School of Biosciences

Division of Food Sciences

EVALUATION OF *MOMORDICA BALSAMINA* AND *MOMORDICA FOETIDA* FROM SWAZILAND FOR THEIR ANTIMICROBIAL ACTIVITY, ANTI-PROLIFERATIVE PROPERTIES AND BIOCHEMICAL COMPOSITION

Thabile Precious Nkambule, BSc., MSc.

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ABSTRACT

Momordica balsamina (*inkakha*) and *M. foetida* (*inshubaba*) are used in several countries, including Swaziland, both for food and as a traditional medicine to treat and cure several diseases. Systematically investigating their bioactivities and identifying the compounds responsible for the curative properties could greatly extend the therapeutic applications of these plant species. The aim of the present study was to investigate antimicrobial, anti-proliferative activities and biochemical composition of extracts of *Momordica* plants.

Methanol-water extracts of *M. balsamina* and *M. foetida* leaf, stem and fruit were screened for antimicrobial activity against a range of bacteria. Gram-positive bacteria were found to be the most sensitive to *Momordica* extracts particularly leaf extracts. The lowest minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were from leaf extracts of *M. balsamina* and MBC was determined to be 2.5 mg/mL against *L. monocytogenes*. Following fractionation of *Momordica* extracts using solid phase extraction (SPE) the methanol eluted fraction showed antimicrobial activity against *L. monocytogenes* and *S. aureus* strains which was comparable to the antimicrobial activity of crude extracts.

The anti-proliferative activity of methanol-water extracts, of *M. balsamina* and *M. foetida* leaf, was tested against human liver hepatocellular carcinoma (HepG2) cells. Leaf extracts from both *M. balsamina* and *M. foetida* displayed anti-proliferative activity at concentrations of 0.25 and 1mg/mL, respectively. Biochemical analysis of the extracts was carried out through Fourier transform

infrared spectroscopy, high performance liquid chromatography, nuclear magnetic resonance and liquid chromatography-mass spectrometry analysis. This study demonstrated the highest total phenolic content in leaf extracts with a range of 34.39 to 47.94 mg GAE/g, followed by stem extracts with a range of 21.02 to 26.19 mg GAE/g and least by fruit extracts with a range of 3.97 to 12.97 mg GAE/g. A good correlation was revealed between the antimicrobial activity of *Momordica extracts* and total phenolic content suggesting that phenolic content could be partly responsible for the antimicrobial activity of the extracts, but this remains to be confirmed.

These results could indicate the potential use of *Momordica* plants as antimicrobial agents as well as anti-cancer agents and also provide the basis for the isolation and identification of the biologically active substances in these extracts.

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CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction

Food-poisoning micro-organisms that contaminate foods and cause illness by infection and by forming toxins are of major concern (Roller 1991). The Centre for Science in the Public Interest (CSPI) reported food-borne diseases in the African region, including Swaziland, as being associated with microorganisms such as *Salmonella* Typhimurium, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* (DeWaal and Robert 2005). These conditions were stated to be linked to poverty resulting in consumption of unsafe food, lack of access to clean water, weak government structures, population growth and rise of HIV/AIDS and other communicable diseases. As interventions, synthetic chemicals are often used as preservatives in food processing and food storage in order to inhibit food-borne pathogens and to extend shelf life (Shetty and Labbe 1998; Lu *et al.* 2011). Antibiotic drugs are also used to treat humans with pathogenic illnesses. These interventions can result in allergic reactions to the antibiotics in humans (Friedman *et al.* 2002) as well as an increase in resistance of some pathogens to these antibiotics (O'Brien 1987; Friedman *et al.*2002; Levy and Marshal 2004). The use of antibiotics such as penicillin has been linked to hypersensitivity reaction (Smith *et al.* 1966; Lin 1992). Neonatal antibiotic treatment with select treatment such as vancomycin has been reported to alter the gut thus enhancing susceptibility to allergic asthma (Russell *et al.* 2012).

In Swaziland about 85% of the population is mainly dependant on medicinal plants to meet primary health care needs (Amusan 2009; Nguta *et al*. 2011)

due to the cost of antibiotics and hospital charges. Increased consumer awareness of potential health risks associated with synthetic food additives has also renewed interest in naturally occurring alternatives (Michaelidou and Hassan 2008).

This trend has led to increased research on natural preservatives (Cuvelier *et al.* 1994). Food preservatives derived from plants are of growing interest (Serra *et al.* 2008; Lou *et al.* 2010) as plant compounds often possess antimicrobial properties as part of the plant's defence mechanism against infections (Lou *et al.* 2010) and may have other applications. The food industry thus has increased interest in extracts of fruits, herbs, vegetables, cereals, and other plant materials because of their potential preservative effects and also their potential ability to retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Javanmardi *et al.* 2003). Consumer pressure is forcing many food companies to reconsider and reduce the levels of usage of certain food additives; the need for effective food preservatives is thus greater than ever and is fuelling a renewed interest in microbial inhibitors (Roller 1991). Plants are the richest bio-resource for drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer *et al.* 1999). Medicinal plants have been used for centuries as remedies for human diseases and offer a new source of biologically active chemical compounds for use as antimicrobial agents (Das *et al.* 2010). Edible and medicinal plants have many naturally occurring compounds and these have been shown to display antimicrobial activities against food spoilage microorganisms and food-borne pathogens. These

phytochemicals must be exploited for food preservation and for improving food safety (Shetty and Labbe 1998).

Momordica belongs to the *Cucurbitaceae* family and is native to the Paleotropics (mainly Africa) (Jeffrey 1980; Robinson and Decker-Walters 1997). The *Cucurbitaceae* family comprises more than 40 species, the majority of which are African (Robinson and Decker-Walters 1997; Bosch 2004a). There are three reported *Momordica* species in Swaziland, *Momordica balsamina*, *Momordica foetida* and *Momordica cardiospermoides* (Long 2005). However, *M. cardiospermoides* is a rare species and thus not widely seen or used. *Momordica* are harvested from the wild and are used both for food and as traditional medicine. Leaves are often cooked and consumed as a relish. To reduce their bitterness, leaves are usually mixed with other vegetables such as spinach and amaranth. They are also added to other dishes such as soups and meat. The antimicrobial properties of Swazi species of *Momordica* plants have not been studied to date. Some reports still rely on traditional use (Amusan *et al.* 2005, 2007; Amusan 2009; Dlamini and Geldenhuys 2011) yet others focus on nutritional value (*M. involucrata*) (Ogle and Grivetti 1985; Bwembya *et al.* 2008). With more information on the attributes of these indigenous vegetables, the potential market value would increase (Aphane *et al.* 2002). There is a clear gap between traditional and scientific knowledge and this signifies the importance of this study. Thus this study is aimed at evaluating some bioactivities of *Momordica* plant extracts. The hypothesis is that *Momordica* species found in Swaziland have bioactive properties which could be used as a natural preservative to control food-borne pathogens and as anti-cancer agents.

1.2 Description and morphology of *M. balsamina* and *M. foetida*

1.2.1 Description

***Momordica balsamina* (*Inkakha*)**

Kingdom – *Plantae*

Superdivision – *Spermatophyta* (Seed plants)

Division – *Magnoliophyta* (Flowering plants)

Class – *Magnoliopsida* (Dicotyledons)

Order - *Cucurbitales*

Family – *Cucurbitaceae* (Cucumber family)

Sub family - *Cucurbitoideae*

Genus – *Momordica*

Species – *balsamina* Linn. www.plants.usda.gov

***Momordica foetida* (*Inshubaba*)**

Kingdom – *Plantae*

Superdivision – *Spermatophyta* (Seed plants)

Division – *Magnoliophyta* (Flowering plants)

Class – *Magnoliopsida* (Dicotyledons)

Order - *Cucurbitales*

Family – *Cucurbitaceae* (Cucumber family)

Sub family - *Cucurbitoideae*

Genus – *Momordica*

Species – *foetida* Schumach. www.oel.org

1.2.2 Morphology

Momordica balsamina is a monoecious, annual or short-lived perennial herb, trailing or climbing with simple tendrils; the stem grows up to 1.5 m long. Leaves (Figure 1-1) are alternate, simple; stipules absent; petiole 0.5–6 cm long, pubescent; blade broadly ovate to orbicular, 1–9 cm × 1–12 cm, deeply palmately 5–7 -lobed, lobes 3–5-lobulate. Flowers are solitary, unisexual, regular, 5-merous; male flowers with pedicel 1.5–10 cm long, receptacle 2–4.5 mm long, sepals 0.5–1 cm long, petals 1–2 cm long, pale yellow, cream or white, and 3 free stamens; female flowers with pedicel up to 0.5 cm long, receptacle 0.5–1 mm long, sepals narrow, up to 0.5 cm long, petals 0.5–1.5 cm long, and inferior, 1-celled ovary (Bosch 2004a). Immature fruit are green (Figure 1-1), ovoid, 2.5-7.5 cm long; narrow at both ends, rostrate, fleshy, smooth or muricate and bitter in taste (Faujdar *et al.* 2013). Mature fruit are a red, berry type and tuberculate in shape, dehiscing with 3 valves, exposing the many seeds embedded in a red pulp (Bosch 2004a; Faujdar *et al.* 2013). Seeds (Figure 1-1) are ash coloured, ellipsoid, compressed, rugulose on the flat faces and with a grooved margin which is tuberculate on the edges (Bosch 2004a; Faujdar *et al.* 2013). Seeds are ovate, compressed, 9–12 mm long (Bosch 2004a).

