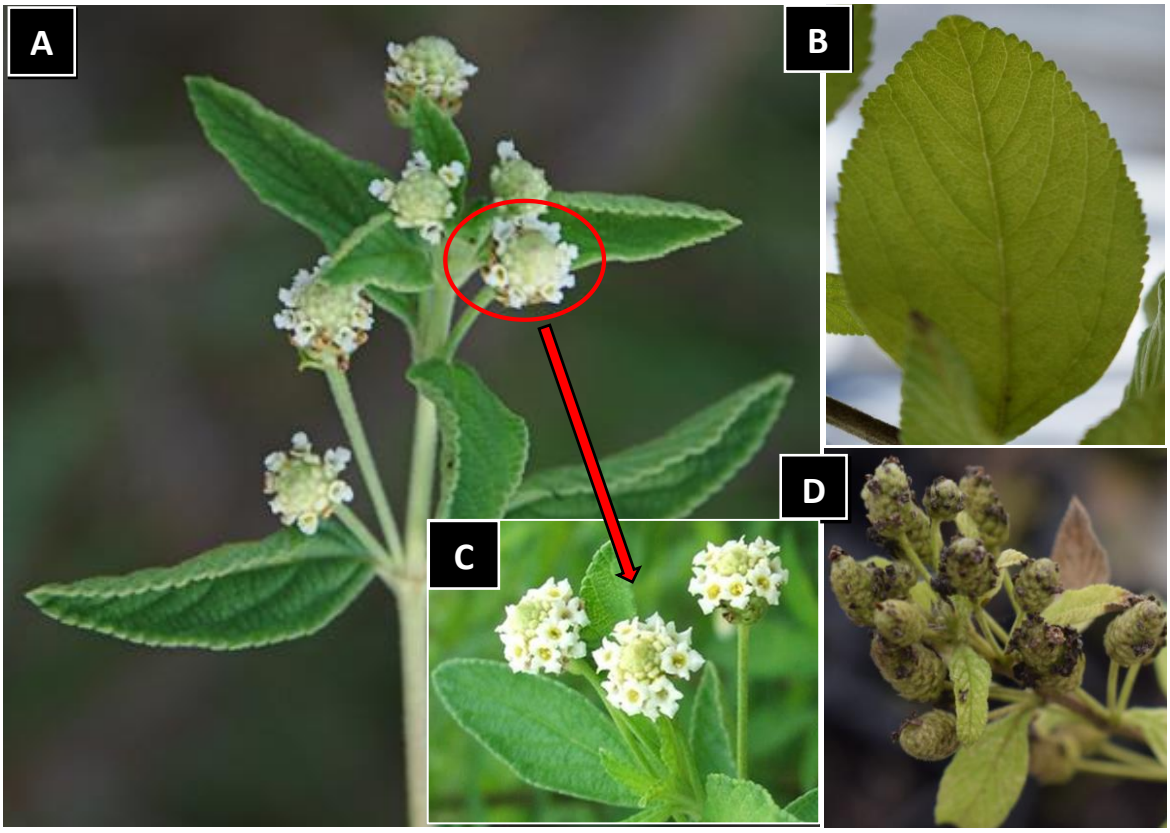
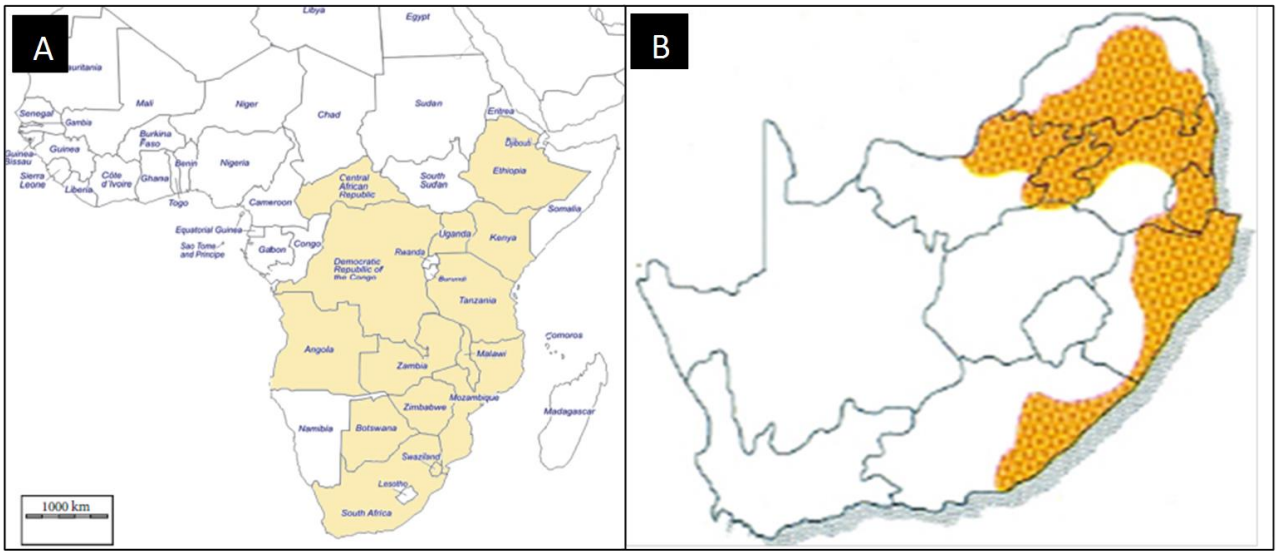


Figure 1. Pictures of *Lippia javanica* taken from wild populations showing the whole morphology (A), the adaxial leaf surface showing pinnate venation (B), flower heads (C) and flower buds (D).

L. javanica is indigenous to South Africa, but its distribution extends towards the tropical African countries, which include Swaziland, Botswana, Zimbabwe, Mozambique, Malawi, Tanzania, Zambia, and Kenya (Fig. 2. A) (Van Wyk, 2008; Asowata-Ayodele, 2015). In South Africa, *L. javanica* grows in Mpumalanga, Limpopo, Gauteng, North West, Eastern Cape and Kwazulu Natal (Fig. 2. B) (Van Wyk, 2002). The shrub is particularly abundant in

the northern provinces of South Africa and Swaziland (Olivier *et al*., 2010). It is also known to grow vegetatively from roots and stems; thus it can be easily propagated (Olivier *et al*.,



2010).

Figure 2. Map depicting countries in Africa where *Lippia javanica* occurs (A) and a distribution map of *Lippia javanica* in South Africa (B) (Adopted from Van Wyk, 2008; Maroyi, 2017).

Raimondo *et al*. (2009) listed *L. javanica* as a species of Least Concern (LC) under the Red List categories of the IUCN (International Union for Conservation of Nature). This listing was because the shrub encroaches reserves and other rangelands, thus easily growing in areas including those with low maintenance (Gordijn, 2010). *L. javanica* has been identified as one of the species found in Savanna and reserve encroachment (Hottman and O'Connor, 1999; Gordijn, 2010) because there are no control mechanisms implemented or introduced in these areas to minimize its growth and spread. Therefore harvesting may be a reasonable control mechanism because, in areas where harvesting is implemented, the shrub will decrease in spread. For example, the shrub has been harvested in Limpopo villages by plant traders and local people who sell it for income generation and as a source of medicine (Raimondo *et al*., 2009), which consequently reduces its populations. Therefore, harvesting whole plants from the wild is a sustainable means of controlling the distribution of the species. What should also be adopted is rather the large-scale harvesting of plants, which is essential in establishing a commercial-scale production.

2.2.2 Medicinal uses of *Lippia javanica*

Lippia species have been historically used as traditional medicines, and some have been validated scientifically (Serrato-Valenti *et al*., 1997; Chagonda *et al*., 2000; Argyropoolou *et al*., 2007; Passos *et al*., 2009; Shikanga *et al*., 2010; Tozin *et al*., 2015). Many African tribes are familiar with the different uses of *L. javanica* in treating various ailments as the shrub has antiviral, antioxidant, antidiarrhoeal, antitrypanosomal, anti-inflammatory, antibacterial, and anticonvulsant essential oil properties (Mujovo *et al*., 2009; Nagavani and Rao, 2010; Shikanga *et al*., 2010; Chagonda and Chalchat, 2015). Although the leaves and stems are commonly used parts of the shrub, roots are also utilised in some circumstances (van Wyk *et al*., 1997). Leaf infusions are prepared and often utilized as inhalants, teas or rubbed onto the skin (Fig. 3) (Mujovo *et al*., 2008). Leaves are also used as food additives or leafy vegetables (Asowata-Ayodele, 2015; Maroyi, 2017). In some cases where roots are used, they are prepared as antidotes to treat food poisoning, sore eyes and bronchitis (Fig. 3) (Hutchings, 1996; Maroyi, 2017).

Traditional healers and pharmaceutical industries all over Africa mostly use *L. javanica* to prepare embodiment formulations for soothing and treating skin disorders such as dermatitis, psoriasis, itchiness and cracking (Fig. 3) (Mujovo *et al*., 2008; Mahlangeni *et al*., 2018). These embodiment formulations can also be prepared as lotions or lotion bars to treat stretch marks in pregnant women (Hutchings, 1996; Maroyi, 2017). In Kenya, it is known that fresh wounds are treated by wrapping approximately 50g of fresh leaves, whereas in South Africa, sprained joints are treated by applying the powder from dried crushed leaves (Fig. 3) (Maroyi, 2017). In Zimbabwe, pneumonia is treated by rubbing the chest and abdomen with leaf ointments (Maroyi, 2017; Mahlangeni *et al*., 2018). Generally, the leaves aid in the healing process along with disinfecting wounds colonized by opportunistic microorganisms (Abubakar, 2009).

Strong infusions made from leaves and stems are commonly used to prepare tea as a cough remedy to treat asthma, chronic coughs, fevers, pleurisy and bronchitis (Fig. 3) (Manenzhe *et al*., 2004; Mujovo *et al*., 2008; Chagonda and Chalchat, 2015). In Botswana, the leaves of *L. javanica* are used as a caffeine-free tea substitute, while in Limpopo and Mpumalanga

provinces of South Africa along with Zimbabwe and Malawi, the tea is used as a nerve tonic or generally as a health tonic (Manenzhe *et al*., 2004; Mujovo *et al*., 2008). The tea is commonly prepared using hot leaf infusions with water or milk (Smith, 1966), and Manenzhe *et al*. (2004) noted that in some places, the tea is consumed daily. Maroyi (2017) and Mujovo *et al*. (2008) stated that the use of herbal teas had increased globally in the past 20 years because of the functional properties of the teas and the increased interest by consumers to produce the tea as this beverage possesses health-promoting properties.

A study on HIV and AIDS patients in Ngwelezane hospital in Northern Kwazulu Natal, South Africa, reported that the primary symptoms of AIDS wear off as patients drink tea infusions made from *L. javanica*'s fresh leaves (Dlamini *et al*., 2006). Other studies reported that *L. javanica* has three compounds that can inhibit the HIV-1 reverse transcriptase enzyme (Manenzhe *et al*., 2004; Mujovo *et al*., 2008). Further studies show that *L. javanica*'s essential oils are being tested in the cream production industries to produce creams that can aid in treating skin conditions common in HIV/AIDS patients (Mokoka, 2005). In addition, *L. javanica* is used with *Artemisia afra* to make remedies that fight against malaria, measles and prophylaxis against lung infections, dysentery and diarrhoea (Fig. 3) (Olivier *et al*., 2010; Maroyi, 2017). These uses indicate that *L. javanica* fights against a wide range of immunosuppressant ailments.

L. javanica possesses other non-medicinal properties that are being thoroughly explored by industries (Fig. 3). Studies have been conducted to validate these non-nutritional uses such as pesticides, insect repellent, and inhibiting bacteria populations. For example, Manenzhe *et al*. (2004) found that the essential oils of *L. javanica* contained several essential compounds such as terpenoids, which promote the plants' effectiveness in inhibiting *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli*. Another study by Muzemu *et al*. (2011) investigated the effectiveness of *L. javanica* as a pesticide and it was found that water extracts from *L. javanica* suppressed up to 66.7% of the aphids and red spider mite population on rape (vegetables) and tomatoes (fruits). The leaves of *L. javanica* are also used as an insect repellent by placing them in cupboards and jars where the aroma can spread (Asowata-Ayodele, 2015). On a commercial scale, large amounts of *L. javanica* are reported to be cultivated in Kenya and South Africa to produce essential oils for the perfume industry and to incorporate in the mosquito repellent candles (Muzemu *et al*., 2011; Asowata-Ayodele, 2015).

These findings show that *L. javanica* has a wide range of uses which include non-medicinal uses such as inhibiting populations of bacteria, pesticides, perfumery, and flying insects.

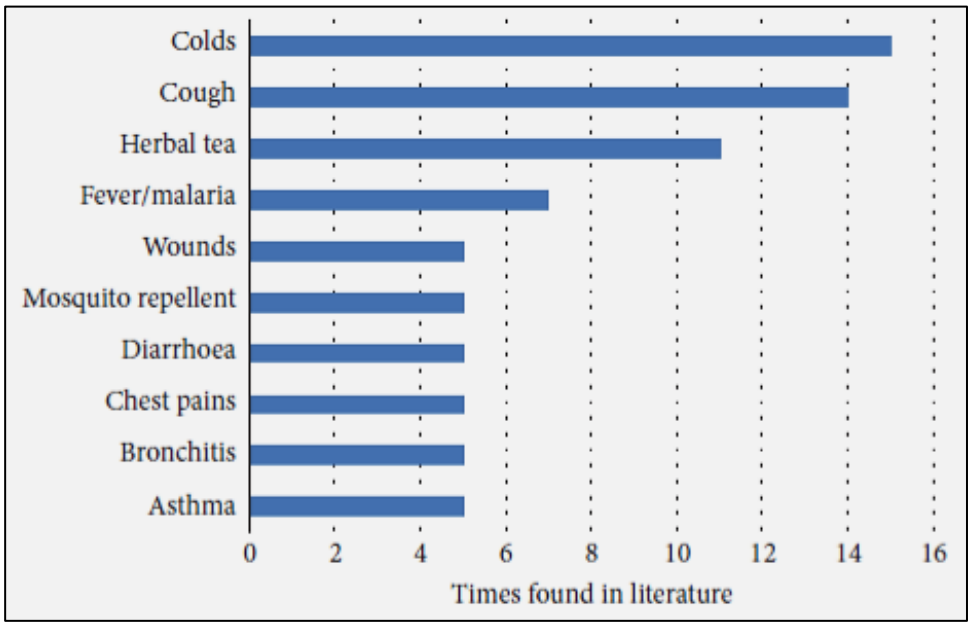


Figure 3. Ethnobotanical uses of *Lippia javanica* counted once per publication in central, eastern, and southern Africa (Adopted from Maroyi, 2016).

2.2.3 Economic importance of *Lippia javanica*

A study conducted by Goggin *et al.* (2009) indicated that almost 80% of the people in South Africa rely on and utilize plants with medicinal properties for health problems. Many South Africans regard medicinal plants as a cheaper, safer and desirable alternative to treating various ailments (Mander *et al*., 2007). Informal plant traders or traditional healers collect *L. javanica* from wild populations or cultivate it for income purposes (Mahlangeni *et al*., 2018). Considerable quantities of *L. javanica* are sold in local markets and informal sectors as herbal teas marketed under the brand name "Zumbani" in Zimbabwe and "Mosukudu" or "Mosukujane" in Botswana (Combrinck *et al*., 2007). Whiteside (1997) reported that during 1994/1995, *L. javanica* tea bags generated an income of up to R20 300 in Botswana. This study indicates that *L. javanica* has been utilised as a source of income in local and informal sectors for a long time.

L. javanica is commercially farmed in South Africa and Kenya, mainly for its medicinal essential oil products for the perfume industry (Van Wyk, 2008). According to Xaba *et al*. (2010), clinical trials on human volunteers regarding the effectiveness of fever tea extracts against malaria have proven to be more effective (repels no less than 95% of mosquitos) than

most available commercial products (repels only 42% of mosquitoes) (Maroyi, 2017). As a result, the South African government's Council for Scientific and Industrial Research (CSIR) set up a rural community partnership to grow *L. javanica* on a commercial scale in Giyani, Limpopo province (Xaba *et al*., 2010). *L. javanica* extracts are processed for large-scale productions of mosquito repellent candles and other insect repellents (Maroyi, 2017). The community products are called "Giyani's Hi Hanyile mosquito repellent and essential oil factory" (Xaba *et al*., 2010; Maroyi, 2017). The large-scale production can produce 400 000 candles per year, and each 250 g candle retails for approximately R20 with a burning capacity of 55 hours (Xaba *et al*., 2010).

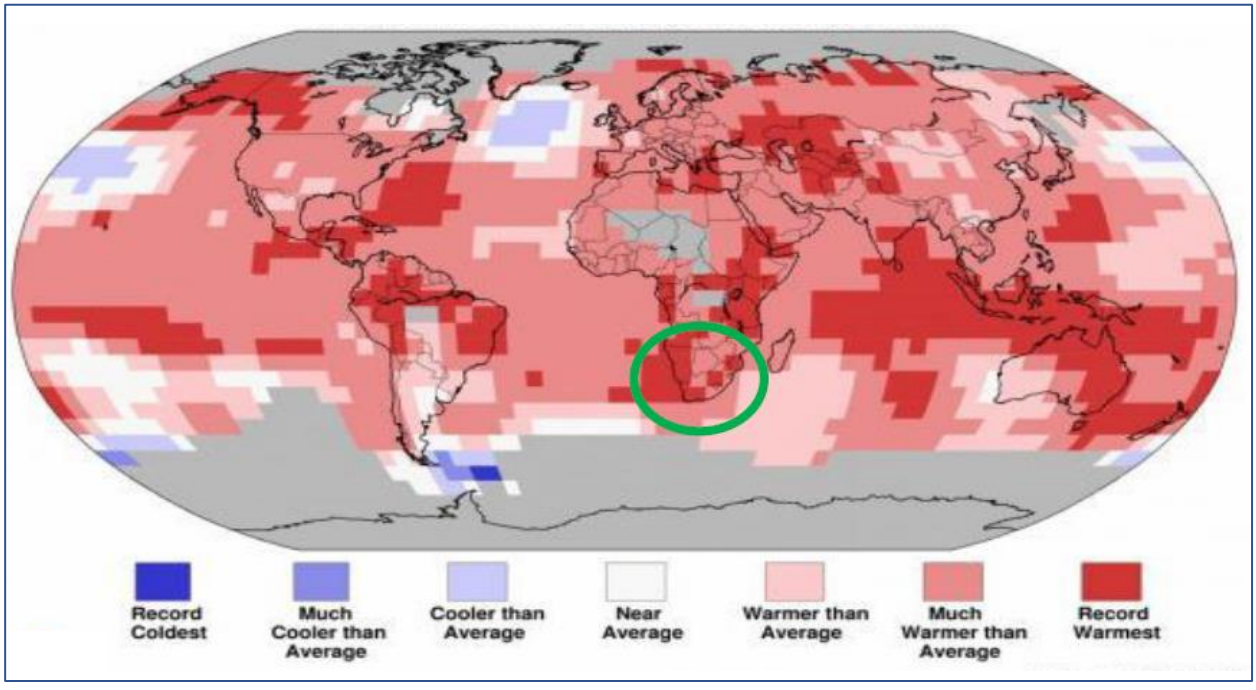
2.2.4 Effects of climate change on leaf morphology and histology of *Lippia javanica*

Climate change is a global environmental problem that results in abiotic stresses, which lead to anomalies in rainfall patterns, a shift in habitat ranges for species, and rapid increase in the earth's average temperature (Kruger *et al*., 2012). The earth's climate changes due to both natural and anthropogenic activities (McMichael *et al*., 2006; Hansen *et al*., 2001). The natural causes are volcanic eruptions, variations in solar radiation, earth's orbital changes, and the movement of crustal plates while anthropogenic activities include deforestation, burning of fossil fuels and livestock production (McMichael *et al*., 2006; Kruger *et al*., 2012). These activities lead to high emissions of greenhouse gases such as carbon dioxide, aerosols, methane and sulphur dioxide (Nakicenovic *et al*., 2000; McMichael *et al*., 2006). These greenhouse gases form a "blanket" in the atmosphere, absorbing the outgoing solar radiation which then gets re-emitted, thus significantly warming the Earth's surface and the lower atmosphere (Nakicenovic *et al*., 2000; United Nations Framework Convention on Climate Change, 2011).

It was predicted by the United Nations Framework Convention on Climate Change (2011) that temperatures will increase by 0.2 ºC per decade (Fig. 4). This incline will result in drought conditions with more hot days and high evaporation rates resulting in lower water tables and high water stress (Aggarwal, 2008; Kruger *et al*., 2012). High temperature stresses have enormous impacts on the growth of plants, mainly, medicinal plants because they are more sensitive to changing environmental conditions (Stinziano and Way, 2014). Heat stress may result in physiological, molecular, morphological, and biochemical changes in the plant,

thus limiting the development and productivity of the plant (Weis and Berry, 1988; Castro-Diez *et al*., 1997; Azhar *et al*., 2011). These changes can also affect the crucial medicinal properties of medicinal plants and given that there is an increase in the global demand for these plants, a comprehensive understanding of the responses of medicinal plants to abiotic stresses is essential.

Human-induced climate change increases heatwaves, which then increase in frequency, intensity and duration (Nakicenovic *et al*., 2000; McMichael *et al*., 2006). Heatwaves are becoming more frequent in different parts of the world, and they may have adverse effects on animals, plants and humans when they experience difficulties in adaptation (Fig. 4) (McMichael *et al*., 2006). The past two decades of the 20th century have been exceptionally warmer than the previous decades. The years 2011 to 2016 were the warmest on record, and the year 2015 mainly was warmer due to the El-Nino, and it was recorded as the hottest year since modern observations in the late 1800s (Fig. 4) (Stinziano and Way, 2014). Africa has predominantly experienced more heatwaves in the last two decades. A heatwave is defined as prolonged periods of extensive heat and this can last for days or weeks and is strongly correlated to climate change (Hansen *et al*., 2001; Kruger *et al*., 2012). Heatwaves affect plant physiology and metabolism which in turn affects plant growth and reproduction with two main consequences of either adaptation or extinction (Duriyaprapan and Britten, 1982; Azhar



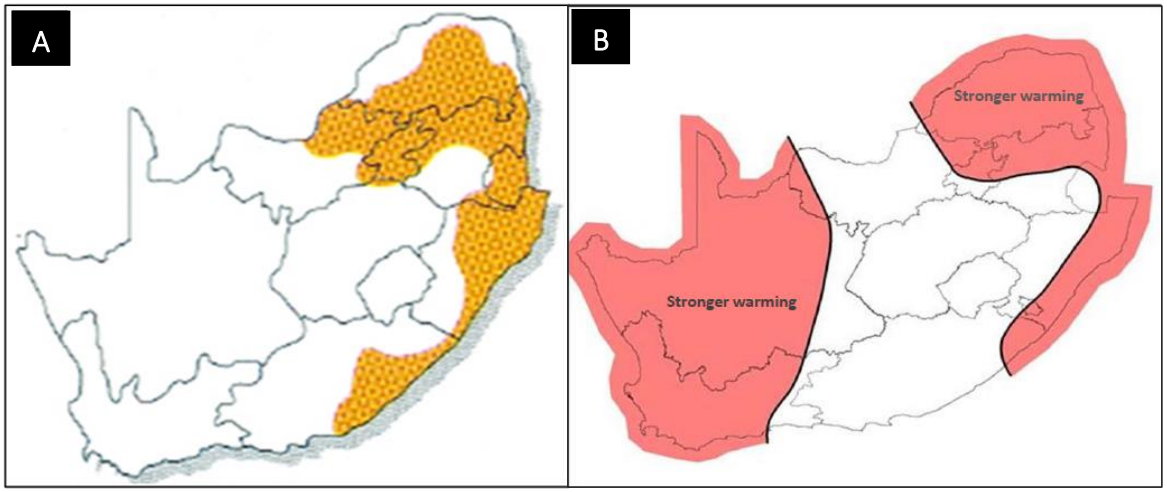
et al., 2011). The prediction made by several studies (Aggarwal, 2008; Kruger *et al*., 2012; Stinziano and Way, 2014) that there will be an increasing intensity and occurrence of weather

extremes means that more effort is required to understand their consequences on indigenous medicinal plants.

Figure 4. Ocean and land temperature percentiles from January to August 2016. The green circle outlines South Africa's temperature percentiles (Adopted from NOAA's national centers for environmental information).

South Africa is one of the countries in the world that has experienced drastic increased temperatures over the last 40 years (Kruger and Sekele, 2012). Several provinces such as Mpumalanga, Limpopo, Gauteng, North West, Eastern Cape and Kwazulu Natal in South Africa have since doubled the average global temperature patterns, meaning that increased temperatures have been evident over the last part of the 20th century (Lugina *et al*. 2005; Smith & Reynolds 2005; Kruger *et al*., 2012). According to Kruger and Sekele (2012), increased temperatures have augmented, whereas cold temperatures have declined with evident patterns in the northeastern, eastern and western parts of South Africa.

L. javanica is highly abundant in the provinces that have experienced increased temperatures over the latter part of the 20th century (Fig. 5) (Kruger and Sekele, 2012). Stinziano and Way (2014) stated that extreme temperature stresses could affect the production of oils and secondary compounds, which are crucial elements in the plant's medicinal attribute. Bennett and Wallsgrove (1994) also stated that when plants are stressed, secondary compounds and oil production increases while growth is inhibited because carbon that is meant to be allocated to growth processes is redirected to the production of secondary compounds and essential oils. However, the effects of high temperatures are plant-specific and chemical-specific, thus, more



attention needs to be directed onto individual species such as *L. javanica*. This will bring more insight towards the specific species and its responses to temperature stress. *L. javanica* is extensively used by both herbalists and traditional practitioners to treat various ailments

(Weis and Berry, 1988; Lugina *et al*., 2005). Thus, it is essential to investigate the effects of increased temperatures on the shrub's phytochemistry and anatomy.

Figure 5. (a) Distribution Map of *Lippia javanica* in South Africa (a) (Adopted from Van Wyk, 2008). (b) The shaded regions are areas experiencing stronger warming in South Africa from 1962 to 2009 (Adopted from Kruger and Sekele, 2012).

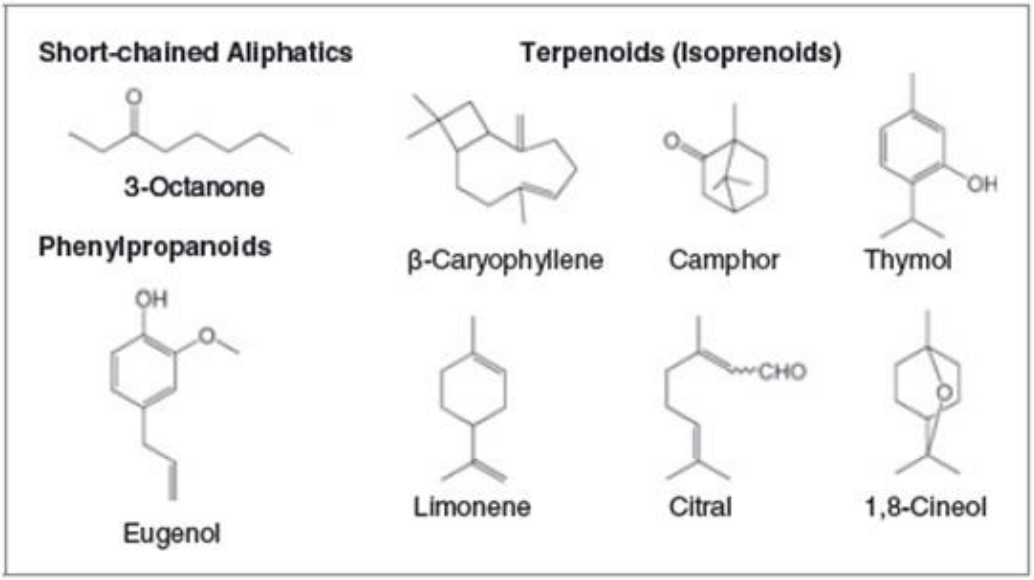
2.2.5 Essential oil composition

Essential oils are hydrophobic phytochemicals and concentrated fluids extracted from plants that consist of volatile compounds (Elsharkawy, 2014). These oils consist mainly of lipophilic and very volatile secondary metabolites (Turek and Stintzing, 2013). According to the International Organisation for Standardization (ISO), the term "essential oils" is defined as a product obtained from plant raw material that is obtained using water or steam distillation through mechanical processes (ISO, 1999) (Turek and Stintzing, 2013). These oils are produced in the leaves and are then transferred to the rest of the plant (Galadima *et al*., 2012), where they are stored in small sacs within flowers, roots, shoots and seeds (Whitton, 1995).

Essential oils are generally composed of oxygen, hydrogen and carbon atoms, which are grouped into oxygenated chemical compounds and hydrocarbons (Fig. 6) (Andogan *et al*., 2002; Turek and Stintzing, 2013; Florczak, 2014). Oxygenated compounds consist of alcohols, esters, aldehydes, phenols, ketones and oxides, although compounds made of sulphur, nitrogen and lactones may also be present (Turek and Stintzing, 2013; Florczak, 2014). On the other hand, hydrocarbons consist of terpenes, which are further divided into mono-, di- and sequi-terpenes (Fig. 6) (Lawless, 1992; Florczak, 2014). These oils tend to give plants a distinctive smell that assists in attracting pollinators as well as providing a defence mechanism against foreign agents such as bacteria and fungi (Whitton, 1995). These oils are also known to help plants adapt during extreme environmental conditions. These properties are further extended to humans when plant extracts are used to treat various ailments (Turek and Stintzing, 2013).

Figure 6. Typical structures of essential oils (Adopted from Turek and Stintzing, 2013).

Essential oils are widely used for flavouring and as a "green" alternative in the pharmaceutical, nutritional and agricultural sectors (Dionisio *et al*., 2012; Stintzing and Turek, 2013). These oils are also used for their antioxidant properties in food preservation and are incorporated in packaging food material (Andogan *et al*., 2002; Florczak, 2014). Essential oils have gained great popularity and there is a need to produce large quantities of these oils due to an ever-growing awareness and demand with regards to the utilization of natural ingredients in food, medicine, and cosmetic products (Galadima *et al*., 2012). Large quantities of essential oils such as lemon and eucalyptus have been traded and up to 1000 metric tons per annum were traded in 2007 which estimates to several hundred million euros (Florczak, 2014; Seol and Kim, 2016). These essential oils possess biological activities that are of health and pharmaceutical benefits.



L. javanica contains essential oils that have anti-inflammatory, antimicrobial and antioxidant activities beneficial in alleviating illnesses and improving health (Maroyi, 2017). However, several studies depicted that there are differences in the essential oil composition of *L. javanica* which questions the biological activity of the plant (Neidlein and Staehle 1974; Chagonda *et al*., 2002 and Viljoen *et al*., 2005, Combrinck *et al*., 2007; Maroyi, 2017). For example, according to Viljoen *et al*. (2005), *L. javanica* depicts qualitative and quantitative deviations both between and within natural plant populations in Southern Africa. The authors demonstrated that these variations are not related to the plant's geographical distribution, but rather due to the differences in the metabolic pathways that secrete the essential oils. Catalan and de Lamposa (2002) found that all *Lippia* species in Southern Africa, including *L. javanica* and *L. scaberrima* depict intraspecific chemical variations in their major essential oils. These chemical variations may suggest different biological activities that justify the variety of ethnopharmacological uses in different cultures (Maroyi, 2017).

Viljoen *et al*. (2005) assessed natural populations from the same area and five distinct chemotypes were observed from these native populations. Neidlein and Staehle (1974) reported that the key compounds in the essential oils of *L. javanica* are; caryophyllene, p-cymene and linalool while, Chagonda and Chalchat (2015) reported that the chemotypes dominating these essential oils are linalool (68.8%), myrcene (54.0%) and limonene (39.9%). Furthermore, Chagonda *et al*. (2002) also found that there were variations in the primary essential oils of *L. javanica* samples from Zimbabwe obtained from the same site. For example, it was found that linalool ranged between 1.7 and 27% in *L. javanica* samples collected from the same location (Chagonda and Chalchat, 2015). These results indicate that *L. javanica*'s essential oil compositional differences need to be taken into consideration to illuminate the possible role of climate change on the volatile constituents of this medicinal plant.

A review study conducted by Maroyi (2006) summed up the chemotypes of essential oils found in *L. javanica* from different authors and concluded that the standard and dominant chemotypes are piperitenone, ipsdienone, carvone, tagetenone, limonene, caryophyllene, p-cymene, linalool, myrcene, myrcenone, ocimenone, sabinene, and ipsenone. These compounds may be considered as the major essential oil components of *L. javanica* in this study since the review summed up findings from 171 studies.

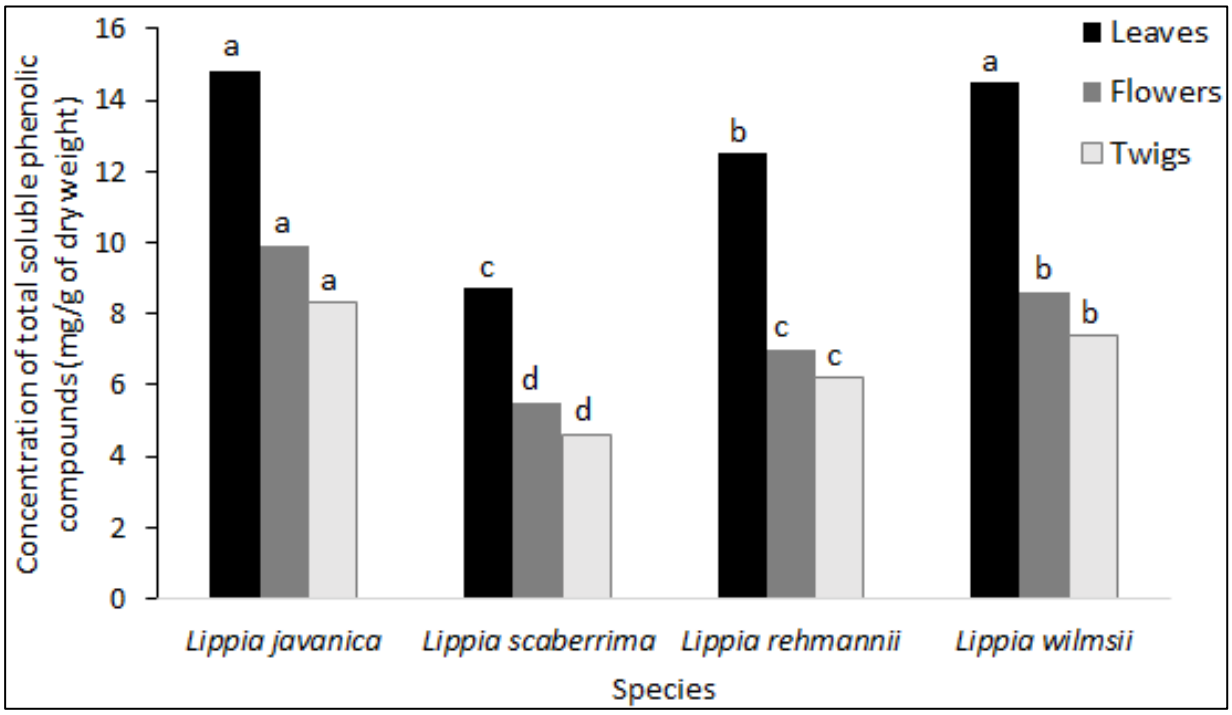
2.2.6 Phenolics and flavonoids

There are four main classes of phenolic compounds, namely; phenolic acids, flavonoids, stilbenes and lignans (Harborne, 1984). Medicinal plants mostly contain phenolics and flavonoids, which possess antioxidant properties (Cai *et al*., 2004; Abdeltaif *et al*., 2018). Antioxidants are chemical compounds that help prevent oxidation of cells (Miguel, 2010). Oxidation is a chemical reaction that reduces chain reactions capable of damaging cells by producing scavenging free radicals (Bhatt *et al*., 2013). Medicinal plants have become of interest to researchers as they contain combinations of different chemical compounds (anti-oxidants) acting synergistically or individually to improve health and treat diseases (Bhatt *et al*., 2013). Studies show that phenolics and flavonoids in medicinal plants contain the highest amount of antioxidants, which are generally responsible for scavenging reactive oxygen species (Ramalakshmi *et al*., 2007; Bhatt *et al*., 2013; Miguel, 2010). In the plant kingdom, phenolic compounds are the largest group of phytochemicals (Harborne, 1984; Bhebhe *et al*., 2016), while flavonoids are the most common and widespread group of phenolic compounds (Bhebhe *et al*., 2016; Matamela, 2014).

Phenolic compounds are bioactive compounds that are produced by plants through the phenylpropanoid, phosphate and shikimate pathways (Randhir *et al*., 2004; Abdeltaif *et al*., 2018). They are derived from phenylalanine, which possesses an aromatic ring bearing one or more hydroxyl groups (Fig. 7) (Sulaiman and Balachandran, 2012; Matshediso *et al*., 2015). They consist of lignins, lignans, simple phenols, tannins, coumarins, flavonoids and phenolic acids (Pereira *et al*., 2009). These compounds are involved in different plant defence mechanisms and physiological processes such as Ultraviolet Radiation (UV), infections and wounding (Alonso-Amelot *et al*., 2004; Bennett and Wallsgrove, 1994). According to a study conducted by Alonso-Amelot *et al*. (2004; 2007), plants that occur at higher altitudes generally contain more phenolic compounds because they need to be protected from the UV-B radiation.

Shikanga *et al*. (2010) found that flavonoids and phenolics were the two main phytochemicals present in *L. javanica*'s leaves. The study found that *L. javanica* has a higher phenolic content in the leaf extracts (14.8 mg/g) than the flowers (9.9 mg/g) and twigs (8.3 mg/g) (Fig. 7). It was also found that *L. javanica* had the highest total phenolic compounds than the other *Lippia* species (*L. scaberrima, L. rehmannii, L. wilmsii*) that are indigenous to South Africa (Shikanga *et al*., 2010). This study proves that amongst the *Lippia* species found in South Africa, *L. javanica* possesses a higher total phenolic content (Fig 7). Another study conducted by Bhebhe *et al*. (2016) showed that the phenolic content of *L. javanica* is higher than that of *Camellia sinensis* commonly sold in Zimbabwe (common black tea) which is known to contain a very high phenolic content.

Figure 7. Total soluble phenolic content of *Lippia* species that are indigenous to South Africa. Different letters [a-d] are significantly different (Tukey test, $P < 0.05$) (Modified results from Shikanga *et al*., 2010).



Flavonoids, on the other hand, are the largest group of naturally occurring phenolic compounds, and they are widely spread in plant tissues (Gottlieb, 1989; Panche *et al*., 2016). They consist of catechin, iso-flavonols, flavones, flavonols, anthocyanidins, proanthocyanidins and anthocyanins (Matamela, 2014; Panche *et al*., 2016). These flavonoid groups all share a basic structure called 2-phenylchromane. They consist of a fundamental structural skeleton called 2-phenylchromane (Fig. 8) (Harborne, 1984). The phenyl ring varies

in its position; it either attaches to the C-2 or C-3 bond (Fig. 8) (Matamela, 2014). Flavonoids are ever-present in photosynthesizing cells; thus they are widely distributed across plant taxa (Gottlieb, 1989). They have numerous biological properties such as anti-inflammatory, antimicrobial and antioxidant activities; therefore, they have become an essential subject of medicinal research (Nagavani and Rao, 2010; Sulaiman and Balachandran, 2012; Dzoyem and Eloff, 2015). These compounds inhibit oxidative and hydrolytic enzymes and scavenge free radicals (Frankeli, 1995). Free radicals in humans are responsible for various disorders including cancer, arthritis, AIDS, and injuries to the central nervous system (Pourmorad *et al.*, 2006).

Flavonoids were considered in this study because they are natural products that are well known for their health benefits, and they are abundant in foods and are widely consumed (Gottlieb, 1989). According to Panche *et al*. (2016), different studies make an effort to isolate the ingredients of flavonoids because of their extensive uses. Flavonoids are considered an essential aspect in a range of pharmaceutical, nutraceutical and medicinal applications (Panche *et al*., 2016). Flavonoids are a cluster of phenolic compounds that are studied alongside phenolic acids because they are an indispensable component due to their vast medicinal properties (Sulaiman and Balachandran, 2012).

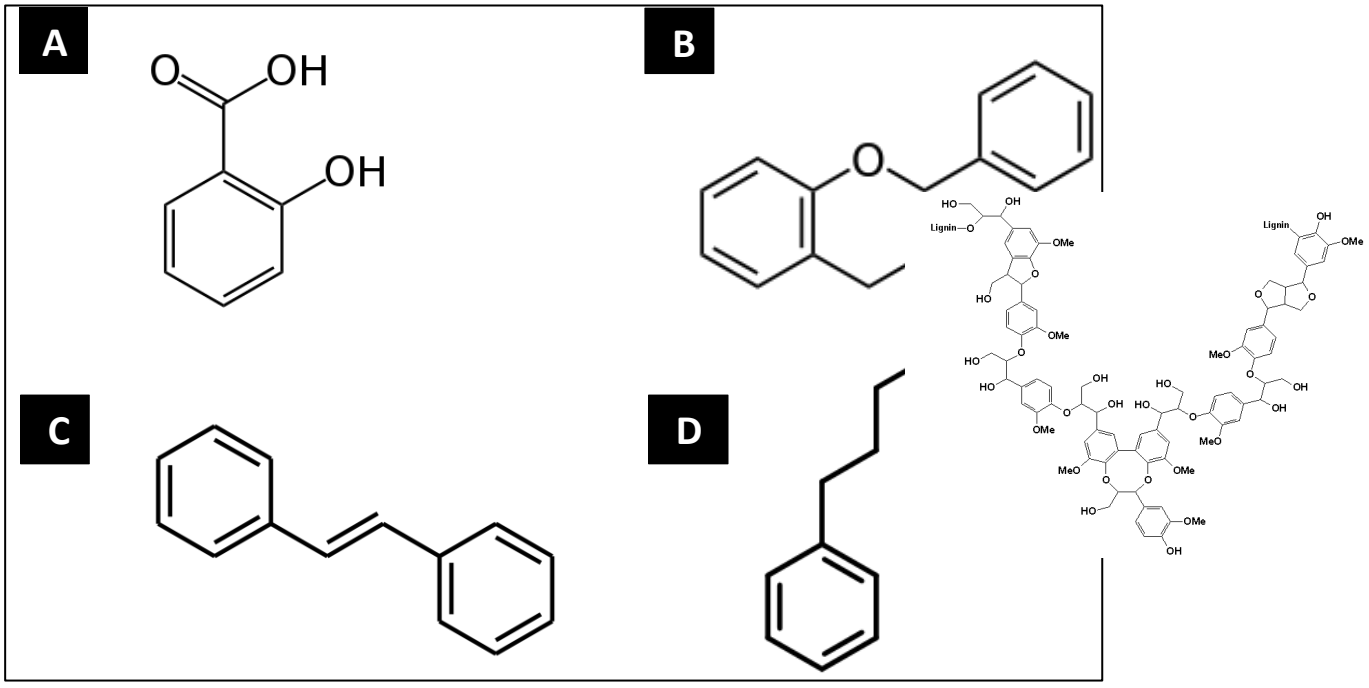


Figure 8. Phenolic compounds structure; phenolics (A), flavonoids (B), stilbenes (C), lignins (D).