Where *Momordica balsamina* and *Momordica charantia* L. both occur, they are not clearly distinguished by the local population. Hence, many vernacular names apply to both (Bosch 2004a). Interspecific taxonomy of *Momordica* genus is not clear, as a result botanical names and common names are often confused and are found to be used incorrectly or interchangeably (Schafer 2005; Joseph John *et al.* 2007).



Figure 1-1. *M. balsamina* leaves, fruit and seeds.

Momordica foetida is a dioecious, perennial herb, nettle, creeping or climbing tendrils simple or bifid; stem grows up to 4.5 m long with dark green spots when young, woody when old, rooting at nodes (Bosch 2004b; Behera *et al.* 2011). Leaves (Figure 1-2) are alternate, simple, stipulates absent petiole 1.5 to 17 cm long, blade broadly ovate-cordate to triangular-cordate. Flowers are unisexual, regular, 5-merous, calyx tube and lobes obconical up to 11 mm long, petals free, obovate-lingula, up to 3.5 cm long, white, pale yellow to orange yellow, with three scales to inside base, male flowers in fascicles from 1-9 on a peduncle 2-23 cm long, with three stamens, anthers coherent in the centre of the flower, female flowers solitary in the axils of leaves, ovary inferior, ovoid, stigma 3-lobed (Bosch 2004b). The fruit (Figure 1-2) is a long stalked ellipsoid berry with densely soft spiny exocarp, 7x5cm (Bosch 2004b; Behera *et al.* 2011), orange when ripe, densely covered with soft spines, dehiscing with 3 valves and exposing the many seeds embedded in red pulp. Seeds (Figure 1-2) are oblong, flattened, about 1 cm long, having a brown

coat, sculpted edges with 2 flutes (Bosch 2004b). It is widely distributed in tropical Africa and occasionally cultivated in its native area for leaves, and tender fruits are used as a vegetable and also in traditional medicine (Behera *et al.* 2011).



Figure 1-2. *M. foetida* leaves, fruit and seeds

1.3 Uses of Momordica plants in Swaziland

M. balsamina and *M. foetida* are used in Swaziland for both food and traditional medicine. Dlamini and Geldenhuys (2011) confirmed that in Swaziland *Momordica involucrata, Momordica clematidea* together with *S. birrea* and *Psidium guajava* are indigenous and naturalized species that are harvested for both food and medicinal uses. Traditional vegetables such as

inkhakha (*Momordica balsamina*) and *inshubaba* (*Momordica foetida*) have been known to combat hypertension and diabetes (Masarirambi *et al.* 2010). These vegetables are often recommended to patients with HIV/AIDS and bacterial diseases such as boils and other skin infections. Medicinal reasons seem to be dominating reasons why the Swazi community are still consuming these vegetables (Dube and Musi 2002) as they are bitter in taste which makes them less favourable.

M. balsamina is an African plant which occurs throughout the South of Sahara. *M. balsamina* has its distribution extending to western India through West Asia (Behera *et al.* 2011). *M. balsamina* is also found in Yemen and Australia (Bosch 2004a). *M. balsamina* is also referred to as 'a hidden gift of nature', as it is considered to be an important medicinal and nutritional plant (Thakur *et al.* 2009) even though Puri (2010) seems to suggest that calling it 'a hidden gift of nature' is inappropriate as there is limited research to make such a conclusion. Young leaves of *M. balsamina* are also frequently cooked and eaten as a vegetable in Cameroon and Sudan (Bosch 2004a). *M. balsamina* leaves are among the most commonly occurring and used greens in home gardens in some parts of South Africa and are usually available most of the year however because of their taste they are the least favoured (High and Shackleton 2000). The bitter young fruits have been reported widely as being edible. In other countries such as India it has been reported that the green fruit are consumed. The bright red fruit pulp of *M. balsamina* is eaten in Namibia (Bosch 2004). The ripe fruit however, has been reported to cause vomiting and diarrhoea, and can be poisonous. It is used for colic, as an emetic and drastic

purgative (Robinson and Decker-Walters 1997). Ripe fruits are also reported as possible causes for the death of dogs and pigs (Bosch 2004a).

M. balsamina fruit is used in traditional medicine in Swaziland as a purgative, emetic, antispasmodic and for promoting digestion (Long 2005). Baartjies (2014) recommended the use of *M. balsamina* (*inkhakha*) in Swaziland for the prevention of constipation and piles. This report recommended that the *inkhakha* leaves be boiled in milk and the mixture should be taken by mouth every morning on an empty stomach, 30 minutes prior to meal consumption, for a month. This report also advised diluting the *inkhakha* juice with honey and grated onions to help fight and improve sore throat, bronchitis and asthma.

M. balsamina have been generally used in traditional medicine as an anthelminthic, against fever and extreme urine bleeding, to treat syphilis, rheumatism, hepatitis and skin disorders, stomach and intestinal complaints (Hutchings *et al.* 1996; Bosch 2004a). *M. balsamina* has also been used as an abortifacient, aphrodisiac and lactogenic, and in treating diabetes (Bosch 2004a).

In recent years, *M. balsamina* has gained popularity as more research has focused on the plant and its properties. Different parts (leaves, stem, fruits and seeds) of *M. balsamina* have been reported to have many pharmacological activities (Behera *et al.* 2011). Stem-bark extract has shown hypoglycaemic properties (Geidam *et al.* 2007). Stem and leaf extracts in South Africa demonstrated some antidiabetic properties (van de Venter *et al.* 2008). Seed extracts of *M. balsamina* in India have also demonstrated a significant anti-hyperglycaemic potential (Kalia *et al.* 2010). Aqueous extracts of fruit pulp

have been shown to increase CD4+ lymphocytes count significantly (Bot *et al.* 2007). Karumi *et al.* (2003) conducted a study which established the analgesic and anti-inflammatory effect of an aqueous leaf extract in mice. Wound healing properties in albino rats have been reported (Ilango *et al.* 2010). Antimalarial activity has been displayed by *M. balsamina* whole plant when used alone or in a traditional mixture in Niger (Benoit-Vical *et al.* 2006). Odhav *et al.* (2007) reported antioxidant activity of *M. balsamina* leaves from South Africa. Otimenyin *et al.* (2008a) established antidiarrhoeal properties of *M. balsamina* leaves and stem extracts from Nigeria. Several antimicrobial studies of *M. balsamina* extracts have reported activity against different bacterial species (Table 1-1).

M. foetida is widespread in tropical Africa and South Africa. Leaves are also harvested from the wild in Gabon, Sudan, Uganda, Tanzania and Malawi and consumed as a vegetable after being boiled (Olaniyi 1975; Bosch 2004b). These plants seem less popular and are thus usually consumed in smaller amounts and usually in times of food scarcity. Currently, the main interest in *M. foetida* is in its medicinal aspects (Bosch 2004b; Oloyede and Aluko 2012). *M. foetida* is used as a traditional medicine in Swaziland to combat stomach problems, boils and skin diseases and high blood pressure (Long, 2005). *M. foetida* has been generally used in traditional medicine to cure stomach ache, other intestinal disorders, for headaches, earache, toothache and as an antidote for snakebites (Bosch 2004b; Behera *et al.* 2011), skin problems and boils, and for the treatment of malaria (Waako *et al.* 2005; Bosch 2004b).

Table 1-1. Previous research on antimicrobial activity of *M. balsamina*

Plant part(s) studied	Solvents used	Origin	Bacteria studied	Antimicrobial activity	Reference(s)
Leaves	Aqueous	Nigeria	Multi-drug resistant *Shigella* species	Shigellocidal potential with inhibition zones ranging from 5.8 to 9.5 mm, an MIC range of 543.8-937.5 µg/mL and a MBC range of 1031.3-1312.5µg/mL	Iwalokun *et al.* 2001
Whole plant	Methanol	Nigeria	*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella* Typhi	Antibacterial activity against all test bacteria	Jigam *et al.* 2004
Whole plant	Aqueous and ethanol	Nigeria	Multi-drug resistant *Salmonella* Typhi	Both extracts were effective	Akinyemi *et al.* 2005
Leaves and stem	Methanolic and water	Nigeria	*Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella* Typhi, *Proteus vulgaris* and *Pseudomonas aeruginosa*	More activity (inhibition zone) observed in methanol extracts against *E. coli*, *B. subtilis* and *P. vulgaris*	Otimenyin *et al.* 2008b

Table 1-1. Previous research on antimicrobial activity of *M. balsamina* (continued)