2.2.7 Histological Background of *Lippia javanica*'s leaves

Histology is the study of detailed microscopic biological tissues and cells of animals and plants (Rebecchi *et al*., 2007). Plant histology, in particular, is concerned with the specialised functions of cells and tissues of plants in relation to their detailed structure and composition (Rebecchi *et al*., 2007; Martinez-Nataren, *et al*., 2011; Asowata-Ayodele, 2015). It provides physiological, phylogenetical and morphological interpretation through cellular and tissue structures (Asowata-Ayodele *et al*., 2016; Rebecchi *et al*., 2007; Barthlott and Neinhuis, 1997). Histology is a useful tool in tracing microscopic changes in plants after being exposed to extreme environmental stresses (Barthlott and Neinhuis, 1997). This tool, therefore, helps determine the resistance and different coping mechanisms of a plant at a microscopic level (Asowata-Ayodele, 2015; Asowata-Ayodele *et al*., 2016). The leaf histology of *L. javanica* has been investigated by many studies (Combrinck *et al*., 20017; Martinez-Nataren, *et al*., 2011; Asowata-Ayodele, 2015; Asowata-Ayodele *et al*., 2016), and research shows that the cellular and tissue structure of *L. javanica* is similar to that of any medicinal plant.

The outermost structure of the leaf surface on *L. javanica* is the cuticle (Fig. 9), and it aids in preventing water loss as it is hydrophobic (Barthlott and Neinhuis, 1997; Centritto, 2002). The cuticle of *L. javanica* is thick, and it acts as a survival mechanism in harsh conditions, especially in high temperature environments (Fig. 9) (Rebecchi *et al*., 2007). The epidermis serves a similar function in preventing water loss (Barthlott and Neinhuis, 1997). The epidermis is present in both the abaxial and adaxial surfaces of *L. javanica* as it is significant for the development of trichomes, wax secretion and stomata (Rebecchi *et al*., 2007). *L. javanica*'s leaves also have palisade and spongy mesophyll cells that are responsible for the secretion of essential oils and other phytochemicals. The vascular bundle is found within the mesophyll layer and is responsible for the transportation of water and food particles throughout the plant.

Glandular and non-glandular trichomes are widely distributed in the Verbenaceae family (Passos *et al*., 2009; Aneta, 2013). Trichomes are unicellular or multicellular foliar appendages that originate from the epidermis. These trichomes are found on the surface of the leaf, and they secrete essential oils and in some plants, secrete phenolics and flavonoids which contain medicinal properties (Fig. 9) (Wagner *et al*., 2004; Passos *et al*., 2009). Trichomes are classified according to their size, shape and function (Asowata-Ayodele *et al*., 2016) and their density on the leaf surface is dependent on environmental conditions, i.e. the harsher the environmental stresses, the higher the frequency of trichomes (Passos *et al*., 2009; Asowata-Ayodele *et al*., 2016). Plants found in hostile conditions increase the density of trichomes which results in a higher production of essential oils, flavonoids and phenolics which are secreted to protect the plant (Aneta, 2013). A study conducted by Werker and Fahn (1981) shows that glandular trichomes reduce the plant's transpiration rate, thus decreasing the leaf temperature while non-glandular trichomes increase the plant's adaptability to changing environmental conditions, particularly in dry conditions (Aneta, 2013). According to Asowata-Ayodele *et al*. (2016), *L. javanica* possesses spike-like, non-glandular, and uniseriate trichomes that are responsible for the secretion of high terpenoid content. These trichomes therefore, directly or indirectly help plants to adapt under extremely harsh environmental conditions (Aneta, 2013).

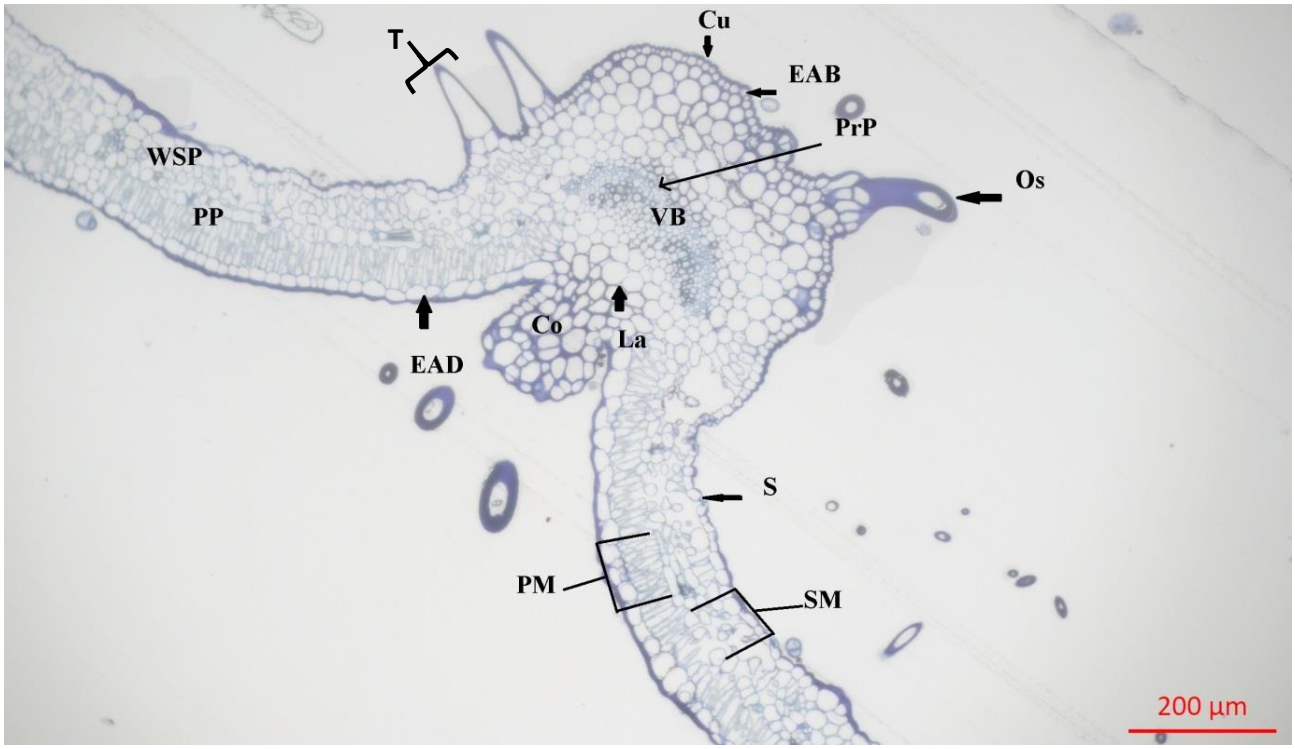


Figure 9. Leaf cross-section of *Lippia javanica* showing primary structures such as; cuticle (Cu), epidermis (EAB), primary phloem (PrP), Ostiole (OS), vascular bundle (VB), laticifers (La), collenchyma (Co), stomata (S), spongy mesophyll (SM), palisade mesophyll (PM),

epidermis of the leaf adaxial surface (EAD), palisade parenchyma (PP), Trichome (T), water storage parenchyma (WSP) (Based on third-year microscopy work of Mark Mhlongo, 2015).

CHAPTER 3

Effects of high temperatures on leaf morphology and histology of *Lippia javanica*

SUMMARY

Lippia javanica is an evergreen shrub with aromatic leaves, commonly used in various rural communities for treating a wide range of ailments. High temperatures significantly affect plant growth and production; thus, this chapter aimed to assess the effects of high temperatures on the leaf morphology and histology of *L. javanica*. This aim was investigated by exposing plant samples to high temperatures in a climate test incubator (47/37 ºC – day/night simulation) episodically for 48, 96 and 144 hours while the control was exposed to ambient conditions in the greenhouse (25/20 ºC– day/night). Morphological assessments showed that there was 80% of leaf browning evident on *L. javanica* samples after exposure to high temperatures (48, 96 and 144 hours). There was also evidence of wilting, curling and rolling, which resulted in a slight decline in leaf size. On the contrary, histological assessments showed that the exposure to high temperatures increased the leaf midrib and blade thickening. The spongy and palisade mesophyll cells were more tightly packed, decreasing the intercellular air spaces and ultimately decreasing transpiration. The trichome average height ($F_{(7, 162)} = 32.09$, $P < 0.001$) and average diameter ($F_{(7, 161)} = 3.56$, $P < 0.01$) also increased significantly after exposure to high temperatures. These findings correlate with the increased production of bioactive compounds, which further protects the plant from harsh conditions. These results suggest that *L. javanica* samples were able to acclimatise under high temperatures by modifying their cellular structures, which helped minimize potential water loss. More evidence of acclimation was present when plant samples recovered after eight

weeks of being returned into the greenhouse under ambient conditions. Therefore, medicinal plants are highly sensitive to environmental stresses, but they display coping mechanisms that enable adaptation under stress for a certain duration of time.

3.1 INTRODUCTION

Summer seasons in South Africa are characterized by extreme weather conditions (Kruger and Sekele, 2012). *L. javanica* and other species of the Verbenaceae family, therefore, experience high solar radiation, high vapour pressure and limited water availability (Johnson and Thornley, 1985). The extremity of these stresses is estimated to increase due to global climate change, resulting in the reduction of plant growth and production, along with a shift in biodiversity (Fischer *et al*., 2001; Centritto, 2002). Plant responses to high temperatures are complex, and they include the deleterious effect or adaptive traits (Chaves *et al*., 2002; Muhl *et al*., 2011; Bita and Gerats, 2013). The most basic morphological characteristics and biological processes that are key to plant growth are sensitive to temperature (Duriyaprapan and Britten, 1982). As temperatures increase, the net photosynthetic rate is negatively affected, which leads to a decline in the plant's performance. Phenology is driven by temperature (Ritchie and Nesmith, 1991; Muhl *et al*., 2011); thus, any moderate inclines in temperature lead to the escalation in developmental processes resulting in the early blooming of plants (Johnson and Thornley, 1985).

Leaves have been identified as the most flexible part of the plant in its response to changing environmental conditions (Nevo *et al*., 2002); thus, the histology of the leaf reveals more dynamics than that of the root and stem. Leaves are an essential part of the plants' survival as they help with food production, gaseous exchange through photosynthesis, and balancing the loss of water, amongst others. Their vast range of functions results in specialised structures such as the epidermis, mesophyll and vascular bundle that carry out different purposes within the leaf. The leaf's morphology and histology reflect functional traits and adaptations which reveal the different types of environments where the plant grew.

Studies show that the effects of environmental stresses on plants may result in physiological, molecular, morphological, and biochemical changes in the plant, which limits the development and productivity of the plant (Weis and Berry, 1988; Castro-Díez *et al*., 1997, Bussoti *et al*., 2002). Other morphological traits that aid in the acclimatisation of plants under high temperatures are; leaf rolling (Schwabe and Lionakis, 1996; Bita and Gerats, 2013), thickening of the cuticle (Richardson and Berlyn, 2002), smaller mesophyll cells and less intercellular air spaces (Mediavilla *et al*., 2001). These traits are mostly accompanied by the increase in the production of secondary metabolites and the accumulation of mucilage, which is as a result of the increased number of glandular and non-glandular trichomes (Ascensao *et al*., 1999). The higher glandular and noglandular trichome density is also known to depict a high temperature tolerance trait (Martínez-Natarén *et al*., 2011). This chapter, therefore, aims to assess the effects of high temperatures on the leaf morphology and histology of *L. javanica*. The study further explores the structural dynamics of glandular and non-glandular trichomes in relation to the production of bioactive compounds and as an adaptational trait against high temperatures.

3.2 MATERIALS AND METHODS

3.2.1 Sample treatment

Mature plants of *L. javanica* were obtained from Mountain Herb Estate in Hartbeespoort (25.7236° S, 27.9653° E) and were maintained in the greenhouse at the University of the Witwatersrand (26.1929° S, 28.0305° E). The plants were kept in the greenhouse at 25/20ºC (day and night simulation) and watered daily for a month to acclimatise before moving them into the climate test incubator (Conviron - CMP6010) for treatment (Fig 10). Three replicates of five sample plants (n = 15) were transferred into a Conviron, where the temperatures were set at 47/37ºC (day/night simulation) for up to 144 hours (under 12 hour light with a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$) (Fig. 10). The temperatures in the Conviron were specifically set at 30ºC from 24h00 to 06h00 and were adjusted to 35 ºC from 06h00 to 12h00 and 40 ºC at 12h00 to 18h00 and back to 35 ºC from 19h00 to 24h00 (Fig. 10). Histological and morphological assessments were conducted every 48 hours (48, 96, and 144 hours) for a total of six days. After performing the assessments mentioned above, the plant samples were then returned to the greenhouse (under ambient temperatures) to recover from the extremely



high temperatures. Samples were returned to room temperatures and watered daily for observation purposes in order to assess recovery.

The control and treated plants were watered twice a day and humidity, soil type and light conditions (12 hour light with a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$) were kept constant throughout the treatments (Fig. 10). Control plants were exposed to ambient temperatures (25/20ºC), and according to Wigge (2013), these ambient conditions do not severely affect plant growth, photosynthetic rate and development. The primary dependent variable that was investigated in this study was the duration of exposure to high temperatures (Fig. 10).

Figure 10. Samples of *Lippia javanica* in the Conviron where temperatures were adjusted to 47/37ºC (day/night simulation) and watered daily.

3.2.2 Morphological assessment

Leaves of *L. javanica* were assessed using a Zeiss dissecting microscope, and images were captured using a Nikon digital camera (D5300) to compare colouration (pigmentation), thickness, and shape changes under different treatments. The exterior leaf surfaces were

viewed under 0.5X (low) and 1X (high) magnification. Plant samples returned to the greenhouse were also photographed to detect possible recovery from high temperatures.

3.2.3 Histology

Leaves of *L. javanica* were harvested, and rectangular sections (1 mm x 2 mm) were cut between the third and the fourth principal vein, 5mm from the mid-vein (mid-section of the plant). A standard glutaraldehyde-osmium method was used to prepare the leaf segments for microscopy with the addition of 1% caffeine for the precipitation of phenolic compounds (Wronka *et al*., 1995) and embedded in Spurr's epoxy resin (Spurr, 1969). Semi-thin (0.5 µm – 0.8 µm) sections of the fresh leaves were obtained using a Reichert Ultramicrotome and were stained with 0.01% (w/v) Toluidine Blue and then heat-fixed onto 22 x 50mm glass slides. These were then mounted in p-xylenebis (N-pyridinium bromide) (DPX), covered with glass coverslips and after that viewed with an Olympus BX63 OFM light microscope and photographed with an attached Nikon DXM1200 digital camera. The details of this method are in appendix 1. The glandular and non-glandular trichome height and diameter were measured using the Olympus BX63 OFM light microscope using the 10X, 40X and 100X objectives for better magnification.

3.2.4 Data Analyses

Qualitative and quantitative analyses of data were performed in this study. Images of the leaf midrib, blade, and trichomes contributed data towards qualitative analyses. The differences in height and diameter of glandular and non-glandular trichomes per treatment were photographed and were also qualitatively analysed. Quantitative data were analysed on R-studio, and the data was first checked for normality using a Shapiro test before performing any further statistical tests. The first test conducted was a Student's t-test, which was used to determine if there was a significant difference between the glandular and non-glandular trichome height. After that, a one-way Analysis of Variance (ANOVA) was performed to determine the statistical difference in the glandular and non-glandular trichome heights between treatments. This step was followed by a Post-hoc Tukey test, which was used to determine where the significant difference lies between the treatments. Lastly, a one way ANOVA was performed to test if there was a statistical significance in the glandular and non-glandular trichome diameters between the four different treatments, which was followed by a

Tukey Post-hoc test which was conducted to determine where the significant difference was. All the statistical analyses were done at a significant level of $P \leq 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Morphological assessment

L. javanica showed several stress responses to the high temperature treatments. Externally, the morphology of *L. javanica*'s leaves changed in pigmentation after being exposed to high temperatures. All the leaves of the control samples were green; however, after the first 48 hours of exposure to high temperatures, 50% of the leaves showed signs of yellowing (Fig. 11 A, D, G, J). The 96 hours treatments had 65% of the total leaves yellowing and browning while the last treatment (144 hours) had approximately 80% of the leaves on the sample plants browning which is a sign of desiccation and loss of chlorophyll (Miller *et al*., 2011) (Fig. 11 A, D, G, J). According to Miller *et al*. (2011), the deficiency in chlorophyll occurs due to the destruction in the plant's food manufacturing systems, causing the leaves to change colour from green, yellow to brown. The findings from Miller *et al*. (2011) imply that, despite not measuring the chlorophyll content in this study, the changes in pigmentation observed in *L. javanica* is a reliable indicator of chlorophyll loss, as a stress response, which results in low food manufacturing for the plant.

After the 48 hours treatment, the harvested leaves showed minor signs of wilting, rolling and curling; however, the 96 hours treatment showed more leaves rolling, wilting and curling up (Fig. 11 A, D, G, J). The 144 hours treatment showed a higher degree of leaves wilting, rolling and curling, which brought about a slight decline in the size of the leaves (Fig. 11 A, D, G, J). The leaf sizes (diameter) of the 48 hours, 96 hours and 144 hours treatment were less than the control by 3%, 7% and 13%, respectively. These morphological changes are similar to a study conducted by Ashraf *et al*. (2004), where they observed that maize and soybeans wilt under extreme heat stress as a result of high water loss. Another study conducted by Tripathi *et al*. (2017) discovered that Soybean and Elodea treated at 47°C to 57°C in four days resulted in wilting and a complete loss of chlorophyll. The cells in those leaves underwent plasmolysis, tonoplast membrane and plasmalemmal disorganisation. The results from Tripathi *et al*. (2017) imply that the leaves of *L. javanica* at a cellular level could have undergone plasmolysis, tonoplast membrane and plasmalemmal disorganisation, as a result of

the intensive wilting, rolling and curling. This shows that exposing plants to high temperatures result in a tremendous change in the leaf morphology, which may reflect histological changes at a cellular level and possible desiccation of the plant. The leaf water content of *L. javanica* after exposure to high temperatures was not measured; however, extreme wilting and rolling as a stress response in this study is a good indicator of high water loss.

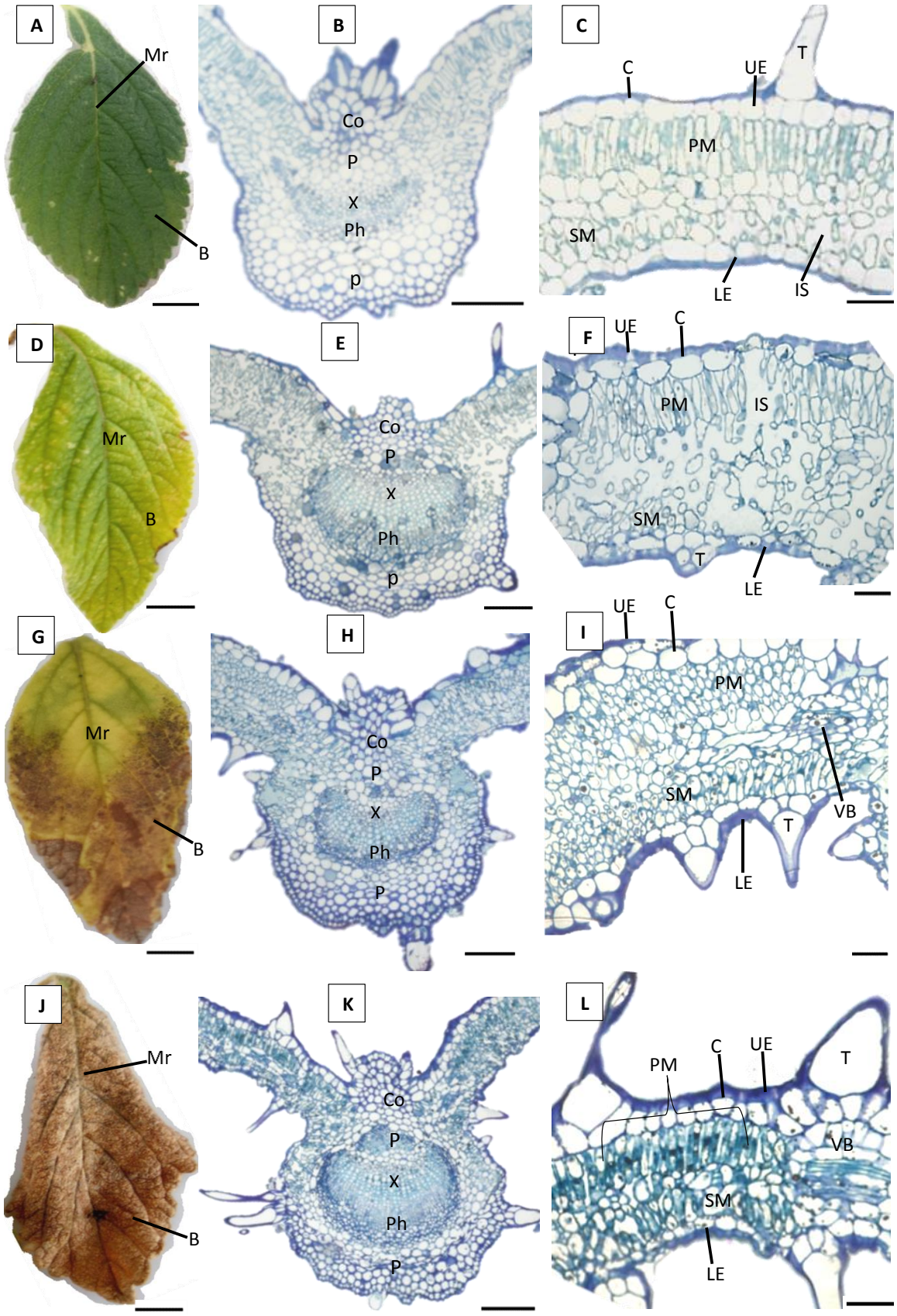


Figure
11. Leaf histology cross section (midrib and blade) and morphology of *Lippia javanica* under ambient conditions (A, B, C) and high temperatures (47/37^{0}C) after 48 hours (D, E, F), 96 hours (G, H, I) and 144 hours (J, K, L). Labels: Co = collenchyma, P = parenchyma, X = xylem, Ph = phloem, E = epidermis, C = cuticle, T = trichome, PM = palisade mesophyll, SM = spongy mesophyll, IS = intercellular space, UE = upper epidermis, LE = lower epidermis, VB = vascular bundle. Scale bars: C, F, I, L = 50 µm; B, E, H, K = 200 µm; A, D, G, J: 1cm = 0.58cm.