Plant part(s) studied	Solvents used	Origin	Bacteria studied	Antimicrobial activity	Reference(s)
Leaves	Petroleum ether, dichloromethane, ethanol (80%) and water	South Africa	*Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.	Ethanol extracts against: *B. subtilis* (MIC – 0.78 mg/mL) *E. coli* (MIC – 1.56 mg/mL) *K. pneumoniae* (MIC – 1.56 mg/mL) *S. aureus* (MIC – 3.125 mg/mL)	Ndhlala *et al.* 2011
Six compounds (triterpernoids) isolated from the aerial parts	Chloroform, methanol, ethanol, aqueous Dissolved in Dimethyl sulfoxide	Mozambique	Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis* Vancomycin resistant strains	Isolated compounds (balsaminol A, balsaminol F, balsaminagenin A, balsaminagenin B, balsaminoside A and karavilagenin C) showed activity against efflux pump systems of Gram-positive bacteria with the MIC range of 25µM to 200 µM against MRSA COL_{OXA} and 100 µM to ≤ 200 µM against *E. faecalis*. Ethanol extracts gave a higher activity followed by methanol, chloroform extracts and the least activity was shown by aqueous extracts	Ramalhete *et al.* 2011

Table 1-1. Previous research on antimicrobial activity of *M. balsamina* (continued)

Plant part(s) studied	Solvents used	Origin	Bacteria studied	Antimicrobial activity	Reference(s)
Aerial parts	Hexane, dichloromethane, ethyl acetate and methanol	Mozambique	*Staphylococcus aureus*, *Enterococcus faecalis* (Gram positive bacteria), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (Gram negative bacteria) and *Mycobacterium smegmatis*	All extracts showed activity against all the bacteria studied with MIC values within the range of 7.5 µg/mL to 250 µg/mL.	Madureira *et al.* 2012

M. foetida is also used by traditional medicine practitioners in the treatment of TB in the form of decoctions and infusions (Tabuti *et al.* 2010). In Uganda the leaves of *M. foetida* are used in the treatment of stomach ache (Hamill *et al.* 2000) and wounds, while the *M. foetida* root is used for the treatment of teething syndrome (Ocheng *et al.* 2014). Other uses include as an aphrodisiac and abortification (Bosch 2004b).

M. foetida has received attention in research focusing on antidiabetic screening of plant extracts from South Africa (van de Venter *et al.* 2008) and West Africa (Baily and Day 1989), effect on blood glucose of a constituent foetidin from *M. foetida* in Nigeria (Marquis *et al.* 1977), antimalaria screening in Uganda and Mozambique (Waako *et al.* 2005; Froelich *et al.* 2007; Tabuti 2008 and Ramalhete *et al.* 2008), TB treatment in Uganda (Tabuti *et al.* 2010), antioxidant activity (Oleyede and Aluko 2012, Acquaviva *et al.* 2013) in Nigeria and Eastern Democratic Republic of Congo, and potential laxative in Nigeria (Odusote and Awaraka 2004). Several antimicrobial studies on *M. foetida* extracts have displayed activity against different bacterial species (Table 1-2).

It is evident from the results shown by the cited studies (Table 1-1 and Table 1-2) that there is a great variation in antimicrobial activity of *Momordica balsamina* and *Momordica foetida* plant extracts and this may be due to several factors including plant part, solvent, extraction method, concentration of extract and plant source. Zaika (1988) summarized some factors that may affect antimicrobial activity of plants. Microorganisms differ in their resistance to a given plant extract, and a given microorganism differs in its resistance to extracts from many types of plants. Generally, bacteria are more

resistant to antimicrobials than fungi. Furthermore, antimicrobial action on spores could be different from that of vegetative cells. Gram-negative bacteria are more resistant than Gram-positive bacteria. The effect of a plant extract may either be inhibitory or germicidal. Plants harbour microbial contaminants, and may serve as substrates for microbial growth and toxin production. The concentration of extract might be too low to prevent spoilage by microorganisms. Active components of plant extracts at low concentrations may interact synergistically with other compounds (NaCl, acids, and preservatives) to increase preservation. Nutrients present in plants may stimulate growth and/or biochemical activities of microorganisms. Lastly, the extent to which plant extracts inhibit microbial growth depends on their source and processing.

Medicinal properties change according to the environment in which the plant is found. Climate and soil play an important role in the concentration of the plant's active ingredients and hence medicinal properties (Geidam *et al.* 2007). Different species of *M. balsamina* and *M. foetida* will therefore exhibit particular properties depending on where they are found. For these reasons effectiveness of *Momordica* plants from area to area will differ. Currently, in Swaziland there have been no studies focusing on antimicrobial properties of these particular *Momordica* plants. There is a clear gap that exists between traditional and scientific knowledge and this signifies the importance of this study.

Table 1-2. Previous research on antimicrobial activity of *M. foetida*

Plant part(s) studied	Solvents used	Origin	Bacteria studied	Antimicrobial activity	Reference(s)
Leaves and stem	Methanol	Rwanda	*Bacillus subtilis* (ATCC 6633), *Candida albicans* (ATCC 10231), *Mycobacterium smegmatis* (ATCC 607), *Pseudomonas aeruginosa* (ATCG 15442), *Salmonella* Gallinarum (ATCC 9184) and *Staphylococcus aureus* (ATCC 13709).	Leaf extracts had activity against *Bacillus subtilis*, *Candida albicans*, *Mycobacterium smegmatis* and *Staphylococcus aureus*, while the stem extracts had activity against *B.subtilis* and *M. smegmatis* only	Boily and Van Puyvelde (1986)
Leaves and stem	Methanol	Uganda	*Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853; *Staphylococcus aureus* ATCC 29213; *Enterococcus faecalis* ATCC 29212; *Candida albicans* ATCC 14053; *Staphylococcus aureus* NCRL; *Staphylococcus epidermidis* NCRL; *Bacillus subtilis* NCRL).	Poor activity against *Candida albicans*, moderate activity against *S. aureus* ATCC NCRL and *S. epidermidis* NCR, and good activity against *S. aureus* ATCC 29213.	Hamill *et al.* (2003)

Table 1-2. Previous research on antimicrobial activity of *M. foetida* (continued)

Plant part(s) studied	Solvents used	Origin	Bacteria studied	Antimicrobial activity
Leaves	Ethanol crude partitioning fractions: dissolved in methanol: water (2:3 v/v) then partitioned with hexane, chloroform, ethyl acetate and butanol.	South Africa	*Escherichia coli* (ATCC 8739), *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 19582), *Staphylococcus aureus* (ATCC 6538), *Ent. faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 10702), *B. pumilus* (ATCC 14884), *Pseudomona aeruginosa* (ATCC 7700), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 10031), *K. pneumoniae* (ATCC 4352), *Proteus vulgaris* (ATCC 6830), *P. vulgaris* (CSIR 0030), *Serratia marcescens* (ATTCC 9986), *Acinetobacter calcaoceuticus* (Aci1), *A. calcaoceuticus* (Aci2) Environmental strains: *K. pneumoniae*, *Bacillus subtilis*, *Shigella flexneri*, *Salmonella* sp., *Staphylococcus epidermidis*, *P. aeruginosa*, *P. vulgaris*, *Enterococcus faecalis*, *E. coli*, *S. aureus*, *Micrococcus kristinae* and *M. luteus*.	Crude ethanol extracts displayed antimicrobial activity with MIC range of 0.039 mg/mL to 5 mg/mL against *E. coli*, *P. aeruginosa*, *S. aureus*, *Ent. faecalis*, *B. cereus*, *B. pumilus*, *K. pneumoniae* and *P. vulgaris*. Fractions of ethyl acetate extracts exhibited the highest broad spectrum antibacterial activity against test bacteria followed by butanol, chloroform, aqueous and hexane extracts at a concentration of 5 mg/mL. MIC exhibited by ethyl acetate, chloroform and hexane fractions ranged between 0.156 and 2.5 mg/mL while those of butanol and aqueous fractions ranged between 0.3125 and 5 mg/mL.

Reference: Odeleye and Oyedeji (2008)

Table 1-2. Previous research on antimicrobial activity of *M. foetida* (continued)

Plant part(s) studied	Solvents used	Origin	Bacteria studied	Antimicrobial activity	Reference(s)
Leaves	Ethanol (70%) and partitioned into hexane, chloroform, ethyl acetate, butanol and aqueous	South Africa	32 bacterial strains for both standard and isolates (as above)	Fractions (3β,7β-dihydroxyl-cucurbita-5,23,25-trien-19-al and Kaempferol-*3*-*O*-β-D-glucopyranoside) showed minimum inhibitory concentration (MIC) values for 25 bacterial strains ranging from 7.8 to 250 µg/mL	Odeleye *et al.* 2009
Roots	Hexane, methanol and water	Uganda	S*treptococcus mutans* (CCUG 6519 T), *Streptococcus sobrinus* (CCUG 25735 T), *Lactobacillus acidophilus* (CCUG 5917 T)**,** *Porphyromonas gingivalis* (CCUG 25226), *Aggregatibacter actinomycetemcomitans* (CCUG 56173), *Tannerella forsythia* (CCUG 21028 AT)	Antibacterial activity of methanol extracts (MIC 1 mg/mL) against *Lactobacillus acidophilus* only.	Ocheng *et al.* 2014

1.4 Drying methods for indigenous vegetables

Indigenous vegetables are used both in fresh and dried form. Some of the common indigenous vegetables in Swaziland are listed in Table 1-3. The fresh vegetables are normally not available throughout the year. Some form of preservation is imperative to broaden their availability, reduce losses and increase their shelf-life. The most common preservation method in the home setting is sun-drying or air drying. Sun-drying is highly dependent on time and weather (Masarirambi *et al.* 2010). A survey in Kenya on processing and preservation of African leafy vegetables showed that sun-drying was the most practiced method (45%) compared to other methods of preservation such as fermentation (32%), solar-drying (13%) and other methods (10%). The study also outlined the disadvantages of sun-drying as it takes a long time and therefore cannot be used to prolong shelf lives of vegetables for commercial purposes (Ayua and Omware 2013). Shitanda and Wanjala (2006) reported that freeze dried fresh jute (*Corchorus olitorius* L.) from Kenya had the best colour, ascorbic acid content and reconstitution characteristics when compared with drying under shade (air-drying), in the sun or by vacuum. Solar drying has been recommended based on its capacity to better retain nutrients in vegetables in Uganda compared to sun drying and oven drying (Kiremire *et al.* 2010).