3.3.2 The midrib

The midrib region of *L. javanica*'s leaves under controlled conditions was similar to that of a typical plant (Fig. 11). The parenchyma cells were globular shaped and had thin cell walls that were smaller in size at the adaxial and more prominent in size at the abaxial leaf surface. According to Bacelar *et al*. (2004), these parenchyma cells are responsible for wound healing and vegetative rejuvenation as they maintain the ability to divide during the course of their lives. The vascular bundle of the control was narrow and did not have a very distinct boundary; however, there was a distinction between the xylem and the phloem cells (Fig. 11 B). According to Drazeta *et al*. (2004), vascular bundles are generally surrounded by the bundle of sheath and it is made up of thin-walled cells. Its primary function is to transport water and minerals to different parts of the lamina (Bacelar *et al*., 2004; Drazeta *et al*. 2004). The xylem dissolves minerals and transports water while the phloem transports food material to the plant (Drazeta *et al*., 2004). The collenchyma cells were more prominent in the control samples with thicker cell walls as they were stained darker than the parenchyma cells (Fig. 11 B). This is because the cell walls of the collenchyma cells contain thick cellulose deposits, which are plastic and flexible, thus providing structural support and protection against breakage.

After exposing the sample plants to high temperatures (from 48 hours to 144 hours), the parenchyma cells shrunk and decreased in size resulting in no difference in cell size of the parenchyma cells positioned at the adaxial and abaxial leaf surfaces (Fig. 11 E, H, L). The vascular bundle became more distinct with a dark blue colour from the 48, 96 and 144 hours treatment with a distinct alignment of the xylem and phloem. These results may be due to the increased transportation of water and food which enables temperature regulation (Drazeta *et al*., 2004). The collenchyma cells reduced in size and had thinner cells after the exposure to high temperatures. This may be because of the reduced deposition of cellulose and thus less plasticity and flexibility of the cells, which may ultimately lead to the plant not being adequately protected against breakage and no structural support. This could be the reason why leaves easily fall off under high temperatures as they start to wilt and lose their elasticity, becoming more brittle (Sam *et al*., 2003).

The overall histological change after exposing *L. javanica*'s leaves to high temperatures showed that the stain (Toluidine blue) that was applied on the leaf midrib got darker (dark

blue) in colour from 48 hours to 144 hours as compared with the control which was light blue in colour (Fig. 11 B-C, E-F, H-I, K-L). There were more dark blue stains on the leaves exposed to high temperatures, particularly around the phloem compared with the xylem, which is evidence of the presence and accumulation of secondary metabolites (Combrinck *et al*., 2007). A study conducted by Combrinck *et al*. (2007) suggested that the darker stains on the spongy mesophyll of *L. scaberrima* are due to the accumulation of secondary metabolites. The study indicated that the secondary metabolites consist of phenolic compounds and some essential oils. Another study conducted by Gechev *et al*. (2012) found that tissue plants under stress typically accumulate phenolic compounds and the accumulation was an indication of a stress response pathway triggered in plants. Essential oils and phenolic compounds such as flavonoids have antioxidant properties and are mainly secreted in plants in the mesophyll layer, vascular bundle and trichomes as a defence mechanism against environmental stress (Peinado *et al*., 2009; Gevech *et al*., 2012; Dzoyem and Eloff, 2015). Therefore, this study suggests that the darker stains present in the midrib section of the treatments are a response to the accumulation of secondary metabolites, which act as a defence mechanism against environmental stress (evidence of this accumulation is further discussed in chapter 4).

3.3.3 The blade

The control had thin cuticle linings and small round epidermis cells, which were equal in size on both the abaxial and adaxial surfaces of the leaf (Fig. 11 C). According to Barthlott and Neinhuis (1997) and Rebecchi *et al*. (2007), these two outer layers protect the leaf from harmful substances and help prevent the loss of water. Another study by Riederer and Schreiber (2001) showed that the cuticle is the main barrier against water loss in a leaf, which means that any extreme environmental stresses that require plants to acclimatise will result in the cuticle thickening. The palisade mesophyll cells in control samples were cylindrical shaped and aligned next to each other at the adaxial side of the leaf leaving very little intercellular spaces (Fig. 11 C). According to Sam *et al*. (2003), the palisade layer is known to have many chloroplast cells that are responsible for photosynthesis. On the other hand, the spongy mesophyll cells were ball-shaped and relatively loosely packed leaving more intercellular spaces than the palisade mesophyll cells. According to Mediavilla *et al*. (2001), these intercellular spaces allow gaseous exchange between different leaf parts. The spongy layer contains less chloroplast cells, and thus it is not responsible for photosynthesis, which

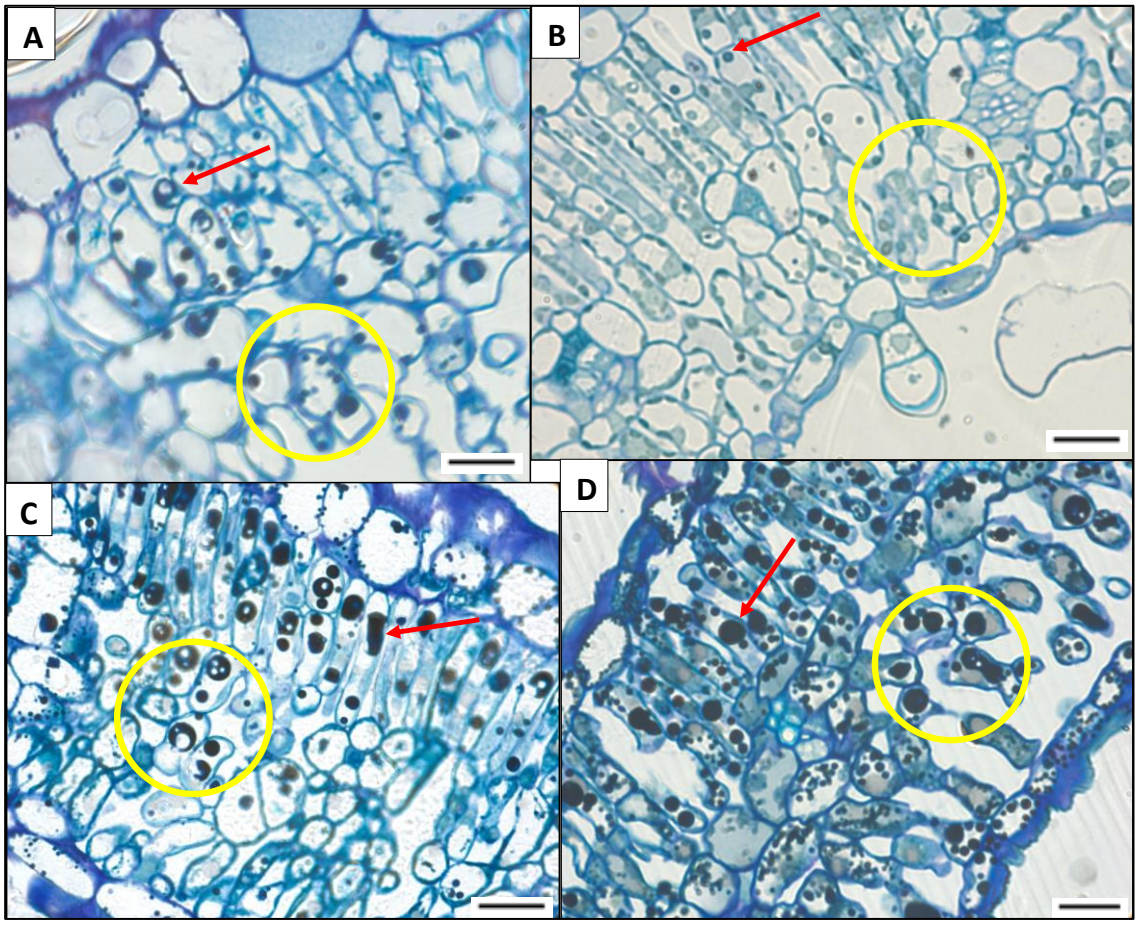
makes sense as the spongy mesophyll cells are positioned close to the abaxial surface of the leaf, which receives less solar radiation for photosynthesis (Sam *et al*., 2003).

The high temperature treatments (48, 96 and 144 hours) showed an increasing trend in the thickness of the cuticle and had bigger epidermal cells on both the adaxial and abaxial leaf surfaces (Fig. 11 F, I, L). This change may be due to the increased protection of the plant against high temperatures. After 48 hours of exposure to high temperatures, the palisade and spongy mesophyll layer started shrinking and there was evidence of more intercellular spaces (Fig. 11 F). According to a study conducted by Hartikainen *et al*. (2014), the increased area of intercellular spaces is an indication of weaker acclimation to high temperatures, which means that *L. javanica*'s first 48 hours of exposure to high temperatures showed signs of weaker acclimation. This inability to acclimatise could be due to the short period of exposure to high temperatures, which resulted in plants expressing a sudden shock in response to the stress.

After the 96 hours treatment, however, the palisade mesophyll cells changed from being cylindrically shaped to small round-shaped and doubled up in numbers, occupying all the empty intercellular spaces on the adaxial surface (Fig 11 I). The mesophyll cells doubled up in numbers and decreased in size, with some cells possessing a cylindrical shape to some being round-shaped. They were densely packed and significantly reduced the intercellular air spaces. The 144 hours treatment had cylindrical-shaped palisade mesophyll cells while the spongy mesophyll cells were globular shaped which was similar to the control, but differed in that both the palisade and spongy mesophyll cells were clustered together occupying all the intercellular spaces (Fig. 11 L). The 96 and 144 hours treatment showed that the plants were able to acclimatise to high temperatures as the leaf histology of the two treatments resembled the histology of the control. According to a study conducted by Mediavilla *et al*. (2001), it was found that the mesophyll layer thickens by increasing the number of mesophyll cells and decreasing the intercellular air spaces, which minimizes the loss of water by plants under harsh environmental factors. This suggests that at 96 hours of exposure to high temperatures, *L. javanica* minimized the loss of water and adapted to high temperature conditions by decreasing cell sizes and increasing the number of mesophyll cells.

The blade, similarly to the midrib section, had darker stains along the mesophyll layer in the treated plants compared with the control. The 144 hours treatment displayed more stains

along the mesophyll layer (both palisade and spongy) (Fig 12 D) than the 96 hours treatment which had more stains along the palisade than the spongy mesophyll (Fig 12 C) and 48 hours treatment which had less stains on both the palisade and spongy mesophyll layer (Fig 12 B). The control also had darker stains on the mesophyll layer but was less than those observed in the high temperature treatments (Fig 12 A). According to a study conducted by Combrinck *et al*. (2007), darker stains found on the mesophyll layer are an indication of the accumulation of secondary metabolites. Therefore, the degree of accumulation of secondary compounds was higher in the 144 hours than the 96 hours and 48 hours treatment. This means that the longer the duration of exposure of *L. javanica* to high temperatures in this study, the higher the production of secondary metabolites, which acted as a defence mechanism against environmental stresses. This means that the leaves of *L. javanica* responded positively and adapted to the stress. However, this does not suggest that the shrub can survive under high



temperatures for a longer duration of time, *L. javanica* may die over a prolonged period of exposure to high temperatures.

Figure 12. Leaf histology of *Lippia javanica* showing dark stains along the mesophyll layer in high temperature treated plants (47/37ºC) after 48 (B), 96 (C) and 144 (D) hours and the control (A). The red arrows show dark stains along the palisade mesophyll and yellow circles outline dark stains along the spongy mesophyll. Scale bars = 20 µm.

3.3.4 Trichomes

Trichomes are important outermost structures on the leaf surface (on the epidermis) known for the production of secondary metabolites and increasing the plant's adaptability to changing environmental conditions (Asowata-Ayodele *et al*., 2016). Trichomes are classified according to their size, shape and function (Werker, 2000; Asowata-Ayodele *et al*., 2016). The two main types of trichomes in this study are glandular and non-glandular trichomes. Glandular trichomes are hairs with one or more cells that have a stalk at the base and enlarged top structures, which are referred to as glands whereas, non-glandular trichomes are hairs that are single-celled or multicellular with a thin apex and can easily be distinguished due to the absence of true glandular heads (Werker, 2002). Glandular and nonglandular trichomes are found on both the abaxial and adaxial leaf surfaces. Higher density and distribution of trichomes are mostly found at the adaxial leaf surface in the Verbenaceae family (Tozin *et al*., 2015).

Glandular and nonglandular trichomes were categorized using the classification reported by Ascensao *et al*. (1999) and Martinez-Nataren *et al*. (2011). In *L. javanica*, glandular trichomes of the control and treated plants were classified into peltate trichomes (Fig. 13 A, E, K, O) and capitate trichomes, which were further divided into short (Fig. 13 B-D, H-J, R) and long (Fig. 13 F-G, L-N, P-Q) capitate trichomes. The capitate trichomes were more abundant than the peltate trichomes and were mostly distributed on the adaxial leaf surface. Peltate trichomes, on the other hand, were characterised by larger sizes, which had short stalks made up of one cell and large spherical-shaped heads (80-130 µm in diameter), which generally had a uniform morphology (Fig. 13 A, E, K, O). These trichomes are known to have large spherical heads and secrete the most significant amount of essential oils because of the formation of large subcuticular spaces that mount up secretory products (Combrinck *et al*., 2007; Martínez-Natarén *et al*., 2011). A study conducted by Sharma *et al*. (2003) found that the peltate trichomes in *Mentha piperita* produced the bulk of essential oils and it was proven that the specimen which had more head cells on the leaf surface produced more essential oils. The peltate trichomes in *M. piperita* had similar features to the ones discovered in *L. javanica*; therefore, although this study did not measure oil content on isolated trichomes, it is possible that peltate trichomes in *L. javanica* may be responsible for producing the bulk of essential oils.

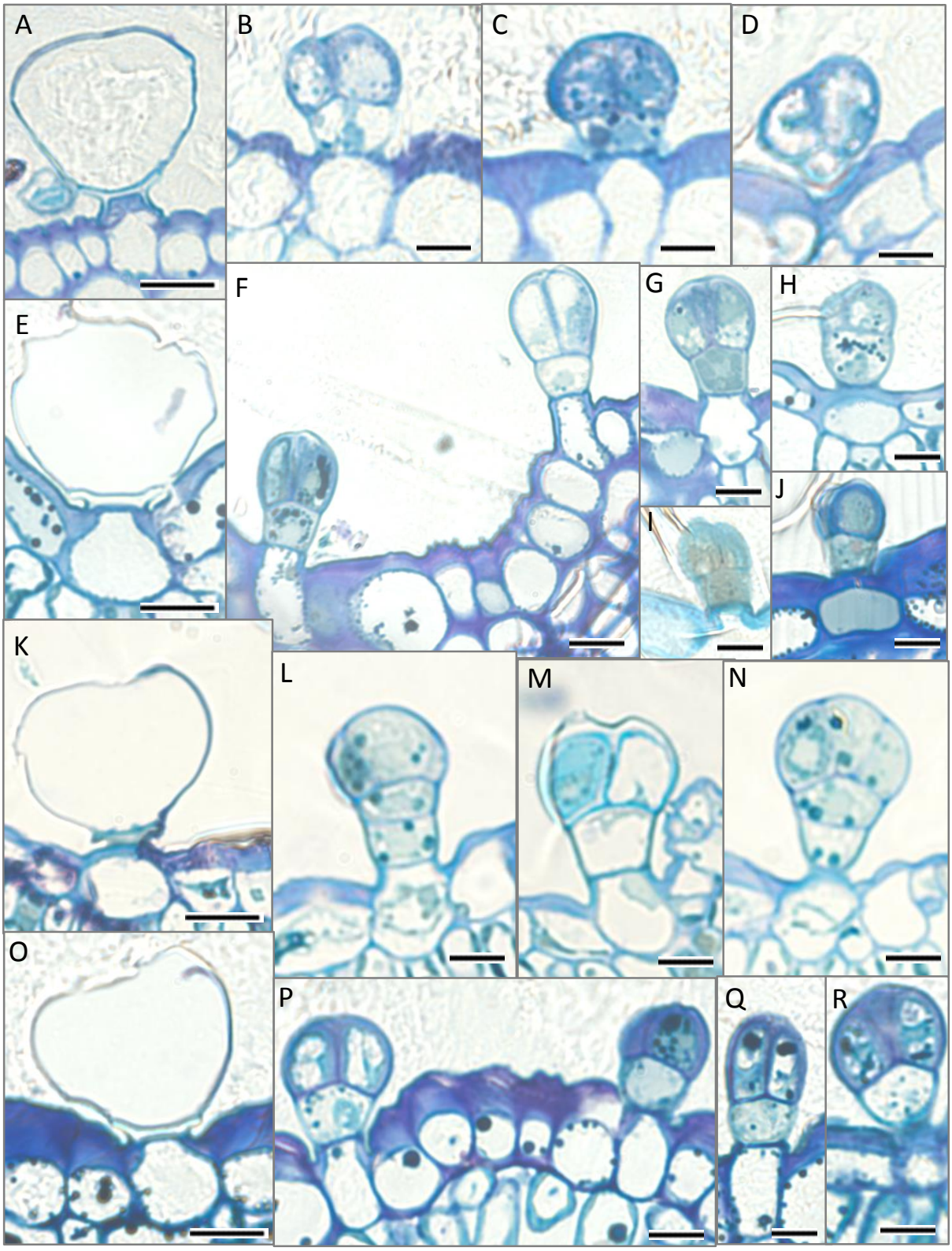


Figure 13. Variety of glandular trichomes found in *Lippia javanica* under ambient temperatures (A-D) and high temperatures (47/37^{0}C) after 48 hours (E-J), 96 hours (K-N) and 144 hours (O-R). Scale bars = 20 µm.

On the contrary, capitate trichomes had a variety of stalk lengths (long and short) and rounded pear-shaped heads (40-60 µm in diameter) made up of one or two cells (Fig. 13). According to studies by Castro-Diez *et al*. (1997) and Ascensao *et al*. (1999), trichomes generally have different morphological characteristics that denote distinct functions and different secretory processes. The two studies reported that capitate trichomes secrete small amounts of essential oils along with flavonoids (Martínez-Natarén *et al*., 2011). Another study conducted by Jia *et al*. (2013) found that capitate trichomes in *Thymus quinquecostatus* are involved in the secretion of essential oils and the oils were observed on the surface of the head cells using stereomicroscopy. The capitate trichomes observed in *T. quinquecostatus* are similar and situated in similar positions to those found in *L. javanica*; therefore, the capitate trichomes in this study may be accountable for secreting essential oils and flavonoids. Glandular trichomes are generally known for their role in secondary metabolite biosynthesis and storage (Combrinck *et al*., 2007). It is, therefore, possible that the trichomes in *L. javanica* play an essential role in secondary metabolite biosynthesis and storage of these compounds as a defence mechanism against environmental stress.

Besides the glandular trichomes, non-glandular trichomes were also found above the epidermal layer. These non-glandular trichomes were relatively bigger in height than the glandular trichomes ($t = -13.75$, $d.f = 102.97$, $P < 0.001$). Non-glandular trichomes in this study were classified into two groups, namely, digitiform (Fig. 14 F, I, D, C, I) and conoidal trichomes (Fig. 14 A, E, D, B, H, G). Digitiform trichomes had elongated finger-shaped heads, while conoidal trichomes had conical-shaped heads. The conoidal trichomes were more abundant on the leaf surface than the digitiform trichomes, but they were both mostly distributed on the adaxial leaf surface. Non-glandular trichomes are known exclusively for their physical protection against biotic and abiotic stresses by forming protective barriers against high temperatures, low humidity and sun radiation (Werker, 2000). However, a study conducted by Santos-Tozin *et al*. (2016) stated that in the Verbenaceae family, glandular trichomes do not only physically protect the plant, but are also involved in the storage and production of biologically active substances such as essential oils and flavonoids. The study used histochemistry analysis to show that non-glandular trichomes produced or stored secondary metabolites, terpenes, alkaloids, lipids and phenolic compounds supplementing the work of glandular trichomes (Werker, 2000; Tozin, 2016). Therefore, the present study suggests that the high number of non-glandular trichomes on *L. javanica*'s leaves is attributed to the extremely high temperatures, which in turn leads to the production of essential oils and

other phytochemicals to further protect the plant in addition to their already known function of physical protection.

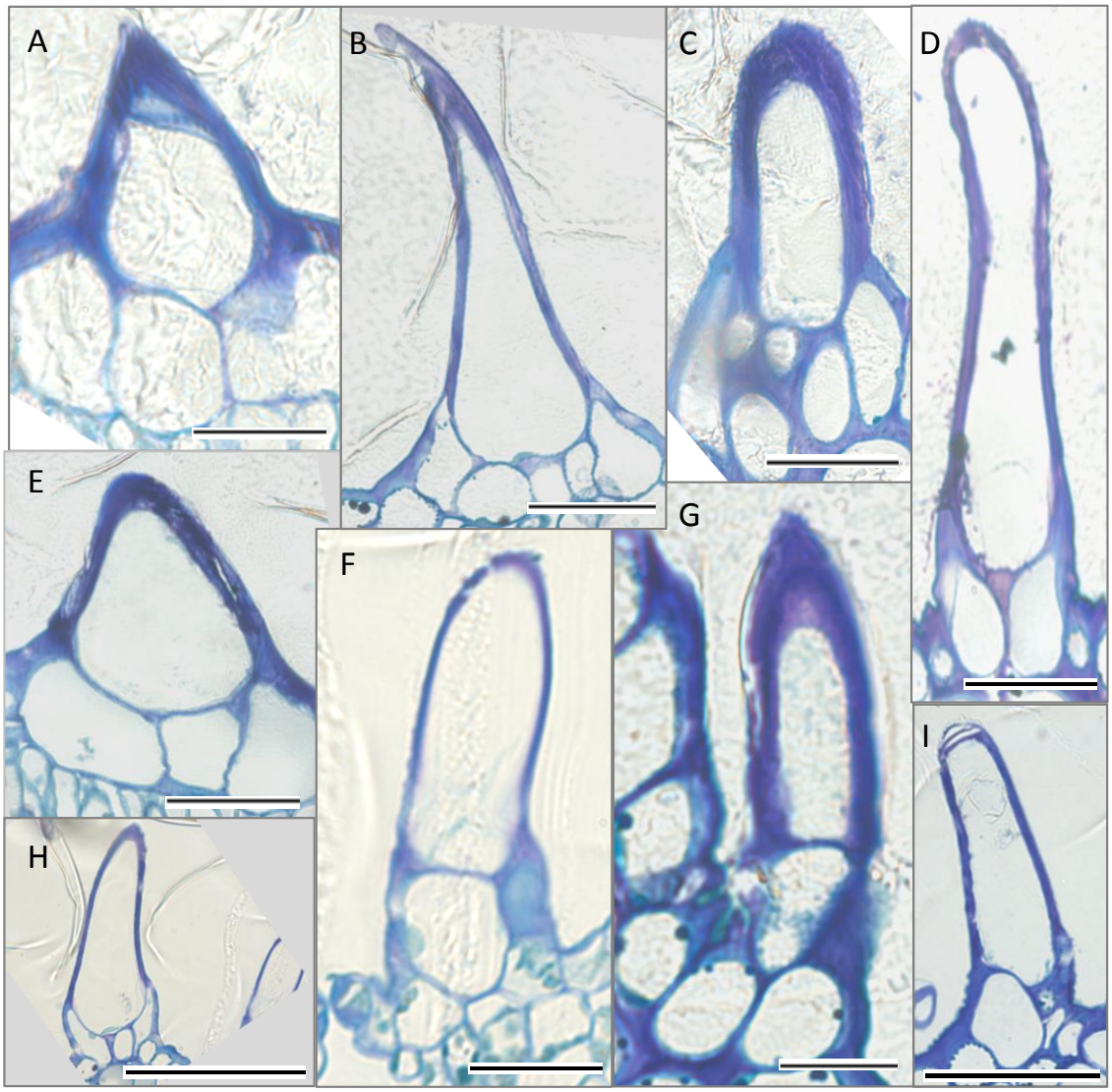
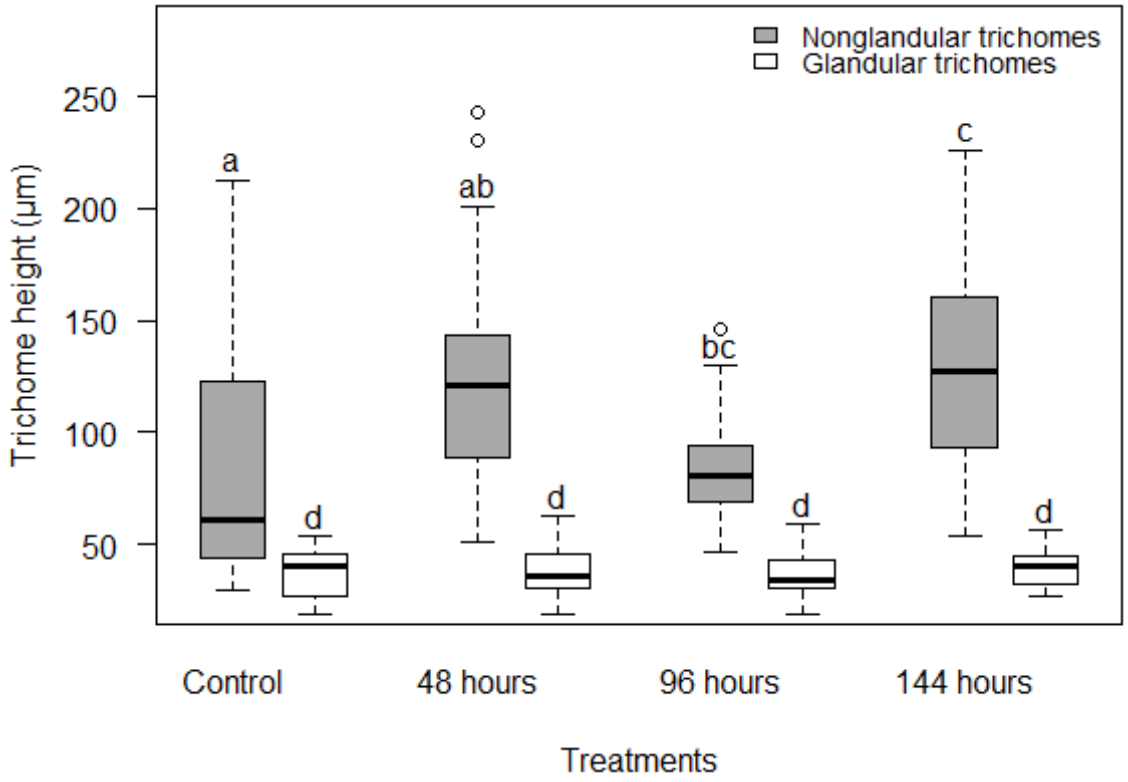


Figure 14. Variety of non-glandular trichomes found in *Lippia javanica* in the control (A) and high temperature treatment (47/37^{0}C) after 48 hours, 96 hours and 144 hours (B-H). Scale bars = 20 µm.

The total number of non-glandular trichomes increased significantly from the control (9) to the 48 hours (25), 96 hours (29) and 144 hours (32) treatment per sectioning while the glandular trichomes fluctuated from the control (22) to the 48 hours (10), 96 hours (15) and 144 hours (29) treatment, with an overall increasing trend. In all the treatments, the total trichome number increased by 51% from the control to the 144 hours treatment. The non-glandular trichome heights under the 144 hours, 48 hours, control and 96 hours treatment had higher averages of 130.1, 123.4, 89.2, 81.6 µm respectively, than the glandular trichome heights of the 144 hours, 48 hours, control and 96 hours treatment which had averages of

39.6, 39, 37.3, 36.6 µm respectively ($F_{(7,162)} = 32.09$, $P < 0.001$) (Fig. 15). Using Tukey HSD test, the control differed significantly from 144 hours and 48 hours from 96 hours, as well as 96 from the 144 hours treatment of the non-glandular trichomes (Fig. 15). On the contrary, the glandular trichomes of the treatments and control were not significantly different from each other. These results show that the duration of exposure to high temperatures increased the quantity and height of non-glandular trichomes on the leaf surface, with little to no effect towards the glandular trichomes. The results in this study correspond to the findings made by Martínez-Natarén *et al*. (2011) and Ascensao *et al*. (1999), which suggests that plants found in more stressful environments depict a greater protection level which is provided by the higher density of non-glandular trichomes. This indicates that the higher production of trichomes by *L. javanica* is due to the need for greater levels of protection against the high



temperatures.

Figure 15. The average height of trichomes along the midline region of *Lippia javanica*'s leaves under ambient conditions (25/20 ºC) and high temperatures (47/37ºC) for 48, 96 and 144 hours. Different letters $^{a\text{-}d}$ are significantly different (Tukey test, $P < 0.05$).

Using a one way ANOVA, there was a significant difference in the trichome diameters per treatment ($F_{(7, 161)} = 3.56$, $P < 0.01$) (Fig. 16). The non-glandular trichome diameter averages under the 48 hours, 96 hours, 144 hours and the control were 44.3, 42.7, 37.6, 32.3 µm respectively, while the glandular trichome diameters averages of the 48 hours, 144 hours, 96 hours and the control were 34.3, 34.2, 31.6, 30.3 µm, respectively (Fig. 16). Using the Tukey HSD test, the non-glandular diameters in 48 hours, 96 hours, 144 hours, control and the glandular diameters in 48 hours, 144 hours and 96 hours treatment were not significantly different from each other. However, the 48 and 96 hours non-glandular trichome diameters were significantly different from the glandular control. These results indicate that high temperatures do not have a significant effect on trichome diameters. Both the glandular and non-glandular trichomes increased their height while maintaining their diameter under extremely high temperatures. These findings suggest that the height of trichomes in *L. javanica* plays a significant role in regulating leaf surface temperatures than the diameter of trichomes.

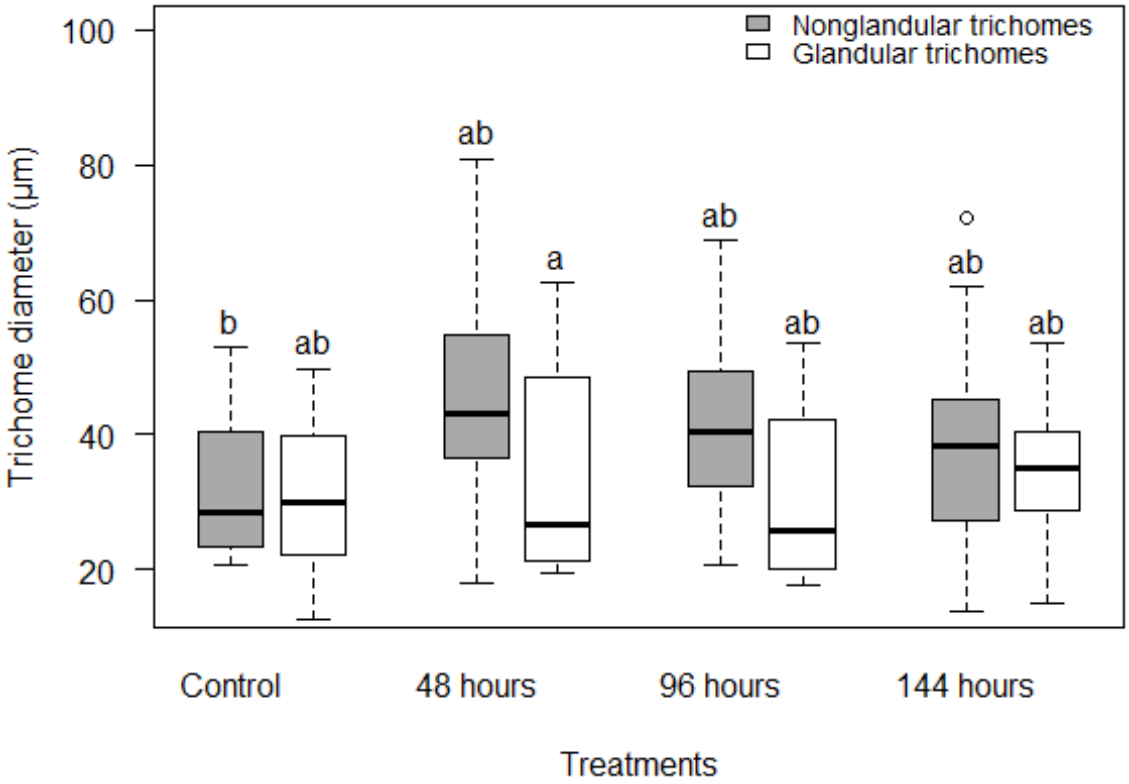


Figure 16. The average diameter of trichomes along the midline region of *Lippia javanica*'s leaves under ambient conditions (25/20 ºC) and high temperatures (47/37ºC) for 48, 96 and 144 hours. Diameter was measured at the widest point of the trichomes. Different letters [a-b] are significantly different (Tukey test, $P < 0.05$).

The increase in trichome height after exposure to high temperatures may suggest that there was an increase in the secondary metabolites produced or stored within the different trichome types and this correlates with the findings in chapter four of our study. The structural modifications of trichomes may imply functional changes. Both glandular and non-glandular trichomes in the Verbenaceae family are known to secrete bioactive compounds (Santos-

Tozin *et al*., 2016) and their secretory organs are located at the bottom, close to the epidermis, of each trichome (Fig. 15 and 16) (Martínez-Natarén *et al*., 2011). The large sac above these secretory organs are known to store the secreted bioactive organs (Martínez-Natarén *et al*., 2011; Santos-Tozin *et al*., 2016). These results, therefore, imply that when *L. javanica* plants were exposed to high temperatures, the increase in trichome height increased the surface area for storage and secretion of bioactive compounds. This means that the higher the number and height of trichomes, the more phytochemicals are produced which ultimately increases the adaptability of the plant by reflecting solar radiation.

High temperatures result from global warming or heatwaves, which are as a result of climate change. These events most likely result in the extinction and/or adaptation of plants. *L. javanica,* in particular, resulted in the adaptation to high temperatures after six days of exposure. *L. javanica*'s samples continued growing after being returned into the greenhouse under ambient conditions (25/20 ºC) (Fig. 17). The recovery was initially observed within six weeks after being returned into the greenhouse (Fig. 17 A). A few green leaves were regenerating on the stems and branches of the plants. After eight weeks, more plants were recovering and there was evidence of more shoots and leaves on the branches and stems of the shrub (Fig. 17 B and C). This means that plants were able to change the cellular structures by increasing trichome numbers, length, and diameter to acclimatise to high temperatures. This was possible due to the short period (144 hours) of exposure to high temperatures and also because plants were not deprived of water during the experiment. The plant samples would not have adapted if the time of exposure was longer and if plants were not continuously being watered. These results suggest that *L. javanica* was able to adapt under high temperatures, which is directly linked to its phenotypic plasticity.

A study by Chagonda *et al*. (2000) found that cultivated plants secrete less phytochemicals than those growing in the wild because of the different stresses the wild populations undergo in comparison to the cultivated plants. This implies that plants grown in the wild experience a wide range of harsh conditions, thus they secrete more phytochemicals to improve their defence and survival mechanism. This means that the secretion of phytochemicals is associated with the increased protection levels required for the survival of plants.



Figure 17. *Lippia javanica*'s recovery to high temperatures after eight weeks in the greenhouse (25/20ºC). The white arrow in picture C shows the growth of new shoots. Picture A was captured after six weeks, B and C, after eight weeks.

3.4 CONCLUSION

The present data suggest that *L. javanica* has possible mechanisms to adapt under high temperatures. This was indicated by the growth of new shoots when samples were returned to the greenhouse. *L. javanica* was able to adapt both structurally and physiologically under high temperatures which was proven by signs of recovery after eight weeks of being returned into the greenhouse under ambient temperatures. Under high temperatures, *L. javanica* responded by wilting, rolling and curling with 80% of the leaves desiccating after the 144 hours treatment. This shows the strenuous effect that high temperatures have on plants growing in the wild and the possible effect of climate change and heatwaves on plants, particularly medicinal plants.

The overall histological analysis showed that the leaves were not able to acclimatise during the first 48 hours of exposure, but managed to adapt after the 96 hours to 144 hours treatment. The adaptation of *L. javanica* was evident as intercellular spaces decreased due to the expansion of palisade and spongy mesophyll cells minimizing water loss and thus decreasing the leaf surface temperatures. The 48 hours, 96 hours and 144 hours treatment showed an incline in dark blue stains, which was evidence for the presence of phytochemicals which assist in reflecting UV rays from sunlight.

Glandular trichomes increased in numbers on the leaf surface under high temperatures and they are known for their role in producing essential oils and other secondary metabolites, which suggests that the total yield of both essential oils and other secondary metabolites increased significantly. Non-glandular trichomes also increased in numbers and in the Verbenaceae family, they are not only known for their role in physically protecting the plant from biotic and abiotic stresses but are also involved in producing biologically active substances. This shows that plants found in more stressful environments are more likely to depict a higher level of protection by secreting more phytochemicals and increasing trichome density.

CHAPTER 4

Effects of high temperatures on essential oil yield, composition, and total phenolic and flavonoid content in *Lippia javanica*

SUMMARY

Lippia javanica is an indigenous medicinal plant used in Southern African countries to treat various ailments. This chapter aimed to investigate the effects of high temperatures on the essential oils and total phenolic and flavonoid content in *L. javanica*. To investigate this aim, plant samples of *L. javanica* were exposed to ambient conditions (25/20 ºC – control) and high temperatures (47/37 ºC – day/night) episodically for 48, 96 and 144 hours. The exposure to high temperatures resulted in a significant increase in the total phenolic content from 191.33 mg GAE/g (48 hours) up to 441.94 mg GAE/g (144 hours) and also, the total flavonoid content significantly increased from 115.91 mg QE/g (48 hours) up to 268.66 mg QE/g (144 hours) ($F_{(7,40)} = 1670$; $P < 0.00$). Control samples of phenolics and flavonoids showed 144.75 mg GAE/g and 81.17 mg QE/g, respectively. The average essential oil yield of *L. javanica* increased significantly from the control (3.41%) to high temperature treatments, 48 hours (5.84%), 96 hours (7.18%) and 144 hours (8.15%) ($F_{(3, 20)} = 16.31$; $P < 0.0001$). The number of essential oil constituents also showed an increasing trend from the control to high temperature treatments. These results suggest that *L. javanica* samples exposed to extreme conditions significantly increase the concentrations of natural secondary metabolites to acclimatise under stress than plants of the same species grown under ambient conditions. These results could help maximize products containing high bioactive compounds for industries, indigenous people and herbal/traditional healers by harvesting *L. javanica* grown under high temperatures.

4.1 INTRODUCTION

Lippia javanica is an indigenous medicinal plant extensively used for cough remedies, embodiment formulations, diabetes and malaria, amongst others (Viljoen *et al*., 2005; Maroyi, 2017). The commonly used parts of the shrub are leaves and stems and sometimes, roots may also be utilized (Van Wyk *et al*., 1997; Viljoen *et al*., 2005). Leaves and stems of *L. javanica* are together used as inhalants, teas, or embodiment formulations, whereas; roots are used as antidotes against eye infections and food poisoning (Hutchings, 1996; Maroyi, 2017). These different parts of the plant contain phytochemicals or bioactive compounds including essential oils, phenolics and flavonoids which are secreted in response to physiological and ecological pressures including pathogens, insects, temperature extremes and UV radiation (Pereira *et al*., 2009; Sulaiman and Balachandran, 2012; Matshediso *et al*., 2015). These phytochemicals also contain medicinal properties that are useful for humans as insecticides, food and for treating various ailments (Hutching, 1996; Viljoen *et al*., 2005; Asowata-Ayodele, 2015; Maroyi, 2017).

Essential oils have become a necessary ingredient in the world's biggest manufacturing industries aiding in aromatherapy, perfumery, cosmetics, food flavouring, cleaning products and healthcare pharmaceuticals (Dionisio *et al*., 2012). Essential oils have healing properties that are mostly used in traditional medicines to treat cancer, skin infections, coughs and malaria, amongst others. *L. javanica*, in particular, has essential oils that have a range of commercially valuable properties. For example, *L. javanica*'s essential oils incorporated in candle wax provide mosquito repellent properties (repels not less than 95% of mosquitoes) better than most available commercial products (repels only 42% of mosquitoes) (Maroyi, 2017). The large-scale candle production yields approximately 400 000 candles per year, and each 250 g candle retails for approximately R20 (Xaba *et al*., 2010). Essential oils of *L. javanica* are known to have antiviral, antioxidant, antidiarrhoeal, antitrypanosomal, anti-inflammatory, antibacterial, and anticonvulsant properties which are due to the individual volatile compounds found in the essential oils of the plant (Combrinck *et al*., 2007; Maroyi, 2017).

Phenolic compounds are the main group of phytochemicals that are widely spread in the plant kingdom (Edeoga *et al*., 2005). They consist of flavonoids, which are the most common type of phenolic compounds and are widely spread in plant tissues (Gottlieb, 1989; Panche *et al*., 2016). These naturally occurring phytochemicals are not only used as a defence mechanism for plants, but their properties also extend to benefit humans, for example, phenolic compounds in *L. javanica* are known for their anti-inflammatory, antimicrobial and antioxidant properties that scavenge free radicals in the body and they tend to be more effective than synthetic vitamin C and E (Frankeli, 1995; Shikanga *et al*., 2010; Sulaiman and Balachandran, 2012). *L. javanica* is known to have the highest total phenolic content amongst the other indigenous *Lippia* species (*L. scaberrima*, *L. rehmannii* and *L. wilmsii*) in South Africa; thus it may be the most effective at treating various ailments (Shikanga *et al*., 2010).