Some of the commonly used drying methods include oven drying, freeze-drying, dehydrator-drying and microwave drying. Another study investigating the effect of drying methods on antimicrobial activity of lemongrass (*Cymbogon citratus*) revealed that oven-dried tissue displayed a better antimicrobial activity compared to freeze dried samples (Nkambule 2008).

Table 1-3. Some common indigenous vegetables found in Swaziland

Local name	English name	*Botanical name*
Ligusha	Vegetable jute	*Corchorus olitorius*
Inkhakha	Bitter gourd	*Momordica balsamina*
Imbuya	Pigweed	*Amaranthus hybridus*
Chuchuza	Black jack	*Bidens bilosa*
Inshubaba	Bitter gourd	*Momordica foetida*
Mandwandwe	Okra	*Abelmoschus esculentus*
Emahala	Common soap aloe	*Aloe saponaria*
Ematsanga	Pumpkins	*Curcubita spp*

Reference: Long (2005)

1.5 Nutritional composition of *Momordica* plants

African communities have a history of using traditional indigenous leafy vegetables to supplement their diet (Odhav *et al.* 2007). Currently, urbanization and influence of urban lifestyle on the rural African population have resulted in a drastic decline in availability of indigenous vegetables as they have been replaced by modern vegetables (Odhav *et al.* 2007; Behera *et al.* 2011). Knowledge of existing nutritionally rich food plants such as *M. balsamina* and *M. foetida* is slowly fading away as they are ignored by the younger generation (Odhav *et al.* 2007). Lack of proper knowledge, especially on their nutritive value, methods of production, preservation and full exploitation are reasons for the decreasing utilization of such vegetables (Karaye *et al.* 2013).

1.5.1 Nutritional composition of *M. balsamina*

The nutritional composition of *M. balsamina* leaves is summarized in Tables 1-4 and 1-5. Preliminary nutritional data of indigenous vegetables, including *M. balsamina* leaves, was presented in Odhav *et al. (*2007). The results from this study showed that *M. balsamina* may be a good source of protein and minerals especially calcium and magnesium. The study provided evidence that *M. balsamina* and other indigenous vegetables could be utilized to improve the diet of rural and urban people. Hassan and Umar (2006) performed nutrient analysis of *M. balsamina* leaves and their results showed that leaves of *M. balsamina* had a moisture content within the range 58.0 ± 2.5% to 93.4 ± 0.7% reported for green leafy vegetables and a higher ash content compared to some other reported leaves such as *Ipomea batatas* (1.8%). This study also indicated a higher protein, lower fat content, and higher carbohydrate content compared to other similar vegetables. This study showed that *M. balsamina* contained 17 amino acids with glutamic acid, leucine and aspartic acid being predominant. The results indicated that *M. balsamina* leaves could be a good supplement for mineral elements especially potassium, calcium, magnesium, iron, copper and manganese. Arnold *et al.* (1985) reported nutritional composition of *M. balsamina* leaves and fruit. From these results *M. balsamina* fruit was found to be high in carbohydrates and fibre but low in calcium, magnesium and potassium compared to the *M. balsamina* leaves.

Bwembya *et al.* (2008, 2014) conducted a micronutrient analysis of *Momordica involucrata* (*inkhakha*) in Swaziland and demonstrated a high vitamin A content followed by calcium, iron and zinc based on % RDA values. These results showed that micronutrients contained in this plant were

significantly higher than other commonly consumed foods such as green beans and pumpkin seeds and thus could provide a better source of micronutrients than some exotic foods purchased from grocery shops. A report by Baartjies (2014) stated that consumption of *M. balsamina* was likely to be beneficial as it is rich in Vitamins A, B, B2, C and minerals such as phosphorus, iron, copper and potassium.

Karaye *et al.* (2013) evaluated nutrient and anti-nutrient contents of selected Nigerian cucurbit seeds including *M. balsamina*. Results of the study showed that *M. balsamina* seeds could be a valuable source of nutrients such as protein (30.86 g/100g DW), fats (33.56 g/100g DW), carbohydrates (28.19 g/100g DW) and fibre (3.49 g/100g DW) which could be exploited in alleviating malnutrition problems. Analysis also revealed the presence of anti-nutritional compounds such as phytate, oxalate, cyanide, nitrate and tannins. When comparing results of the nutritional value of *M. balsamina* leaves from different researchers, there were some differences. A good example is the study by Hassam and Umar (2006) who reported a high energy content (189.22 kcal/100g) of *M. balsamina* leaves while Odhav *et al.* (2007) reported a much lower energy content of 53 kcal/100g. It is evident that nutritional value of the same plant will vary depending on environment as it may be affected by soil and climate (Geidam *et al.* 2007). Other factors such as plant maturity could also result in different nutrient content, this was confirmed in a study by Flyman and Afolayan (2008). This study showed that mineral content of *M. balsamina* and *Vigna unguiculata* subsp. *sesquipedalis* in Botswana may be influenced by stage of maturity.

Table 1-4. Proximate composition of *M. balsamina* leaves (g/100g DW)

Reference	Moisture content	Ash	Crude protein	Crude lipids	Crude fibre	Available carbohydrates	Calorific value (kcal/100g)
Hassan and Umar 2006	71.0	18.0	11.3	2.7	29.0	39.1	189.2
Arnold *et al.* 1985	89.4	ND	3.0	0.1	0.9	3.6	ND
Odhav *et al.* 2007	85.0	2.1	5.0	0.5	2.8	6.8	53.0

ND- Not done

Table 1-5. Mineral Composition of *M. balsamina* leaves (mg/100g DW)

Reference	Sodium	Calcium	Magnesium	Phosphorus	Manganese	Iron	Copper	Zinc
Hassan and Umar 2006	122.5	941.0	220.0	130.5	11.6	60.3	5.4	3.2
Arnold *et al.* 1985	ND	340.0	87.1	ND-	ND	12.7	ND	0.9
Odhav *et al.* 2007	376.0	2688.0	613.0	356.0	10.0	23.0	3.0	12.0

ND- Not done

1.5.2 Nutritional composition of *M. foetida*

Nutritional composition of *M. foetida* leaves consumed in South Africa is presented in Tables 1-6 and 1-7 (Nesamvuni *et al.* 2001). Results from these tables show that *M. foetida* leaves seem to be a good source of protein and vitamins such as ascorbic acid and folate. Ogle and Grivetti (1985) also reported *M. foetida* leaves to be among those wild plants of Swaziland that have good protein content. The researchers further suggested that edible wild plants should be considered a vital component of the Swazi diet because they contribute to maintaining nutritional quality throughout the nation. It is worth noting that there is not much literature available on nutritional content of *M. foetida*. This could be attributable to the fact that more focus on this plant lies in its medicinal uses.

Based on the available information, *M. balsamina* seems to have a higher nutritional value as compared to *M. foetida*. This may be particularly the case for mineral concentration of calcium, iron and zinc. *M. foetida* leaves were particularly rich in ascorbic acid (20.6 mg/100g DW) (Nesamvuni *et al.* 2001) whilst *M. balsamina* leaves had ascorbic acid of 0.4 mg/100g DW (Arnold *et al.* 1985).

From the results of these analyses, it is evident that *M. balsamina* and *M. foetida* could both be an important source of nutrients to supplement other sources. Chemical analysis alone however, should not be the only criterion for judging the nutritional importance of a plant. Thus, it becomes vital to consider other aspects such as presence of anti-nutritional and toxicological factors and biological evaluation of nutrients' form, availability and utilization (Hassan and Umar 2006; Behera *et al.* 2011). Also, it is important to note that

there is a wide variation in nutritional composition from the different studies above. These results may suggest differences within *Momordica* species.