The production of secondary metabolites (essential oils and phenolic compounds) is an energy and carbon costly process that plays a significant role in protecting plants against biotic and abiotic stresses (Bennett and Wallsgrove, 1994). High temperatures are abiotic stresses that are more frequent due to global warming (McMichael *et al*., 2006; Aggarwal, 2008; Kruger *et al*., 2012) and they trigger complex responses in plants which may have adaptive or deleterious effects (Bennett and Wallsgrove, 1994; Chaves *et al*., 2002). Medicinal plants, in particular, are susceptible to temperature changes, and under high temperatures, they tend to increase the density of trichomes on the epidermis, which ultimately increases the production of essential oils and phenolic compounds (Werker, 2000; Tozin, 2016). These compounds protect the plant by reflecting sun rays and absorbing high temperatures, which cools the leaf surface and reduces water loss (Werker, 2000; Tozin, 2016). For example, a study conducted by Stinziano and Way (2014) stated that high temperatures significantly increases the production of bioactive compounds, which are crucial elements in assisting plants to acclimatise.

L. javanica is known to contain a wide range of essential oil properties along with a high production of phenolics and flavonoids, which are of interest to large manufacturing industries and there is a high need to increase or maintain the production of these secondary metabolites. Thus this study aims to better understand the chemical compositions of *L. javanica*'s essential oils and the total phenolic and flavonoid content. This study also aims to investigate the possible effect of high temperatures on the yield and chemical composition of

essential oils and the total phenolic and flavonoid content found in *L. javanica*. The results found in this study will help industries, farmers and traditional/herbal healers to determine the suitable time to harvest *L. javanica* along with the possible changes in the major essential oils which may impact the range of ailments treated by the shrub.

4.2 MATERIAL AND METHODS

As previously mentioned in chapter 3, mature plants of *L. javanica* were maintained in the greenhouse at the University of the Witwatersrand after being obtained from Mountain Herb Estate in Hartbeespoort. The plants were left in the greenhouse for a month to acclimatise before moving them into the climate test incubator (Conviron-CMP6010) for treatment. Three replicates of eight sample plants (n = 24) were transferred into a Conviron where the temperatures were set at 47/37ºC (day/night simulation) for up to 144 hours (under 12 hour light with a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$). The temperatures in the Conviron were specifically set at 30ºC from 24h00 to 06h00 and were adjusted to 35 ºC from 06h00 to 12h00 and 40 ºC at 12h00 to 18h00 and back to 35 ºC from 19h00 to 24h00. Essential oils and the total phenolic and flavonoid content were assessed every 48 hours (48, 96, and 144 hours). After performing the above-mentioned assessments, the plant samples were then returned to the greenhouse (under ambient temperatures) to recover from the extremely high temperatures. Samples were returned to room temperatures and watered daily for observation purposes to assess recovery.

The control and treated plants were watered twice a day and humidity, soil type and light conditions (day and night simulation) were kept constant throughout the treatments. Control plants were exposed to ambient temperatures (25/20ºC) and according to Wigge (2013), these ambient conditions do not severely affect plant growth, photosynthetic rate and development. The primary dependent variable that was investigated in this study was the duration of exposure to high temperatures.

4.2.1 Extraction and Analysis of essential oils

The essential oil composition of *L. javanica* is known to differ between and within species found in one area (Mwangi *et al.*, 199; Terblanche and Kornelius, 1996; Chagonda *et al.*,

2002; Maroyi, 2006; Chagonda and Chalchat, 2015). Thus to investigate the possible role of high temperatures on the volatile constituents of this medicinal plant, the same set of plants were used per treatment (n = 15). Harvested leaf samples of *L. javanica* were air-dried (300 g of leaf material) and a Clevenger-type apparatus was used for the hydrodistillation process (Serrato-Valenti *et al*., 1997). The hydrodisillation method was chosen because it is a cheaper, greener and a more commonly used technique of extracting essential oils. Hexane and methanol are the two solvents that were used during the extraction procedure. Hexane was utilised to extract non-polar compounds as well as medium polarity compounds, which are known for antimicrobial activities, while methanol (MeOH) was used to detect polar compounds such as flavonoids and triterpenoids. The extracts were filtered using Pasteur pipettes to enable the separation of plant residue and the extracts. After the extraction process, the oil was transferred into an empty bottle and the final mass was noted. The total yield was calculated using the formula below:

Percentage (%) yield$= \left[\frac{B-A}{C}\right] X100$

A = Mass of empty bottle (g)

B = Mass of bottle plus oil extracted (g)

C = Mass of distilled material (g)

Essential oils were analysed using the Gas chromatograph/ Mass spectrometer (GC-MS) (Hewlett-Packard G1800A GCD system). An Innowax FSC column (60 m × 0.25 mm diameter) was utilised and helium was the carrier gas, with a flow rate of 0.8 ml/min (Serrato-Valenti *et al*., 1997). The GC-MS was maintained at an oven temperature of 60 °C for the first 10 minutes and then temperatures were programmed to rise to 220 °C at a rate of 10 °C/min, and it was kept constant at 220 °C for 10 minutes and then again, the temperature was programmed to rise to 240 °C at a rate of 10 ◦C/min. Split flow was adjusted to 50 ml/min whereas the injector and detector temperatures were adjusted to 250 °C. The mass spectra/ionization was taken at 70 eV and the mass range was from 35 to 425 m/z. The Total Ion Chromatogram (TIC) was used to determine the percentage amounts of the separated compounds from peak areas (percentage areas), which gave the relative percentage amounts calculated from the computer.

4.2.2 Assessment of flavonoids and phenolics

Extraction and estimation of total phenolics

The extraction of phenolics from the harvested leaf samples of *L. javanica* was carried out using the methodology reported by Siddhuraju and Becker (2003) with modifications applied by Pakade *et al*. (2013). The harvested leaf samples were air-dried at room temperature until a constant mass was achieved and then crashed into powder using a waring blender or a pestle and mortar. The powdered leaf samples were extracted using a sonication bath for 25 minutes with 20 mL of 80% acetone in water. The solution was then obtained and centrifuged for 10 minutes at 6000 rpm and the supernatants were saved. The process was repeated to re-extract and the supernatants were merged. The total volume of the extract was made up to 50 mL with 80% of aqueous methanol (MeOH) solution. The Total Phenolic Content (TPC) was estimated according to the methodology developed by Folin-Ciocalteu (1927).

TPC was estimated from the methanol extracts of *L. javanica* using the Folin-ciocalteu reagent assay. A solution of 750 µL 1:10 Folin – Ciocalteu reagent and 2 mL of 7.5% aqueous sodium carbonate was added to the methanol extracts of *L. javanica* (200 µL) which were reacted in an Erlenmeyer flask fitted with a rubber stopper (Pakade *et al*., 2013). The mixture was diluted with 7 mL of deionised water. The mixture was positioned in a dark cupboard for two hours at ambient conditions and afterwards, the absorbance of the phenolic content in the extract was measured using a spectrophotometer at a wavelength of 765 nm (Iqbal *et al*., 2006). The total phenolic content of the different methanol extracts was calculated using the following linear equation obtained from the calibration curve constructed using Gallic acid working stock solution:

y=3E-05x+0.0007, $r2$ = 0.9958----------Equation 1

(For more details, see appendix 4)

Extraction and estimation of total flavonoids

The extraction of total flavonoids was done using the methodology developed by Siddhuraju and Becker (2003). Powdered leaf material of *L. javanica* (1 g) was extracted in an apparatus consisting of a reflux condenser and a round-bottomed flask with 100 mL of 80% aqueous methanol (MeOH) for three hours. The extract was centrifuged for 15 minutes at 5000 rpm

and was filtered at 0.45µm using a filter paper. The extract was made up to 100 mL with 80% aqueous methanol, which was used to estimate the Total Flavonoids Content (TFC).

To estimate TFCs, the methodology published by Siddhuraju and Becker (2003), with some modifications mentioned by Phakade *et al*. (2012), were followed. A 10 mL volumetric flask was used to dilute 0.3 mL of the extract with 4 mL of deionised water. The following chemicals were added to the diluted extract at different time intervals; firstly, 0.3 mL sodium nitrate ($NaNO_3$) was added and properly mixed. After 5 minutes, 3 mL of aluminium chloride ($AlCl_3$) solution was added. After a further 6 minutes, 2 mL of 1 M sodium hydroxide solution was placed inside the volumetric flask. After the sequential addition of these chemicals, the reaction mixture was made up to 10 mL with deionised water and stirred for 15 minutes. A UV-visible spectrophotometer measured the TFC on the extract mixture at a wavelength of 510 nm (Iqbal *et al*., 2006). The total flavonoid content of the different methanol extracts was calculated using the following linear equation obtained from the calibration curve constructed using quercetin standards:

$y=0.0283x+0.0236$, $r2 = 0.9735$----------Equation 2

(For more details, see appendix 4)

Methanol was chosen as a solvent in this study because it is a polar solvent (amphiphilic compound) used for the extraction of bioactive compounds. It was chosen because many studies have compared different solvents and methanol was determined as a better solvent because it extracts a greater quantity of plant material and is less toxic than the other solvents (acetone, chloroform and cheksan) (Masoko and Eloff, 2007). For this study, 80% aqueous methanol was prepared and it is known to have an intermediate polarity; hence, it can extract triterpenoids, flavonoids and stilbene because these compounds have an intermediate polarity (Taylor *et al*., 2007). A study by Masoko and Eloff (2007) stated that there is no perfect solvent that extracts all polar, nonpolar and bioactive compounds, among others, without destroying some class of compounds, which means that the type of phytochemical groups that are of interest are an important factor that governs the choice of solvents used in an extraction.

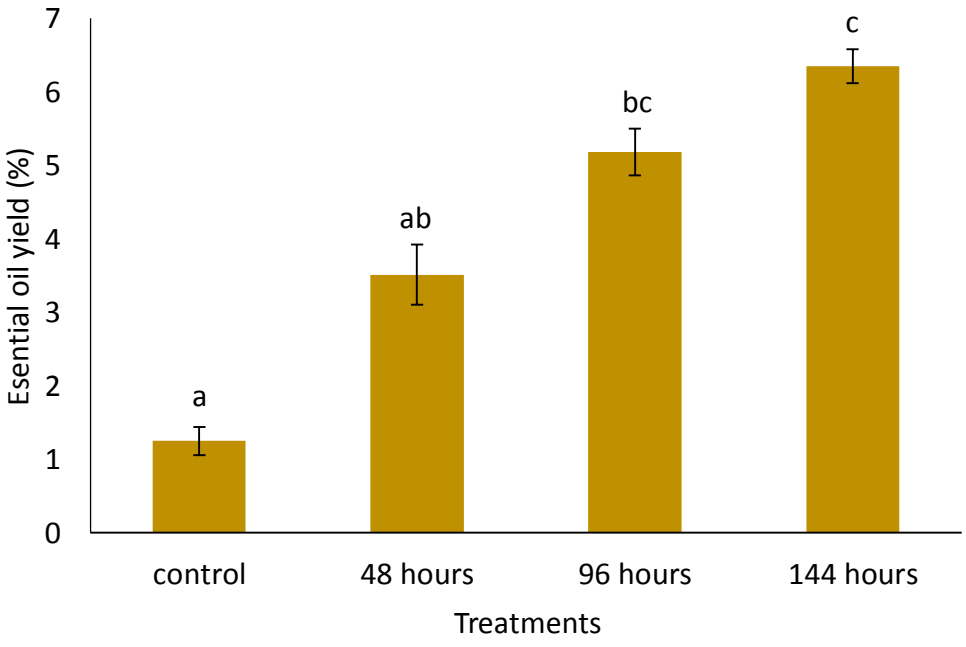
4.2.3 Statistical analysis

Quantitative and qualitative data were analysed. For qualitative analysis, essential oil compounds were categorised into three main groups; oxygenated chemical compounds (alcohols, esters, aldehydes, phenols, ketones and ethers), terpenes (hydrocarbons) and non-terpenoids. Quantitative data, on the other hand, were analysed on R-studio and excel. All quantitative data were tested for normality using a shapiro test and all the tests were performed at a significant level of $P \leq 0.05$. Percentage areas of the same component that were recorded twice or thrice by the GC-MS were added together to make up a total percentage area for that component. A one-way Analysis of Variance (ANOVA) was performed between the essential oil yields per treatment and a two way ANOVA was conducted between total phenolic and flavonoid content per treatment. A Tukey HSD posthoc test was then performed to determine where the difference lies after the one way ANOVA and two way ANOVA, respectively.

4.3 RESULTS AND DISCUSSION

4.3.1 Essential oil yield

The average essential oil yield of *L. javanica* increased significantly from the control (1.2%) to the high temperature treatments; 48 hours (3.5%), 96 hours (5.2%) and 144 hours (6.3%) ($F_{(3, 20)} = 16.31$; $P < 0.0001$) (Fig. 18). The oil colours ranged from lemon yellow (control) to yellowish brown (144 hours treatment). A study by Chagonda and Chalchat (2015) indicated that the average essential oil yield found in *L. javanica* under ambient conditions was 1.1% while studies by



Chagonda *et al*. (2000) found a total yield of 0.3 to 0.7%. The essential oil yield found in the control of this study using hydrodistillation is therefore similar to the yield obtained by Chagonda and Chalchat (2015) using steam distillation. The results found in this study, after exposing *L. javanica* to high temperatures, show an increase in the essential oil yield which is of high economic significance because industries and pharmaceutical companies aim on increasing the essential oil yield of the shrub because of its wide range of medicinal properties.

Figure 18. Essential oil yield of *Lippia javanica* under ambient conditions (25/20 ºC) and exposed to high temperatures (47/37 ºC) for 48, 96 and 144 hours. (n = 24).

Studies show that both essential oil yield and quality of components are largely affected by environmental factors such as altitude, light intensity and seasonality, among others (Chagonda and Chalchat, 2015; Tozin *et al*., 2015; Argyropoulou *et al*. 2007). This effect is more evident in this study because high temperatures significantly increased the essential oil yield secreted in *L. javanica* after six days. A study conducted by Clark & Menary (1980) found that the oil yield of peppermint (*Mentha piperita*) increased significantly under high light intensity. Additionally, recent studies conducted by Manukyan (2011) and Forouzandeh *et al*. (2012) found that the concentration and yield of essential oils from *Nepeta cataria* and *Ocimum basilicum* increased significantly after exposure to drought conditions, respectively. These studies show that plants exposed to extreme environmental stresses tend to secrete more phytochemicals as a survival mechanism inorder to acclimatise (Edeoga *et al*., 2005; Al-Gabbiesh *et al*., 2015). It is therefore clear from these results that the production of essential

oils in *L. javanica* is temperature sensitive and that the secretion of essential oils is a positive response to environmental stresses.

A study by Al-Gabbiesh *et al*. (2015) stated that plants exposed to environmental stresses reveal not only a higher concentration but also a higher yield of essential oils than those that are cultivated under ambient conditions. In this context, however, the exposure to environmental stresses significantly reduces the growth of plants because carbon that is meant to be allocated to growth is redirected to the production of secondary metabolites for the survival of the plant (Bennett and Wallsgrove, 1994). The result may be detrimental for the plant, depending on the duration of exposure to extreme environmental stresses. Most environmental stresses are periodic; thus some plants are able to adapt under stress by increasing the production of secondary metabolites and then later returning to their normal biological metabolism. *L. javanica* in this study was able to recover after being exposed to high temperatures for a maximum of 144 hours when they were returned into the greenhouse for eight weeks. This finding could mean that *L. javanica* was able to acclimatise by secreting large concentrations of essential oils and other phytochemicals under stress and returned to its normal metabolism in the greenhouse under ambient conditions which allowed carbon atoms to be redirected to growth.

4.3.2 Essential oil compositions

The two solvents, 80% aqueous methanol and hexane, which were used in the extraction of essential oils yielded the same essential oil components. It was observed that methanol yielded more essential oil components and contained all components that were yielded by hexane. This, therefore, means that 80% aqueous methanol was a better solvent than hexane at extracting both polar and nonpolar components (see appendices 2 and 3 for more details).

The analysis of essential oil composition in the control yielded a total of 29 essential oil components, representing 99.68% of the total oil constituents, which consisted mainly of linalool (33.11%), carvone (24.53%), piperitenone (6.68%) and tagetenone (5.16%) while the 48 hours treatment yielded a total of 14 essential oil components, representing 97.63% of the

total oil constituents, which consisted mainly of camphor (42.97%), eucalyptol (10.86%), linalool (10.43%) and caryophyllene (10%) (Table 1). The 96 hours treatment produced 37 essential oil components, representing 99.76% of the total oil constituents, which consisted of eucalyptol (20.41%), camphor (15.71%), carvone (12.4%) and fenchone (9.66%) whereas, the 144 hours treatment produced 38 essential oil components, representing 95.11% of the total oil constituents, which consisted of eucalyptol (34.1%), p-cymene (15.79%), caryophyllene (10.16%) and terpinen-4-ol (4.29%) (Table 1) (For more details, see appendix 2). These results show that there is a general increasing trend in the number of essential oil components secreted by *L. javanica* after exposure to high temperatures.

The 48 hours treatment, however, did not follow this general trend. It showed a 48% decline from the control in the number of essential oil components. This could be *L. javanica*'s response to the unexpected increase in temperatures and the sudden shock experienced and thus decreased the number of constituents but increased their concentration. A second possible reason might be because the plants, after 48 hours, were promoting maximum absorption of high temperatures due to the sudden shock; thus, they increased the concentration of essential oil constituents to cool down the leaf surface temperatures (Lee and Ding, 2016). At 96 and 144 hours, *L. javanica* started producing more essential oil components with lower concentrations. This might suggest that plants were able to acclimatise to the high temperatures after the 48 hours treatment and more essential oil constituents were being produced for further absorption of high temperatures.

Exposing *L. javanica* to high temperatures resulted in the secretion of new major essential oil components such as eucalyptol, camphor and p-cymene, which were not previously present in the control (Table 1). Eucalyptol is mostly produced by eucalyptus and it is known for its role in treating chronic diseases as it has antioxidant and anti-inflammatory properties (Dzoyem and Eloff, 2015; Seol and Kim, 2016). These properties are also present in the control of the shrub such that *L. javanica* is commonly used for treating diabetes, fever and asthma. On the other hand, p-cymene is an aromatic compound that has anti-microbial, anti-inflammatory and antioxidant properties and is used in medicine or food (Marchese *et al*., 2017) while camphor is generally used for its scent to repell against mosquitoes and for embodiment formulation to treat different skin diseases (Lukwa *et al*., 2009; Hamidpour *et al*., 2013). The secretion of new essential oil constituents might be because high temperatures trigger different metabolic pathways within plants enabling other compounds to be secreted, which would not have been

secreted under normal environmental conditions (Viljoen *et al*., 2005; Combrinck *et al*., 2007; Maroyi, 2017). From these new major essential oil components produced by *L. javanica* under high temperatures, there might be an expansion or a complete change in the range of ailments treated by the shrub.

Some major essential oil constituents such as carvone, tagetenone and piperitenone, which were present in the control ended up disappearing in the high temperature treatments. This might be because certain chemotypes are not produced at specific temperature ranges; thus temperature alterations have a significant impact on the essential oil composition produced by plants. A study by Lee and Ding (2016) found that the specific ratio of essential oil constituents determines the therapeutic and wellness-enhancing properties of the oil. However, a study by Nakatsu *et al.* (2000) suggested that although there are differences in the composition of essential oils of various plants, there is considerable overlap in their overall properties. For example, *Tetradenia riparia* and *Virola surinamensis* have different essential oil profiles, but they are reported to treat malaria symptoms (Al-Gabbiesh *et al*., 2015). Therefore, the changes observed in the oil constituents of *L. javanica* after exposure to high temperatures may not suggest changes in the biological activity of the shrub.

Various authors have identified the major essential oil compounds of *L. javanica*; however, there have been different major compounds determined for *L .javanica* populations distributed in different areas including those that were not geographically isolated (Terblanche and Kornelius, 1996; Neidlein and Staehle, 1974; Chagonda and Chalchat 2015). It was then established that there are quantitative and qualitative variations in essential oil constituents both between and within natural plant populations due to the differences in the metabolic pathways that secrete essential oils (Maroyi, 2006; Chagonda and Chalchat 2015). A review study by Maroyi (2006), however, summed up the constituents of essential oils found in *L. javanica* from different authors and concluded that the standard and dominant chemotypes are piperitenone, ipsdienone, carvone, tagetenone, limonene, caryophyllene, p-cymene, linalool, myrcene, myrcenone, ocimenone, sabinene, and ipsenone. These constituents are similar to the major compounds found in the control of this study. However, four major components (eucalyptol, camphor, fenchone and terpinen-4-ol) found in the high temperature treatments did not match with the components found in these studies. This might be because high temperatures trigger new metabolic pathways in plants, which results in the production of new chemotypes to enable adaptation under stress. This means that *L. javanica* displayed coping

mechanisms by secreting new essential oil components, which may be helpful for adaptation under stress.

Table 1. Essential oil composition and component percentage area of *Lippia javanica* under ambient conditions (control) and exposed to high temperatures (47/37^{0}C for 48 96 and 144 hours). A dash (-) represents no components found.