Table 1-6. Proximate composition of *M. foetida* leaves

Parameters	Concentration (g/100g DW)
Protein	3.3
Fibre	3.2
Calorific Value (kcal/100g)	92.0

Source: Nesamvuni *et al.* 2001

Table 1-7. Vitamin and Mineral Composition of *M. foetida* leaves

Element *	Concentration (mg/100g DW)
Calcium	1.1
Iron	3.0
Zinc	0.4
B-Carotene	5.4
Folate (µg/100g)	40.0
Ascorbic acid	20.6

Source: Nesamvuni *et al.* 2001

1.6 Toxicity and cytotoxicity of *M. balsamina* and *M. foetida*

1.6.1 Toxicity of *M. balsamina*

Behera *et al.* (2011) reported that there are no published reports concerning any fatal or serious effects of *Momordica* in humans at normal doses of 50mL (juice) given orally. Toxicity has however, been observed in some animal studies. Leaves and fruits of *M. balsamina* from Nigeria were found to be toxic to various organs and tissues of rats at very high doses (Shettima *et al.* 2001). *M. balsamina* stem bark at very high doses (LD_{50} 3750 mg/kg) caused weakness, sleepiness, depression, dilated pupils, urination and death within 24 hours in rats (Karumi *et al.* 2006). van de Venter *et al.* (2008) concluded that aqueous and organic extracts (dichloromethane and methanol 1:1) of *M. balsamina* stem had some degree of toxicity against Chang liver, 3T3-L1 adipose and C2C12 muscle cells, with organic extracts giving a higher toxicity when cells were exposed to 12.5 µg/mL. The results of this study confirmed that *M. balsamina* extracts, particularly organic extracts, possess activity in hepatocytes.

A study by Geidam *et al.* (2004) concluded that aqueous stem extracts of *M. balsamina* had no significant effect on serum electrolytes and by extension on kidneys and other organs in alcohol fed and normal rats. A study by Geidam *et al.* (2007) could not establish complete safety of aqueous extracts of *M. balsamina* stem bark when extracts were administered for two weeks at a dose of 0.56 mg/100 g body weight. *M. balsamina* extracts at lower doses were found to be safe in rats (Shettima *et al.* 2001; Karumi *et al.* 2006). Benoit-Vical *et al.* (2006), reported that *M. balsamina* extracts at high concentrations (200 and 400 mg/kg/day) had no toxic effects on orally treated mice.

According to Bosch (2004a) and Welman (2004), there are contradictory reports on toxicity of *M. balsamina* plant parts and therefore this should instil caution when transferring uses from one region to another. As in many other cucurbit species, different types may occur: bitter and toxic as well as non-bitter and edible ones. Welman (2004) reported that the green fruit contains a resin, toxic alkaloids, and a saponic glycoside that causes vomiting and diarrhoea. These substances are however denatured during cooking.

1.6.2 Toxicity of *M. foetida*

Different parts of *M. foetida* have also been studied for their toxicity. Nguta *et al.* (2011) reported that organic extracts of *M. foetida* leaves from Kenya were shown to be the most toxic compared to other plant extracts against brine shrimp, *Artemia salina* L and exhibited potent activity with an LC_{50} value of 8µg/mL. The authors concluded that the toxicity data obtained from this study suggested that *M. foetida* leaves are not safe for use as antimalarial remedies. van de Venter *et al.* (2008) concluded that organic (dichloromethane and methanol 1:1) extracts of the whole *M. foetida* plant had some degree of toxicity against Chang liver and C2C12 muscle cells, however toxicity was shown to be lower than *M. balsamina* extracts when cells were exposed to 12.5 µg/mL. A study performed by Waako *et al.* (2005) suggested that a *M. foetida* water extract was well tolerated by mice within a dose range of 10 to 1000 mg/kg/day given in two divided doses. This study also showed that survival time of *Plasmodium berghei* (Anka) infected mice increased with extract dose over the experimental dose range. The authors, however, suggested that there was a need for further studies on the water extracts of *Momordica foetida* especially observational field studies to document its

efficacy and safety in humans before it can be regarded as a remedy for malaria.

1.7 Phytochemical composition of *M. balsamina* and *M. foetida*

Phytochemicals are naturally occurring plant compounds and many have the potential to inhibit some diseases (Akinmoladun *et al.*, 2007). These include a wide variety of secondary metabolites including, but not limited to, flavonoids, alkaloids, tannins and terpenoids (Cowan, 1999). There are more than 8000 phenolic structures currently known, some are simple structures such as phenolic acids whilst others have very complex structures such as tannins. These structures are to some extent responsible for the organoleptic properties of plant foods. Plant phenolics consist mostly of phenolic acids, tannins and flavonoids. The most abundant in our diet are the flavonoids.

1.7.1 Phytochemical composition of *M. balsamina*

Screening of *M. balsamina* leaves, stem, seeds and fruit showed the presence of many phytochemicals including resins, alkaloids, flavonoids, glycosides, steroids, terpenes, cardiac glycoside, and saponins (Thakur *et al.* 2009). Kalia *et al.* (2010) reported that *M. balsamina* seeds contain saponins, alkaloids and a range of phenolic compounds. Chloroform- ethanol extracts of the aerial tissues of *M. balsamina* in Saudi contained flavonoids, tannins, cardiac glycocides, sterols and saponins (Akbar and Al-Yahya 2011). Phytochemical screening of *M. balsamina* fruit from India showed the presence of steroids and alkaloids in petroleum ether extracts; phenolic compounds, flavonoids and

alkaloids in 90% methanolic extracts; and phenolic compounds, flavonoids, alkaloids and saponins in aqueous extracts (Faujdar *et al.* 2013). In this study aqueous extracts contained more phytochemicals than those of petroleum ether and 90% methanol extracts. In another study, the extract from dried fruit pulp powder contained resins, tannins, alkaloids, flavonoids, saponin, glycosides, steroidal ring and terpenes (Bot *et al.* 2007).

The bitter taste of *Momordica balsamina* tissues may be caused by cucurbitacins, as in many other *Cucurbitaceae*, but may also be caused by saponins. The ribosome-inactivating protein momordin II has been isolated, as well as the caffeic acid ester rosmarinic acid, which is of pharmaceutical interest because of its anti-inflammatory, antiviral, antibacterial and antioxidant activities (Bosch 2004a). Karumi *et al.* (2004) studied petroleum ether, methanol and distilled water extracts of *Momordica balsamina* leaves and reported that these extracts had high concentrations of alkaloids followed by saponins, tannins, reducing compounds and flavones. In this study, aqueous extracts displayed the highest concentrations of all the compounds identified.

1.7.2 Phytochemical composition of *M. foetida*

Curcubitane triterpenoids and polyphenolic compounds have been isolated from leaf extracts of *M. foetida,* while alkaloids and glycosides have been isolated from whole plant extracts (Acquaviva *et al.* 2013). Odusote and Awaraka (2004) conducted a phytochemical analysis of *M. foetida* extract and the results obtained showed the presence of alkaloids, saponins and cardenolides. Geyid *et al.* (2005) reported the presence of polyphenols and unsaturated sterol/triterpens in methanol extracts from the aerial parts of *M. foetida*. Odeleye and Oyedeji (2008) reported the presence of alkaloids,

flavonoids, terpenoids, steroids, saponins and tannins in *M. foetida* leaf extracts. Odeleye *et al.* (2009) used ethyl acetate partitioning fractions to isolate phytochemical compounds and results showed four curcubitane triterpenoids and flavonoid compounds from chloroform and ethyl acetate fractions, respectively. Ndam *et al.* (2014) performed a phytochemical screening of bioactive compounds in some Cameroonian medicinal plants and showed that acetone extracts of *M. foetida* leaf had low concentrations of phenolics and saponins, moderate concentrations of steroids and glycosides, and high concentrations of flavonoids.

1.8 Antioxidants and phenolic acids in *Momordica* plants

Antioxidants, which include phenolic compounds, are important in maintaining health as well as preventing some diseases (Kähkönen *et al*. 1999). There is increasing interest in polyphenols due to their antioxidant properties, their wide spread availability in plant foods and their possible effects in prevention of diseases including cardiovascular and neurodegenerative diseases and cancer (Manach *et al.* 2004). Phenolic acids may be classified into two major classes: firstly, derivatives of benzoic acid (gallic acid and tannins), and secondly derivatives of cinnamic acid (*p*-coumaric, caffeic, ferulic and sipanic acids) (Manach *et al.* 2004; Dai and Mumper 2010). The derivatives of cinnamic acid are rarely found in the free form, except in cases where the food has undergone some form of processing such as freezing, sterilization and fermentation. The bound forms are glycosylated derivatives or esters of quinic acid, shikimic acid, and tartaric

acid. The most abundant phenolic acid in many fruits and vegetables is caffeic acid. Caffeic acid is often esterified with quinic acid to form chlorogenic acid which is found in coffee (Manach *et al.* 2004). Ferulic acid is abundant in cereal grain and is present mainly in the outer parts of the grain. Flavonoids found in foods are mainly in the form of flavonols (quercetin and kaempferol); flavones are another form, however these are less common in fruits and vegetables. The flavonols are normally present at low concentrations in fruits and at high concentrations in foods like curly kale, broccoli and blueberries. These are also normally present glycosylated with sugars such as glucose or rhamnose (Manach *et al.* 2004).

Momordica plants extracts including those of *M. charantia* and *M. foetida* have been evaluated for antioxidant activity and phenolic content by previous researchers. Some studies have shown a relationship between antioxidant activity and phenolic content whilst some have shown that antioxidant activity does not exactly correlate with the amount of phenolics present. Only little research has focused on the antioxidant capacity and phenolic content of *M. balsamina*. Compared to other related species such as *M. charantia*, *M. balsamina* have been less studied (Grover and Yadav 2004).

Momordica charantia is a hybrid species that is mostly found in India and some other Asian countries such as China and Thailand. Thus it is one of the plants that has been extensively studied for its bioactivity. A study by Zheng and Wang (2001) reported total phenolic content of 0.43 mg gallic acid equivalents (GAE)/g of fresh weight and antioxidant activity, oxygen radical absorbance capacity (ORAC) of 3.43 µmol of trolox equivalents (TE)/g of weight of *M. charantia* phosphate buffer extracts. Kubola and Siriamornpun

(2008) found a strong association between antioxidant activity and total phenolics (R^2 = 0.948) in water extracts of *Momordica charantia* found in Thailand. In this study leaf extracts had the highest total phenolic content followed by green fruit, stem and ripe fruit. The main phenolic acid that was identified was gallic acid and others were tannic acid, catechin and caffeic acid. Horax *et al.* (2005) reported gallic acid, catechin and epicatechin as being the main phenolics found in methanol extracts from four varieties of *M. charantia* fruit (China white, China green, India white and India green). In this study oven-dried tissue provided the highest total phenolics compared to freeze-dried tissue. Also fruit flesh had the highest amount of phenolics. Antioxidant activity of the fruit showed no significant difference between varieties as well as between drying methods. Seeds however, had the lowest antioxidant activity.

Singh *et al.* (2011) analysed roots, stems, leaves and fruits of three Indian cultivars of *M. charantia* (Pusa Vishesh, Kalyanpur Barasati and Priya) for the presence of phenolic acids. This study showed that the phenolic acid profile differed between cultivars. Pusa Vinesh cultivar (small fruits) had gallic acid as the most abundant phenolic acid, especially in seeds (27.154 µg/g fresh weight), other phenolic acids were ferulic acid (5.425 µg/g fresh weight in fruit peel), tannic acid (detected only in leaves 3.48 µg/g fresh weight and stem 3.015 µg/g fresh weight) and caffeic acid (0.554 µg/g fresh weight in root). The Kalyanpur Barasati cultivar (long thin fruits) displayed tannic acids as the most abundant phenolic acid in seeds (43.08 µg/g fresh weight), others were gallic acid (2.143 µg/g fresh weight in peel), caffeic acid (1.412 µg/g fresh weight in stem), ferulic acid (0.689 µg/g fresh weight in fruit pulp) and

trace amounts of cinnamic acid (in stem) and chlorogenic acid (in seeds). In the third cultivar, Priya (bold fruits), gallic acid was the most abundant phenolic and was present in all parts of the plants but highest in seeds (113.053 µg/g fresh weight). Ferulic acid was mainly found in seeds (6.366 µg/g fresh weight), cinnamic acid was only detected in stem and leaves (0.102 µg and 0.028 µg/g fresh weight, respectively). This paper concluded that Pusha Vishesh and Priya were better for human health considering that the edible part of these cultivars were the fruits.

Several studies have measured antioxidant activity and phenolic content of *M. foetida* extracts. A study comparing phenolic content and antioxidant properties of aqueous and ethanolic extracts of *M. foetida* leaves from Nigeria found that ethanolic extracts had higher total phenolic content (5.55 ± 0.46 mg/mL) and total flavonoids (3.94 ± 0.07 mg/mL) than the aqueous extracts which had phenolic content (3.93 ± 0.17 mg/mL) and total flavonoids (2.88 ± 0.04 mg/mL). This study also revealed that the ethanolic extracts had higher antioxidant activity, as measured by the ferric reducing antioxidant power (FRAP) method, (8.95 mg ascorbic acid equivalents (AAE)/g) than the aqueous extracts which had reducing ability of 5.85 mg AAE/g (Molehin and Adefegha, 2014). Acquaviva *et al.* (2013) studied the antioxidant activity of *M. foetida* aqueous, methanolic and dichloromethane leaf extracts in relation to total phenolic and total flavonoid content. This study evaluated the free radical scavenging capacity of the extracts. Their results showed that aqueous extracts had the highest total phenolic content, 50 µM, followed by methanolic extracts which had phenolic content of 15 µM and the lowest content in dichloromethane with 5 µM. Scavenging activity of these three extracts were

consistent with their polyphenol content. Aqueous extracts showed the most scavenging activity at 175 µg/mL which was comparable to the inhibition by 80 mU superoxide dismutase (SOD). The ethanolic extracts of *M. foetida* leaf were also studied to determine their antioxidant potential using the production of thiobarbituric acid reactive substances (TBARS) and iron chelating assays. The results of this study revealed that these extracts displayed good antioxidant activity against pro-oxidant in liver and brain tissues. The extracts also proved to be capable of inhibiting lipid peroxidation (Oloyede and Aluko, 2012).

1.9 Objectives of the study

The aim of this study was to evaluate systematically the bioactivities of *Momordica balsamina* and *Momordica foetida* extracts from plant varieties found in Swaziland. Specific objectives of the study were:

1**.** To establish and compare antimicrobial properties of different solvent extracts of *Momordica* plants.

Hypothesis: The antimicrobial activity of *Momordica* plants extracts will be greatly affected by the solvent used for extraction.

2. To evaluate antimicrobial activity of methanol-water *Momordica* extracts by:

a) Comparing glass-house produced and those extracts sourced from Swaziland.

Hypothesis: Antimicrobial activity of methanol-water *Momordica* extracts will be affected by environmental conditions.

b) Establishing antimicrobial activity of SPE extracts and comparing activity of the phenolic fraction (fraction 3) of the extracts with activity of crude extracts.

Hypothesis: Antimicrobial activity of crude extracts and phenolic fraction of SPE extracts will be similar in *Mormordica* extracts.

c) Studying differences in bacterial growth curves in liquid cultures treated with the extracts.

Hypothesis: Growth curves of bacteria will be affected by treatment with *Momordica* extracts.

3. To determine and compare anti-proliferative activity of plants grown in a UK glasshouse and those grown in Swaziland;

Hypothesis: *Momordica* extracts have anti-proliferative activity which may contribute towards cancer prevention.

4. To determine the biochemical compounds present in methanol-water extracts of *M. balsamina* and *M. foetida.*

Hypothesis: *Momordica* extracts have biochemical compounds which influence its bioactivities.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant material

Momordica balsamina and *M. foetida* tissues were obtained either air dried under shade or oven dried (50°C for 72 h) from the Malkerns research centre, Swaziland or from plants grown in the tropical glass house at the University of Nottingham. For planting in the glass house, *M. balsamina* and *M. foetida* seeds were obtained from ripe yellow wild fruits collected at Malkerns research station, Swaziland. These were air dried and the red coating was removed before seeds were further dried. Dried seeds were pre-treated by soaking on filter paper soaked with distilled water for 24 h. Plants were grown in a glass house with temperatures between 27 – 31°C. Seeds were sown into high nutrient compost (Levington R M3) in 9 cm round pots. These were then maintained by irrigating every other day to keep the compost moist until plants germinated and were ready for transplanting. Transplanting took place between 4-6 weeks when plants were about 10-15 cm tall or when 4 true leaves were showing. Coarse potting compost (C2) with a pH 5.5 to 6 was used in 10 inch pots (young plants of *M. balsamina* and *M. foetida* are shown in Figure 2-1). Seedlings were removed from the 9 cm pots together with compost to avoid disturbing the roots. Plants were watered every two days. At two to three weeks after plants were transplanted, they were supplied with a patterned release fertiliser (Osmocote exact tablet, Everris Ltd, Ipswich, UK). Five tablets were supplied to each pot. Plants were also provided with support by cane sticks (trellis). Once plants had started producing both male and female flowers, pollination was performed manually.

For plant material preparation, plant samples (leaves, stem and fruits) were harvested when plants were about three months old. Tissue samples were washed with running water, drained to remove excess water and spread on oven trays covered with foil paper. Plant samples were then dried in an oven-drier set at 50°C for 72 h. After drying, leaves were carefully separated from the stem and each were crushed and weighed separately. Fruits were also weighed and all samples were stored in airtight containers at -20°C.

2.2 Preparation of plant extracts

2.2.1 Hot water extracts

Ten grams of *Momordica* tissue was weighed and added to a 500 mL beaker with 100mL of distilled water. Samples were brought to the boil for 5 minutes then cooled to about 30°C. Extracts were filtered through Mira cloth 22-25 µm, (VWR) then centrifuged at 1167 x g for 7 minutes. The supernatant containing the extract was then transferred to foil plates (Lakeland, UK). Extracts were then stored at -40°C before they were freeze dried at -40°C at pump readings between 7 x 10^{-2} and 2 x 10^{-2} mbar (Super Modulyo, Thermo Electronic, UK). Freeze dried samples were then weighed, transferred to a sterile container and stored at -20°C until required.



Figure 2-1. *M. balsamina* (A) and *M. foetida* (B) young plants at 6 weeks of planting in 10 inch round pots.