Groups	Components	Control (%)	48 hours (%)	96 hours (%)	144 hours (%)
Alcohol	Terineol	-	-	-	1,15
	Terpinen-4-ol	1,56	1,37	0,31	4,29
	Terpineol	4,27	5,77	3,54	2,83
	Thymol	0,17	-	-	0,1
	trans-Carveol	0,34	-	2,29	0,38
	1-octen-3-ol	-	1,64	-	-
	4-Heptyn-3-ol	-	0,55	-	-
	Isopinocarveol	1,53	-	-	-
	Linalool	33,11	10,43	3,71	0,83
	Myrtenol	-	0,5	0,28	-
	Nerolidol	-	-	0,06	-
Aldehyde	a-Thujenal	0,54	-	0,37	-
	Graniol	-	-	0,68	0,05
	Lilac aldehydeB	0,12	-	0,03	0,05
Ketone	(-)-Carvone	24,53	-	-	0,57
	Tagetenone	5,16	-	-	-
	Camphor	0,53	42,97	15,71	0,79
	Carvone	-	-	12,4	-
	Fenchone	1,23	1,44	9,66	1,46
	Isophorone	-	-	0,01	0,1
	Myrcenone	3,23	-	-	-
	Pinocarvone	0,3	-	1,15	0,64
	Piperitenone	6,68	-	-	-
Ester	Bornyl acetate	-	-	0,09	0,42
Ether	Caryophyllene oxide	-	-	1,06	0,52
	cis-Linaloloxide	2,36	3,96	2,03	1,84
	Eucalyptol	-	10,86	20,41	34,1
	Longipinene epoxide	0,31	-	-	0,56
Hydrocarbon	(-)-Aristolene	-	-	0,14	0,28
	á-Copaene	-	-	3,4	0,05
	á-Myrcene	-	-	0,15	-
	à-Phellandrene	-	-	0,21	0,37
	a-Pinene	-	-	0,4	0,86
	Camphene	-	-	0,12	-
	Caryophyllene	4,3	10	9,31	10,16
	cis-Calaminene	-	-	0,09	0,09
	Eicosane	1,18	-	0,42	1,31
	Mesitylene	1,33	-	0,07	1,58
	o-Cymene	0,28	-	-	0,16
	p-Cymene	-	-	-	15,79
	Pentadecane	0,32	-	-	0,18
	R-Limonene	-	-	-	1,3
	Styrene	0,54	-	-	-
Non-terpenoid	Amphetamine	0,32	-	0,11	0,18
	cis-Myrtanylamine	-	-	0,24	-
	Fomepizole	-	0,69	-	0,86
	nor-Mephendrone	-	-	0,54	-
Phenol	(-)-Myrtenol	2,81	-	2,38	3,83
	(-)-trans-Isopiperitenol	0,54	-	0,29	0,48
	.tau.-Cadinol	-	-	0,39	0,18
	à-Cadinol	-	-	0,39	0,57
	cis-Verbanol	1,4	-	-	-
	endo-Borneol	-	3,45	3,71	2,35
	Fenchol	0,21	4	2,21	3,85
	Isoborneol	0,26	-	-	-
Total (%)		99.68	97,63	99,764	95,11

Essential oil components were grouped into three categories, according to Andogan *et al*. (2002) and Stintzing (2013), namely; terpenes, non-terpenoids and oxygenated compounds (Fig. 19). Terpenes are naturally occurring hydrocarbons that contain one or more carbon to carbon double bonds made up of isoprene units, while non-terpenoids are naturally occurring compounds made up of sulphur, nitrogen and lactones. Oxygenated compounds, on the other hand, are naturally occurring compounds containing oxygen in various functional groups. The essential oils of *L. javanica* were mostly made up of oxygenated compounds followed by terpenes and lastly, by non-terpenoids in all four treatments (Fig. 19). This is because oxygenated compounds and terpenes are responsible for the plant's antioxidant properties which aid in the adaptation to high temperatures. Non-terpenoids, on the other hand, are predominantly made of toxic compounds which contain halogen substituents. These compounds slightly increased under high temparatures in this study which raises the question of the quality of the oil secreted by the plant under high temperatures.

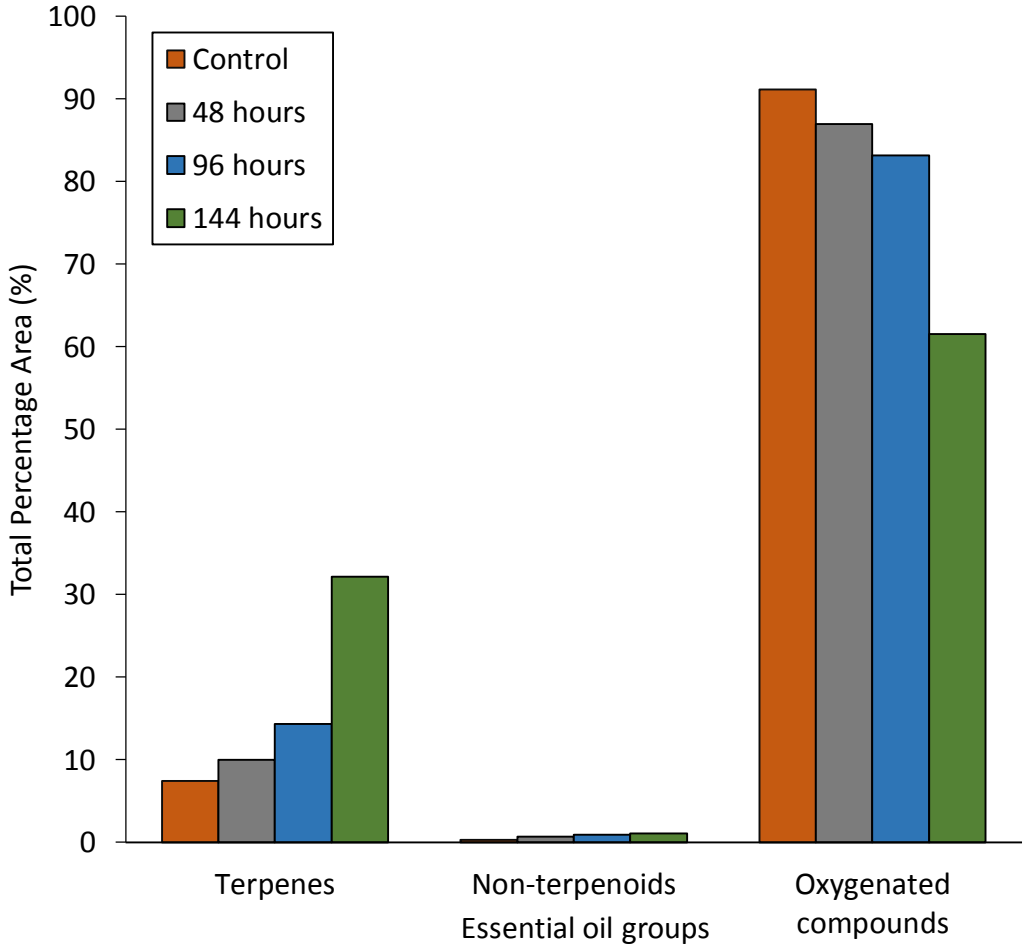


Figure 19. Average percentage area of grouped essential oil chemotypes of *Lippia javanica* under control (25/20 ºC) and exposed to high temperatures (47/37 ºC) for 48, 96 and 144 hours.

Oxygenated compounds were largely dominated by linalool, carvone, camphor and eucalyptol. Linalool is an alcohol commonly found in *L. javanica*'s essential oils and it is responsible for scent or fragrance and industries use it when formulating soaps, perfumes and lipsticks, among others (Peana *et al*., 2008; Lukwa *et al*., 2009). Carvone, on the other hand, is a ketone commonly used in garden insecticides and in the flavour industry for beverages, food and oral hygiene products (Sedlakova *et al*., 2001; Dionisio *et al*., 2012). Camphor is also a ketone and it is used for its scent (mosquito repellent), as an ingredient in cooking (mostly in india), and for embodiment formulation for treating different skin diseases (Lukwa *et al*., 2009; Hamidpour *et al*., 2013). Conversely, eucalyptol is an ether commonly found in eucalyptus and it is used as an insect repellent, incorporated in hygiene products, flavouring agent and treats bronchial and vascular diseases (Soares *et al*., 2005; Dionisio *et al*., 2012). These major oxygenated compounds depict the common uses of *L. javanica* by laypeople who may suggest that the biological activity of *L. javanica* remains the same in all the treatments and as a result, high temperatures may not affect the range of ailments generally treated by the plant. However, it is also possible that when major compounds are found in combination with certain minor compounds, changes in the biological activity of the medicinal plant may occur. This raises questions of the biological activity of *L. javanica* after exposure to high temperatures.

Although oxygenated compounds were dominant in all four treatments, they had an overall decreasing trend from 48 hours to 144 hours, with the control having the highest (Fig. 19). Contrarily, terpenes and non-terpenoids had an increasing trend from 48 hours to the 144 hours treatment, but they were significantly lower than the oxygenated compounds (Fig. 19). These suggest that oxygenated compounds are sensitive to high temperatures; thus, they evaporate, or the plant subtly reduces secreting the compounds when exposed to high temperatures. A study by Lukwa *et al*. (2009) found that *L. javanica* contains a high content of oxygenated compounds, which corresponds to the findings in this study. This is because oxygenated compounds are responsible for the antioxidant activity within medicinal plants; thus, many medicinal plants have high amounts of oxygenated compounds than any other group of compounds (Lukwa *et al*., 2009). The increased secretion of terpenes and non-terpenoids in this study may have contributed to the absorption of high temperatures and cooling down the leaf surface temperatures.

4.3.3 Total phenolic and flavonoid content

The total phenolic and flavonoid content of *L. javanica* showed an increasing trend from the control to the high temperature treatments (48 hours to 144 hours) ($F_{(1,46)}$ = 13.14; P = 0.000721) (Fig. 20). The total phenolic content of *L. javanica* was determined as a form of gallic acid equivalents (GAE). A one way ANOVA showed that the total phenolic content of the 144 hours treatment (441.94 mg GAE/ g) was significantly higher than the 96 hours (271.41 mg GAE/g), 48 hours (191.33 mg GAE/ g) and the control (144.75 mg GAE/ g) (Fig. 20). The incline in the secretion of total phenolics in *L. javanica* under high temperatures suggests that phenolic compounds similar to the essential oils were secreted as a defence mechanism against high temperatures in order to adapt under stress. Equally, studies conducted by Azhar *et al*. (2011) and Jaafar *et al*. (2012) found that the total phenolic content of *Trachyspermum ammi* and *Labisia pumila* increased significantly by 100% and 50% respectively under drought conditions. Another study conducted by Pascual *et al*. (2001) stated that *Lippia* species have an abundance of phenolic compounds that can largely be variable in constitution because of the range of environmental conditions that the plant survived in. Therefore, *L. javanica*'s phenolic compounds fluctuate in quantity depending on the environmental conditions exposed to the shrub in order to adapt to a vast range of environmental conditions. In this study, we can, therefore, suggest that *L. javanica* secreted high amounts of phenolics to acclimatise to the long duration of high temperatures.

Similarly, the total flavonoid content showed an increasing trend from the control to the high temperature treatments (48 hours, 96 hours and 144 hours). The total flavonoid content of *L. javanica* was determined as a form of quercetin equivalents. A one way ANOVA showed that the flavonoid compounds of the 144 hours treatment (268.66 mg quercetin/ g) were significantly higher than the 96 hours (178.56 mg quercetin/g), 48 hours (115.91 mg quercetin/ g) and the control (81.17 mg quercetin /g) (Fig. 20). Similarly, a study conducted by Nogues *et al.* (1998) found that the total flavonoid content of *Pisum sativum* increased by 45% after exposure to extreme drought conditions. This shows that *L. javanica* produced high amounts of flavonoids to enable acclimation under environmental stress and in this study, the shrub was able to adapt under high temperatures.

There was a significant difference between the total phenolic and flavonoid content per treatment. The total phenolic content of *L. javanica* was significantly higher than the total flavonoid content per treatment ($F_{(7,40)}$ = 1670; P < 0.0001). This is because flavonoids are a sub-group within phenolics, thus when phenolics increase, flavonoids also increase, but flavonoids are significantly less than phenolics (Cai *et al*., 2004; Abdeltaif *et al*., 2018). Photoreceptors are responsible for the biosynthesis of phenolics and flavonoids and are therefore responsible for protecting plants against biotic and abiotic stresses (Cai *et al*., 2004; Samanta *et al*., 2011). Phenolics and flavonoids play an important role in absorbing harmful UV radiation, absorbing high temperatures, the colouration of flowers and protecting plants from insects and microbes (Samanta *et al*., 2011; Abdeltaif *et al*., 2018; Alonso-Amelot *et al*., 2007). These phytochemicals filter severe sunlight radiation and absorb increased temperatures in order to protect tissues from damage and enable the acclimation of plants under extreme environmental stresses (Pascual *et al*., 2001).

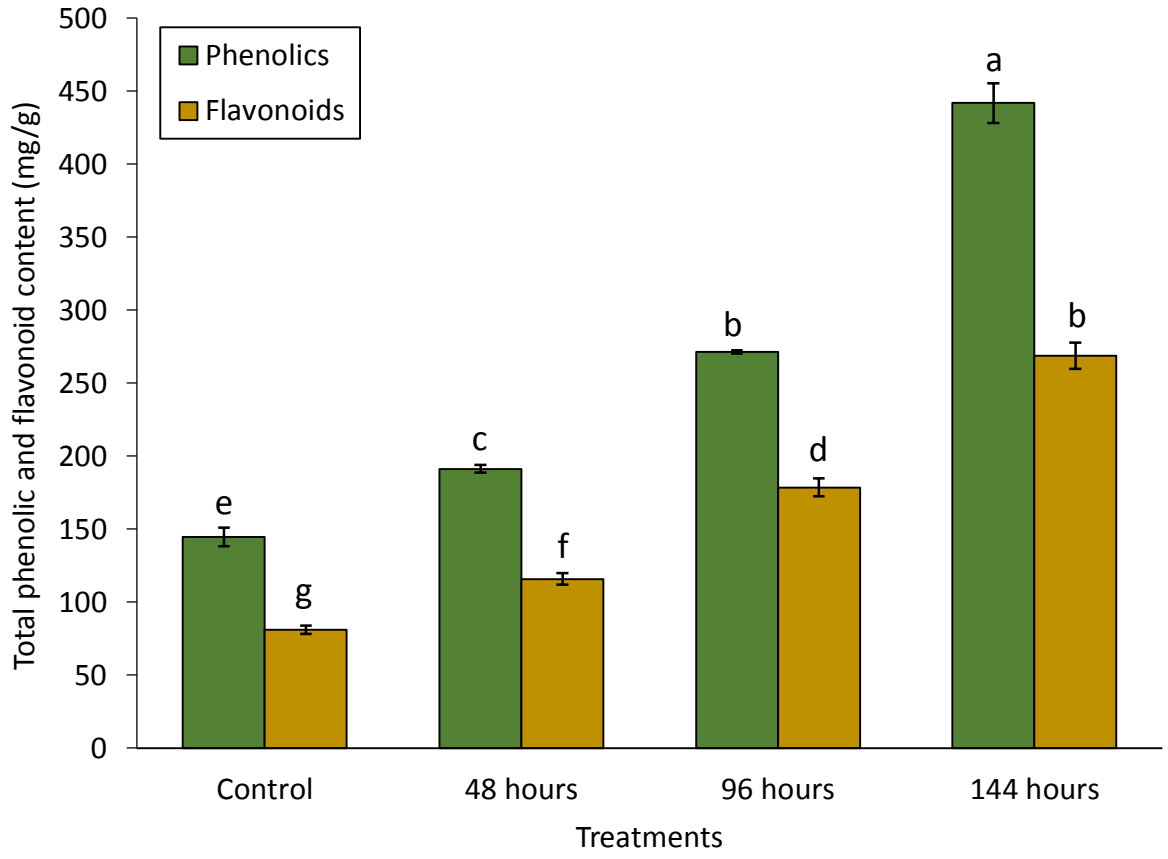


Figure 20. Total phenolic (mg GAE/ g) and total flavonoid content (mg quercetin/ g) of *Lippia javanica* exposed to ambient conditions (25/20 ºC) and high temperatures (47/37 ºC) for 48, 96 and 144 hours. Data represents mean ± standard deviation (n = 24). Means with different letters, $^{a\text{-}d}$, are significantly different (Tukey test; P < 0.05).

The present data shows that the total phenolic and flavonoid content increased after exposure to high temperatures. The increase in total phenolic and flavonoid content in *L. javanica* correlates to higher plant antioxidation, antimicrobial, anticancer, anti-inflammatory, antalgic and neuroprotection properties (Pakade *et al*., 2013; Swargiary *et al*., 2016). This suggests that the higher the phytochemical content found in *L. javanica* under high temperatures, the higher the resulting medicinal properties of the shrub. This would mean that *L. javanica* may be used to treat other ailments that were not previously known to be treated by the shrub and thus increasing the efficacy of the medicinal properties. This could be useful for commercial production, where high temperatures could be a valuable parameter to consider in order to attain maximum output of *L. javanica* products. However, further tests need to be done to verify the biological activity of these phytochemicals.

Given that temperatures are increasing at an alarming rate due to global warming which is as a result of climate change, the production of secondary metabolites in medicinal plants will invariably increase. The increase in essential oils, phenolics and flavonoids will enable plants to acclimatise by absorbing high temperatures, which cools down the leaf surface temperatures. The increase in secondary metabolites will also benefit humans as the biological activity of *L. javanica* increases. These high temperatures may cause extinction in the long run because not all plants may be able to adapt. Also, plants at different stages of life may not respond similarly i.e; mature plants may be able to survive better than juvenile plants, thus the possibility of local extinction. The laboratory conditions used in this study may not reflect the actual environmental conditions in the wild; however, farmers and industries can adopt the methods used in this study to enhance essential oil yield and total phenolic and flavonoid content of *L. javanica*.

4.4 Conclusion

Medicinal plant properties depend on the presence of a wide range of bioactive compounds such as essential oils and the total phenolic and flavonoid content. There is a need to increase these bioactive compounds as they are responsible for treating a wide range of ailments and are extensively used by industries, indigenous people and herbal/traditional healers. High temperatures in this study significantly increased the essential oils and total phenolic and flavonoid content as a defence mechanism against environmental stress. The increase in these

bioactive compounds provides resistance against high temperatures and other physiological and ecological pressures, including pathogens, insects and UV radiation. These properties further extend to humans who benefit from the increase in medicinal properties from phytochemicals. The increase in bioactive compounds may also suggest an increase in the biological activity of *L. javanica*. This could be exploited for financial gains via process optimization where industries and herbal/traditional healers maximize their products by harvesting when temperatures are high. Better products may also be achieved by growing *L. javanica* under high temperature laboratory conditions in order to get the required secondary metabolites. In the wild, this could suggest that in order to attain the maximum yield of phytochemicals, indigenous people and traditional healers should harvest *L. javanica* in summer when the temperatures are at the highest.

General conclusions and recommendations

The morphological, histological and phytochemical data obtained in this study showed that *L. javanica* was able to acclimatise under high temperatures. Histological assessments showed that samples of *L. javanica* under high temperatures modified their cellular structures by increasing the palisade and spongy mesophyll cells in order to decrease intercellular spaces, which then decreased the loss of water and also decreased the surface area for transpiration. Trichome numbers and height also increased significantly which contributed towards adaptation as these structures are not only known for their role in physically protecting the plant from biotic and abiotic stresses but are also involved in producing biologically active substances. Additionally, morphological assessments showed that *L. javanica* decreased water loss by undergoing wilting, rolling and curling which then decreased the leaf sizes. Phytochemical assessments, on the other hand, showed that the production of essential oils, phenolics and flavonoids increased significantly after exposing *L. javanica* to high temperatures. These modifications occurred to promote high temperature absorption and helped minimize water loss.

The dark stains that were found on the plant tissues suggested a high accumulation of phytochemicals. These compounds are of high economic value in formal and informal markets. Industries are always trying to find ways of increasing these phytochemicals, thus measuring other variables such as drought, soil nutrients and light may also be useful to determine the different factors that could potentially lead to a higher yield. High temperatures in this study proved to increase the secretion of phytochemicals, which could be used in industries to produce maximum products containing high phytochemicals. This, therefore, means that industries and farmers who harvest *L. javanica* for its medicinal properties should harvest the shrub during hot summers to acquire maximum essential oils, higher total phenolic and flavonoid content or other bioactive compounds. *L. javanica* extracts are an under-estimated economic resource that could be greatly utilised by industries and scientists as an alternative source of raw material and orthodox medicine.

More studies should be conducted to further investigate the pharmacological activities of *L. javanica* exposed to high temperatures in order to elucidate any changes in the healing properties and the range of ailments treated by the shrub. This will determine the

effectiveness of *L. javanica* in treating various ailments. There is a need to identify the active components in the extracted oils responsible for the different pharmacological activities displayed by *L. javanica* as it will assist in the oil therapeutic application. There is also a need for a detailed characterization of all the oil components as it will assist in improved chemotyping accuracy.

Future studies should aim on characterizing both pure and crude extracts to evaluate the possibility of developing nutraceutical products and commercialization based on the ethnopharmacological uses of *L. javanica* by different cultures. The toxicity levels also need to be studied to determine any possible toxins secreted under high temperatures. Little or no studies have focused on the stems or any other part of the plants except for the leaves (Maroyi, 2006). Consequently, studies should be conducted on the different plant parts such as roots, flowers and stems, as well as the effects of seasonal variations on the phytochemical content and biological activity. There is also a need to conduct in vivo experiments to prove the validity of the existing ethnopharmacological activities. However, *L. javanica* is known to contain potentially toxic compounds, therefore, the levels of toxicity need to be determined through quality control of developing products for toxic compounds to be retained under tolerance levels. *L. javanica* is an under-estimated indigenous plant that could generate foreign revenue and could be a source of income for many.

Appendix 1

Light microscopy fixation and staining

0.1 M Sodium Phosphate buffer

Solution 1- Sodium hydroxide (NaOH): 0.88g.100ml^{-1}

Solution 2- Sodium dihydrogen orthophosphate dehydrate (NaH_2PO_4): 2.4g.100ml^{-1}

To make 0.1 M Sodium Phosphate Buffer solution, mix 35ml of solution 1 with 50ml of solution 2. Then top up the mixture with distilled water to make up to 100ml.

Glutaraldehyde Fixative (3% v/v)

2ml of 25% glutaraldehyde solution is diluted with 88ml of the sodium phosphate buffer.