2.2.2 Chloroform-methanol extracts

Ten grams of *Momordica* tissue was weighed and added to 500 mL Duran bottles containing 100mL of chloroform-methanol (5:3v/v). Samples were left on a stirrer overnight then filtered through Mira cloth and poured into a 500 ml round flask. Samples were then concentrated using a rotary evaporator (Buchi, Switzerland) with a rotation speed of 70%, water bath temperature 40°C for about 25 minutes or until the volume of concentrate was about 20 ml. The remaining solvents were evaporated under nitrogen for about 2 hours or until the samples were dry. *Momordica* samples were then reconstituted using 50 mL of deionized water. Samples were centrifuged at 1167 x g for 7 minutes. The supernatant containing the *Momordica* extract was then transferred to foil plates. Extracts were stored at -40°C before they were freeze dried as before. Freeze dried samples were then weighed, transferred to a sterile container and stored at -20°C until required.

2.2.3 Methanol-water (7:3 v/v) extracts

Twenty grams *Momordica* tissue was weighed and added to a 500 mL Duran bottle containing 200 mL of methanol-water 7:3 (v/v). Samples were left on a stirrer overnight then filtered through Mira cloth and poured into a 500 ml round flask. Samples were then rotary evaporated at a rotation speed 70%, water bath temperature 50°C for about 35 minutes or until the volume of concentrate was about 30 ml. The remaining methanol was evaporated under nitrogen until only the aqueous part remained. An appropriate volume of millipore water was added to make a final volume of 100 mL. Samples were

centrifuged at 1167 x g for 7 minutes. The supernatant containing the *Momordica* extract was then transferred to foil plates. Extracts were stored at -40°C before they were freeze dried as before. Freeze dried samples were then weighed, transferred to a sterile container and stored at -20°C until required.

2.3 Solid phase extraction (SPE)

Waters Oasis HLB:[27] Oasis HLB SPE cartridges (Waters, 3 cc/60 mg) were conditioned with 4 mL methanol followed by 2 x 2 mL of deionised water. Oasis HLB is an all purpose, strongly hydrophilic, water-wettable polymer with an exceptional hydrophilic-lipophilic balance. Oasis HLB maintains high retention and capacity even if it runs dry after conditioning. This reversed-phase sorbent is ideal for acidic, basic and neutral analytes (2016 Waters). Two mL of the sample was loaded onto the cartridge and the non-retained compounds were eluted. The cartridge was washed with 2 x 2 mL of water. The polyphenolic fraction was eluted using 4 mL methanol, thus yielding three fractions: fraction 1 being very polar compounds that were eluted immediately from the column; fraction 2 being compounds that eluted after washing with water; and fraction 3 being compounds that were eluted after washing with methanol (Savage et *al.* 2011). For antimicrobial studies, fraction 3 was evaporated to dryness under nitrogen then reconstituted with PBS. For biochemical analysis the eluted fraction was used in methanol.

2.4 Bacterial cultivation

The bacterial strains used for this study were *Escherichia coli* O157:H7 Sakai Shiga toxin minus (*stx*-), *Salmonella* Typhimurium LT2 (Gram negatives), *Staphylococcus aureus* NCTC 10652 Enterotoxin A, *S. aureus* NCTC 10655 Enterotoxin C and *Listeria monocytogenes* 11944 (Gram positives). These were obtained from the laboratory stock of Food Sciences Division, University of Nottingham, United Kingdom.

For routine culture bacteria were streaked onto Tryptone Soy agar (TSA) (CM 0131, Oxoid) to obtain pure isolated colonies and incubated at 37°C for 18-24 h following a standard aseptic technique and the four-way streak plate inoculation (Cappuccino and Sherman 2005).

For the antimicrobial assays, an inoculum was prepared by inoculating a well isolated single colony from a TSA agar plate into a universal bottle containing 10 mL of Mueller Hinton Broth (MHB) (CM 0405, Oxoid) and incubated overnight at 37°C. A spectrophotometer (Ultrospec 2000, Pharmacia Biotech) was used to prepare a standard suspension (McFarland standard of 0.5; absorbance 600 nm of approximately 0.1) and cell density of 1.5 x 10^{8} cfu/mL (Ahmad *et al.* 2009).

2.5 Biochemical characterization

2.5.1 Selective agars

Each bacterium was streaked onto selective agar to confirm morphological characteristics (selective agars in Table 2-1). This was done following a standard aseptic technique and the four-way streak plate inoculation.

Table 2-1. Selective agars used for the different test bacteria.

Bacterial strain	**Selective agar**
Salmonella Typhimurium LT2	MacConkey #3 and XLD
Escherichia coli O157 SAKAI stx-	MacConkey #3 and Sorbitol MacConkey
Staphylococcus aureus RN4220, *S. aureus* NCTC 10652 Tox A and *S. aureus* NCTC 10655 Tox C	BAIRD-PARKER (BP) Medium
Listeria monocytogenes	Listeria Modified Oxford agar (L/MOX)

This table shows the different selective agars that were used to identify the test bacteria. One colony of each bacterium was streaked onto the agar following a standard aseptic technique and the four-way streak plate inoculation and this was incubated at 37°C for 18-24 hours.

2.5.2 Gram staining

The Gram staining procedure involved placing a loopful of maximum recovery diluent (MRD) (CM 0733, Oxoid) on a microscope slide. Using a sterile loop, a single colony was transferred to the MRD drops and the mixture was emulsified. The film was left to dry for about 30 seconds. The dried film was fixed using a Bunsen flame by passing briefly in the flame 2 to 3 times. The slides were then placed in crystal violet (primary stain) for 1 minute. Excess stain was washed off with water and slides were then placed in iodine (mordant) for 30 seconds and then excess was washed off. The slides were then placed in ethanol (decolourizer) for 1 minute and then washed in water. Slides were then placed into carbol fuschin (red counterstain) for 30 seconds

and excess stain was washed off with water. Slides were viewed under the microscope using oil immersion (final magnification x1000).

2.5.3 Motility test

The method used to check motility was the wet mount. The tests are conducted by adding a drop of saline or MRD (CM 0733, Oxoid) to a slide, adding a specimen, adding a slowing agent (optional), and adding a cover slip. The water helps support the sample and it fills the space between the cover slip and the slide thus allowing light to pass easily through the slide, the sample, and the cover slip. The cover slip is added such that there are no bubbles forming and obscuring the object. In studying a wet mount, it is important to differentiate between Brownian motion, evaporation, and true motility. Brownian motion is when a very small object wobbles back and forth in a random movement under the microscope. This is caused by water molecules colliding with the object. Evaporation is when water starts to evaporate from the slide, which is usually observed in about two minutes. Evaporation will always be greater from one part of the slide, so that it will cause all objects in the field of view to move towards that one point (streaming). Once evaporation begins, it will be impossible to detect true motility and a fresh slide should be prepared. True motility is movement by an organism that has a pattern or a purpose.

Procedure

Bacteria were inoculated by obtaining one colony using a Bunsen-flamed sterile loop and transferring it to a Universal bottle containing 10 mL of Tryptone Soy Broth (TSB) (CM 0129, Oxoid). This was incubated in an

environmental incubator shaker (New Brunswick Scientific, USA) at 37ºC for 24 hours. After incubation, using a sterile loop, 1 to 2 loopsful of inoculum were placed onto a microscope slide and smeared out to cover an area of 2 cm x 1 cm. A coverslip was placed gently on top of the suspension avoiding trapping air bubbles. Slides were then examined using oil immersion (final magnification x1000) under phase contrast lighting.

2.5.4 Catalase test

Catalase catalyses the breakdown of hydrogen peroxide into oxygen and water and is present primarily in strict aerobes and facultative anaerobes and rarely in aerotolerant anaerobes. The presence of catalase is assayed by production of bubbles of oxygen when cells are mixed with hydrogen peroxide.

Procedure

The catalase test was performed by using a sterile loop and placing a small amount of bacterial growth on the base of a petri dish. A drop of 3% hydrogen peroxide (Sigma, UK) was then placed onto the growth and the petri dish was covered with the lid immediately and the reaction was observed. The development of bubbles (effervescence) indicated a positive result.

2.5.5 Oxidase test

The oxidase test detects the presence of the enzyme cytochrome oxidase (cytochrome c) which is in the electron transport chain of some bacterial groups. The presence of oxidase is determined by a colour reaction with

oxidase reagent (N,N-dimethyl-p-phenylenediamine oxalate) in the stick method. The Oxidase detection strips (Microbact) are a convenient form of this method. These strips are impregnated with NNN'N' tetramethyl-p-phenylenediamine dihydrochloride for the detection of bacterial cytochrome oxidase enzyme.

Procedure

The colony to be tested was touched with the Oxidase Detection Strip and this was observed for up to 5 seconds. A deep blue/violet colour indicated a positive reaction.

2.5.6 Coagulase test

This test was done for anticipated *Staphylococcus aureus* strains. *S. aureus* strains in general produce coagulase (although they are not the only species that produce coagulase) and this distinguishes them from the non-coagulase species of staphylococci. Coagulase causes agglutination of blood plasma. This can be examined by mixing the organism with blood plasma on a microscope slide and observing any agglutination reaction. There are a number of commercially produced kits available which make the reaction more readily observable.