The solution is then poured into small bottles with the specimen and refrigerated overnight. This is done to fix sample.

Osmium tetroxide (OsO_4) (2%)

Dilute one vial of osmium oxide with an equal amount of 0.1 M sodium phosphate buffer in the fume cupboard. The specimen is then covered with OsO_4/buffer in the cupboard and is left for one hour.

Uranyl acetate (UA) (1% w/v)

0.25g of uranyl acetate is dissolved in 25ml of 75% ethanol. The solution is light-sensitive thus, the bottle should be covered with foil. This solution should be prepared immediately prior to being used.

Epoxy resin

Components must be at room temperature for use.

Vinylcyclohexene dioxide (VCD) (Resin): 23g

Nonelyl succinic anhydride (NSA) (Hardener): 62g

DER 736 (Plasticiser): 14g

Accelerator- dimethylaminoethanol (DMAE): 1g (must be the last component to be added after the mixture of the above listed).

Fixing and embedding procedure:

- Place samples in Glutaraldehyde fixative overnight
- Change from glutaraldehyde to the buffer, making one double wash followed by five further changes, 15 minutes each.
- Change to Osmium oxide and keep in the cupboard for one hour
- Make uranyl acetate
- Change from osmium oxide to buffer. Change the buffer, making two double washes over half an hour
- Prepare alcohol series: 10%, 25%, 50%, 75% and 100% ethanol
- Change to 10% then to 25% and 50% ethanol after 15min in each solution
- Replace the 50% ethanol with uranyl acetate solution and wrap the bottle with foil keeping it in the fridge for an hour
- Change uranyl acetate to 75% ethanol making two double washes (10min each)
- Change 75% ethanol to 100% ethanol making two changes (10min each)
- Remove 100% ethanol and replace with two changes of propylene oxide (10min each)
- After the second change of propylene, add equal amounts of propylene oxide and resin mixture, leaving it for five hours
- Change to whole resin and leave for overnight
- Remove whole resin and place samples into a resin tray. Fill the compartments where samples are placed with fresh resin.
- Incubate in the oven at 70 degrees Celsius for 8-12hours

Appendix 2

Chromatograms of *Lippia javanica*'s essential oils (Methanol)

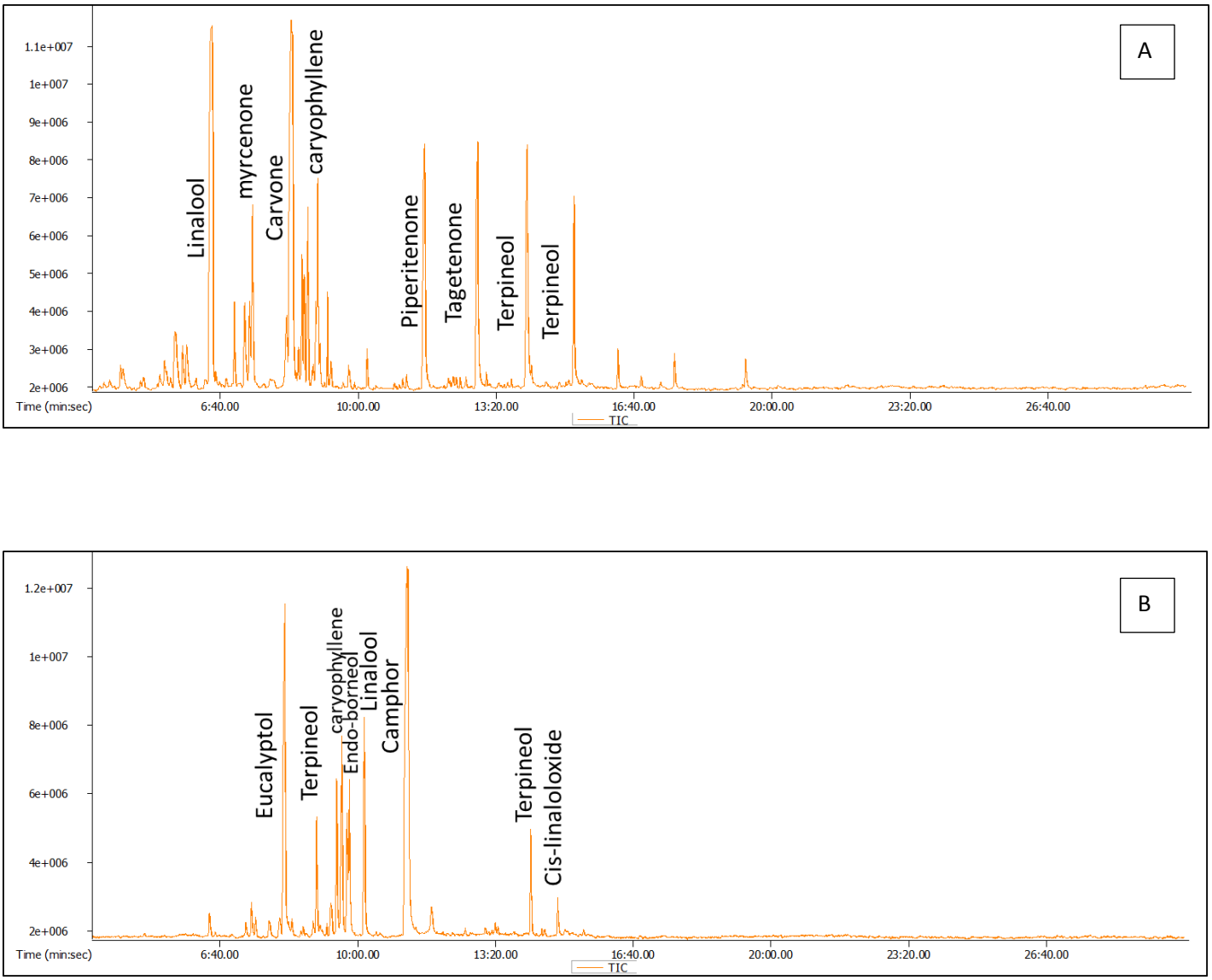
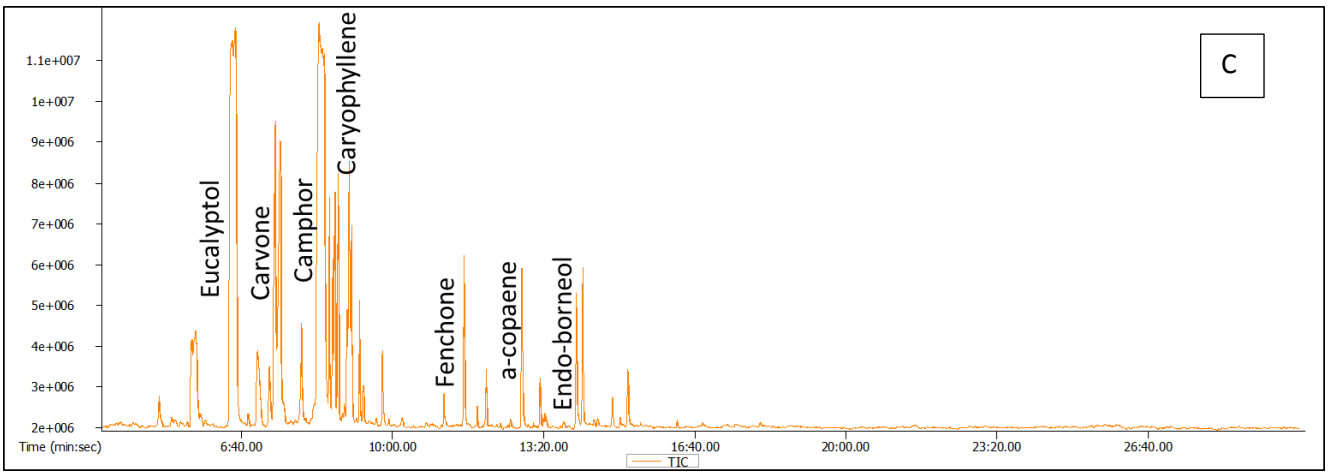


Figure 2.1 Chromatogram of *Lippia javanica*'s essential oils under ambient conditions (control) (A) and after 48 hours of exposure to high temperatures (B).



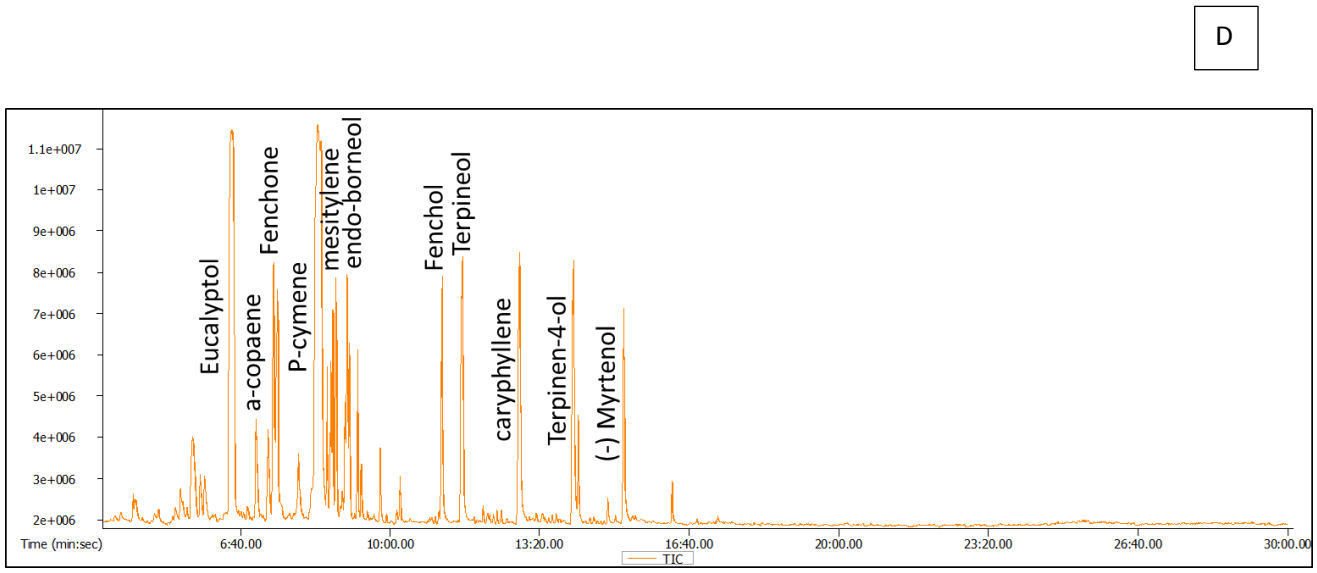
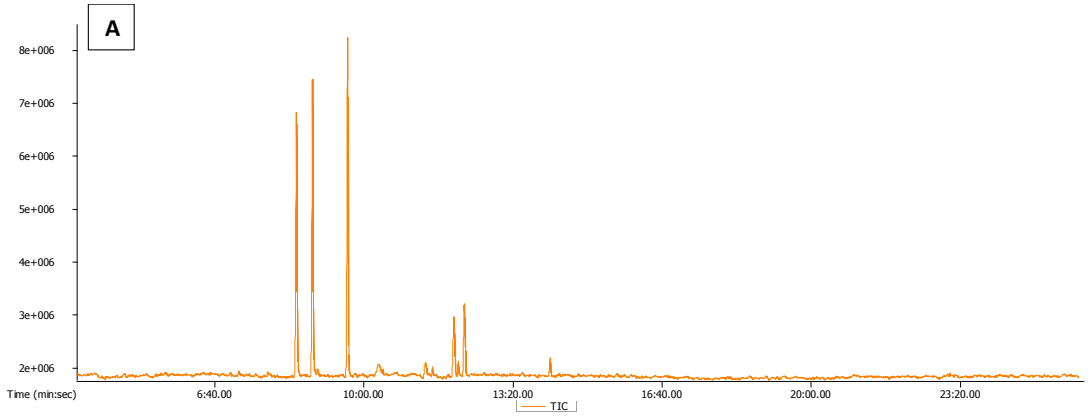
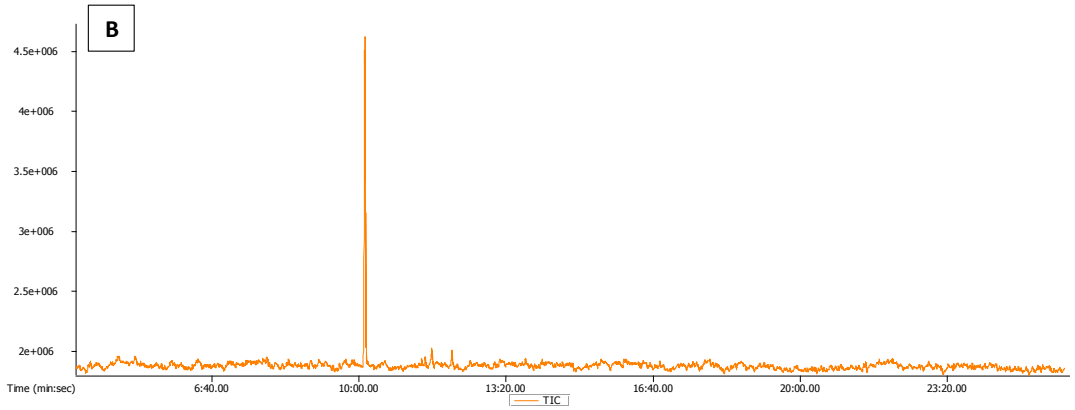


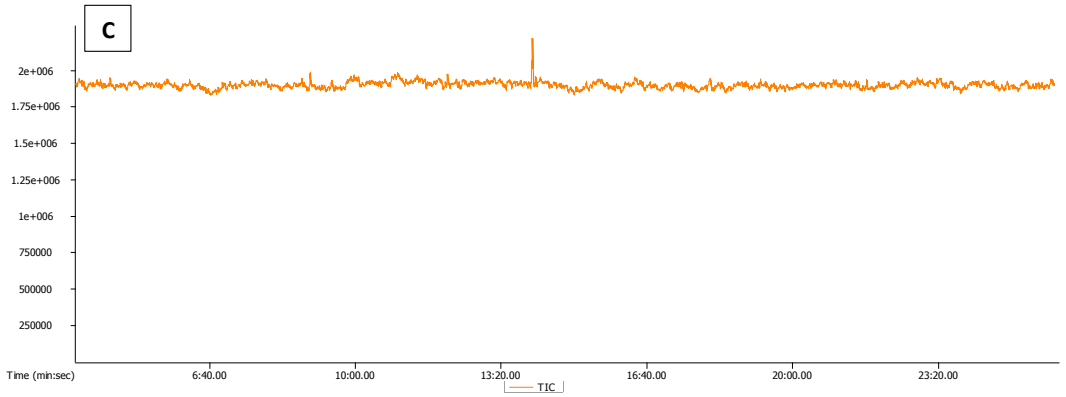
Figure 2.2 Chromatogram of *Lippia javanica*'s essential oils after 96 hours (C) and 144 hours (D) of exposure to high temperatures.

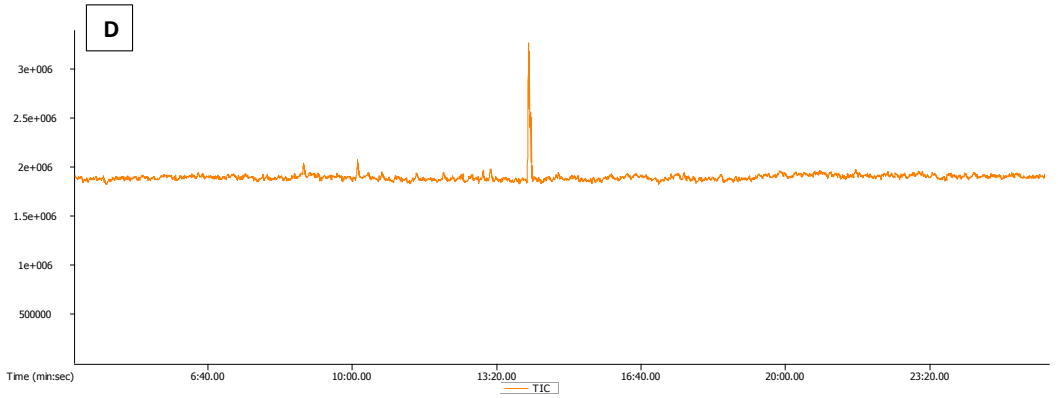
Appendix 3

Chromatograms of *Lippia javanica*'s essential oils (Hexane)


A
8e+006
7e+006
6e+006
5e+006
4e+006
3e+006
2e+006
Time (min:sec)
6:40.00
10:00.00
13:20.00
16:40.00
20:00.00
23:20.00
TIC


B
4.5e+006
4e+006
3.5e+006
3e+006
2.5e+006
2e+006
Time (min:sec)
6:40.00
10:00.00
13:20.00
16:40.00
20:00.00
23:20.00
TIC


C
2e+006
1.75e+006
1.5e+006
1.25e+006
1e+006
750000
500000
250000
Time (min:sec)
6:40.00
10:00.00
13:20.00
16:40.00
20:00.00
23:20.00
TIC


D
3e+006
2.5e+006
2e+006
1.5e+006
1e+006
500000
Time (min:sec)
6:40.00
10:00.00
13:20.00
16:40.00
20:00.00
23:20.00
TIC

Appendix 4

Gallic acid and Quercetin calibration curves

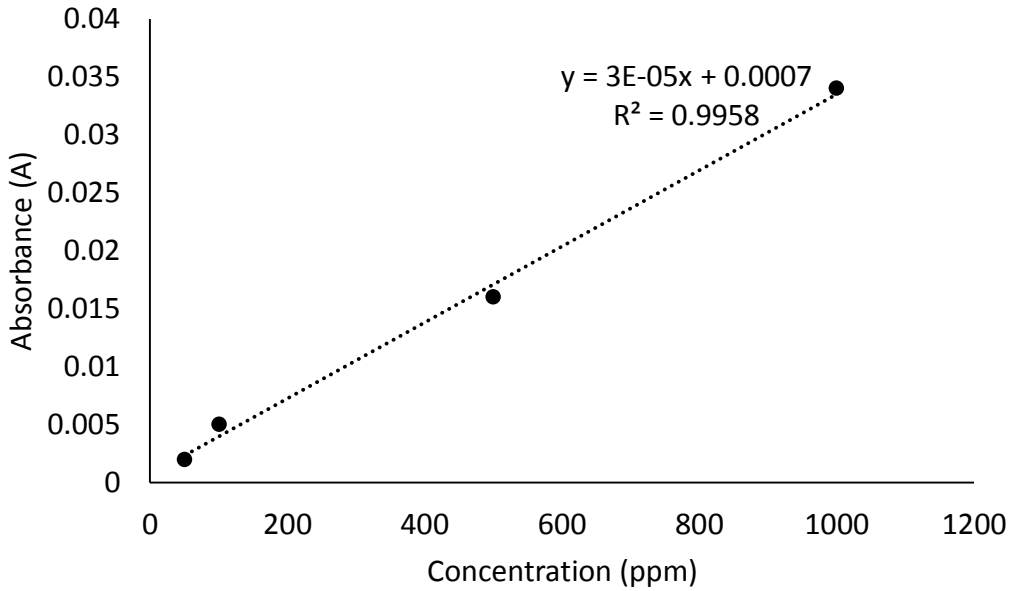


Figure 4.1 Gallic acid standard calibration curve.

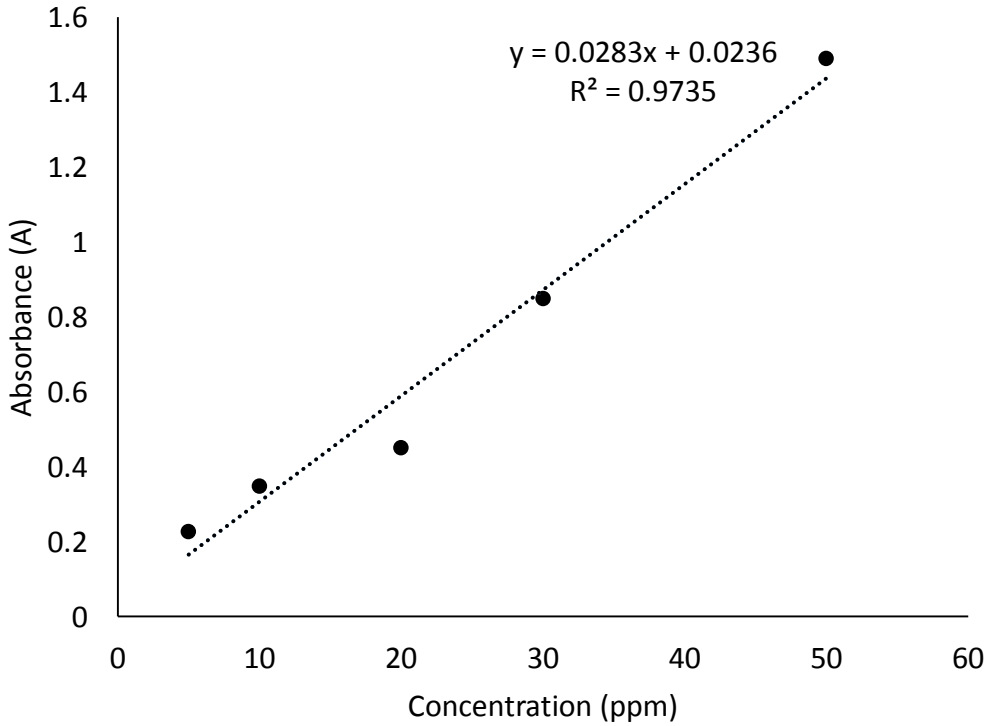


Figure 4.2 Quercetin standard calibration curve.

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