For this study the Staphytect test ((DR0850M, Oxoid, UK) was used. This test detects bound coagulase (clumping factor), specific capsular polysaccharides and Protein A. This is achieved by agglutination of blue latex particles when *S. aureus* is present. The latex particles are coated with fibrinogen (binds

coagulase), immunoglobulin G (binds with protein A) and specific polyclonal antibodies against MRSA capsular polysaccharides.

Procedure

The isolate tested in this section was first confirmed as Gram-positive catalase positive cocci as per manufacturer's instructions. One drop of test reagent (shaken and at room temperature) was placed onto a reaction card circle and one drop of control reagent was placed onto another circle. Five colonies were smeared onto the control circle and five onto the test circle. This was mixed and spread to cover each of the circles. Care was taken to separate loops for the test and control circles. The reaction card was rocked for 20 seconds and this was examined for agglutination. Agglutination in the test circle only meant results were positive for coagulase and thus the organism was probably *S. aureus*.

2.5.7 *Salmonella* speciation test

The Kauffmann-White scheme differentiates the genus *Salmonella* into serotypes based on the somatic (O) and flagella (H) antigens that are present. Each serotype or serovar was in the past given a species designation. To confirm an organism is *Salmonella* it must react with an antiserum containing *Salmonella*-speciation antigens. A polyvalent H antiserum (mixture of a range of H antisera; Oxoid *Salmonella* test kit) was used for the test.

Procedure

The isolate tested was initially confirmed as Gram-negative catalase positive and oxidase negative rods. One drop of saline was placed onto a reaction card

circle. One colony of bacteria was emulsified in the saline using a mixing stick (loop). The suspension was observed for agglutination or clumping. This was the control, if no clumping was observed then it meant proceeding to the next stage. One drop of Salmonella Latex Reagent was added to the suspension and this was mixed for 30 seconds. The reaction card was then gently rocked 2 or 3 times and agglutination was observed within 2 minutes. Agglutination (clumping) indicated the presence of flagella (H) antigens.

2.5.8 Identification of *Salmonella* Somatic Antigen

Polyvalent O (somatic) antisera are intended to aid initial sero-grouping. Full identification of 'O' antigens can be achieved using monovalent specific 'O' antisera. The serotype of *Salmonella* isolates can then be determined by the use of polyvalent and monovalent 'H' (flagella) antisera. Serological identification of *Salmonella* somatic antigens involves mixing the presumptive organism with 'O' antiserum. The bacteria will agglutinate (clump) in the presence of homologous antiserum. These tests were used only once the organism had been shown to be *Salmonella* by the poly-H antigen kit.

Procedure

Two separate loopfuls of normal saline (0.85% sodium chloride) were placed on a clean glass slide. A small part of colony was taken from an overnight culture plate and mixed thoroughly with both drops of normal saline on the slide to obtain a smooth suspension. One loopful of antiserum was added to one of the bacterial suspension drops on the slide; to the other (control) one loopful of normal saline was added. The antiserum was mixed with the

bacterial suspension using a sterile loop. The slide was then gently tilted back and forth for one minute and this was then observed for agglutination under normal light, and with a black object underneath to aid observation. Agglutination meant presence of the test somatic 'O'antigen.

2.5.9 Biochemical profiling

The API (bioMérieux) system is a standardized system for the identification of bacteria, which uses miniaturized tests, as well as a database. The API consists of microtubes containing dehydrated substrates which enable the performance of enzymatic tests or sugar fermentations. During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the Reading Table and the identification is obtained by using the identification software. For this study API version 4.1 was used to identify bacteria. All test kits and supplementary reagents were obtained from bioMérieux Ltd (Hampshire, UK) and used following the manufacturer's instructions. The manufacturer sets specifications for pre-tests which must be complied with before the kit can be used.

API testing was performed using API Listeria for the identification of *Listeria*, API 20E for the identification of *Enterobacteriaceae* and other non-fastidious, Gram-negative rods and API Staph for the identification of the genus S*taphylococcus.*

Procedure

An incubation box was prepared by distributing either 3 mL or 5 mL of distilled water or demineralized water into the honeycombed wells of the tray

to create a humid atmosphere. The strain reference was recorded on the elongated flap of the tray. A strip was removed from its individual packaging and placed in the incubation box. An inoculum was prepared by using a pipette or PSIpette to pick up a few well isolated colonies from a young culture (18-24 hours old) and preparing a suspension using API suspension medium as specified by the manufacturer. This suspension was used immediately after preparation. The strip was inoculated by distributing bacterial suspension into each tube, avoiding the formation of bubbles (the strip was tilted lightly forward and the tip of the pipette was placed against the side of the cupule). For individual API tests the following steps were then followed:

API *Listeria*

The tube and cupule of the DIM test was filled (about 100 µL) avoiding the formation of a convex meniscus. Only the tube parts of tests ESC to TAG were filled (about 50 µL). The incubation box was closed and incubated at 37°C for 18-24 hours. After incubation a drop of ZYM B reagent (bioMérieux) was added to the DIM test. All reactions were read within 3 minutes by referring to the reading table and the positive and negative test guide (Figure 2-2). Results were then entered in the identification software and read.

API 20E

For CIT, VP, and GEL tests both tube and cupule were completely filled. For the other tests only the tube (and not cupule) was filled. For the tests ADH,

LCD, ODC, H2S and URE, anaerobiosis was created by overlaying with sterile mineral oil (liquid paraffin). The incubation box was closed and this was then incubated at 37°C for 18-24 hours. After the incubation period, the strip was read with reference to the reading table (Table 2-2). If three or more tests (GLU test + or -) were positive, all the spontaneous reactions were recorded on the result sheet. For tests that required addition of reagents: TDA test one drop of TDA reagent was added and results were read; IND test one drop of JAMES reagent was added and results were read; and VP test one drop of VP1 and VP2 reagents were added and the results read after at least 10 minutes.

Results were then entered in the identification software and read. This did not include the additional nitrate or nitrite tests done on the glucose tube.

API Staph

Only the tube portion of microtubes was filled. Anaerobiosis was achieved in ADH and URE tests by filling the cupules with mineral oil (liquid paraffin) to form a convex meniscus. The incubation box was closed and this was then incubated at 37°C for 18-24 hours.

After incubation some reactions were developed by adding one drop of VP1 and VP 2 to VP test, one drop of NIT1 and NIT2 to NIT test and one drop of ZYM A and ZYM B reagents to PAL test. All the above reactions were observed after 10 minutes. All other reactions were read by referring to the reading table (Table 2-3). Results were then entered in the identification software and read.

NEGATIVE TESTS



POSITIVE TESTS



Figure 2-2. API Listeria negative and positive results guide. Bacterial inoculum reacts with dehydrated substrates in the API strip to give a colour reaction that is either positive or negative. The reactions are read according to the Reading Table. The above examples are not intended to replace the reading table in the package insert. Taken from http://www.biomerieux.com.

2.6 Antimicrobial sensitivity/resistance assays

2.6.1 Disc diffusion method

A stock solution of each extract at 200 mg/mL was prepared using phosphate buffered saline (PBS: 0.16M NaCl; 0.003M KCl; 0.008M Na_2HPO_4 and 0.001M$KHPO_4$) pH 7.3 at 25ºC. PBS was used as a negative control and Ampicillin (10µg/disc) (Oxoid) was used as a positive control.

The pipette delivery method was used to impregnate discs with extract. Six millimetre diameter sterile filter paper discs were placed in a petri dish approximately 5 mm apart.

Table 2-2. API 20E results Table

TESTS	SUBSTRATE	REACTION TESTED	- RESULTS *negative*	+ RESULTS *positive*
ONPG	ONPG	beta-galactosidase	colourless	yellow
ADH	arginine	arginine dihydrolase	yellow	red/orange
LDC	lysine	lysine decarboxylase	yellow	red/orange
ODC	ornithine	ornithine decarboxylase	yellow	red/orange
CIT	citrate	citrate utilization	pale green/yellow	blue-green/blue
H2S	Na thiosulfate	H_2S production	colourless/grey	black deposit
URE	urea	urea hydrolysis	yellow	red/orange
TDA	tryptophan	deaminase	yellow	brown-red
IND	tryptophan	indole production	yellow	red (2 min.)
VP	Na pyruvate	acetoin production	colourless	pink/red (10 min.)
GEL	charcoal gelatin	gelatinase	no diffusion of black	black diffuse

Table 2-2. API 20E results Table (continued)

GLU	glucose	fermentation/oxidation	blue/blue-green	yellow
MAN	mannitol	fermentation/oxidation	blue/blue-green	yellow
INO	inositol	fermentation/oxidation	blue/blue-green	yellow
SOR	sorbitol	fermentation/oxidation	blue/blue-green	yellow
RHA	rhamnose	fermentation/oxidation	blue/blue-green	yellow
SAC	sucrose	fermentation/oxidation	blue/blue-green	yellow
MEL	melibiose	fermentation/oxidation	blue/blue-green	yellow
AMY	amygdalin	fermentation/oxidation	blue/blue-green	yellow
ARA	arabinose	fermentation/oxidation	blue/blue-green	yellow

The table shows different reactions tested giving rise to colours which either give a positive or negative result for each test on API 20E. This table is used to read the reaction colours given by bacteria (*Enterobacteriaceae*) inocula once they have reacted with dehydrated substrate in the API 20E strip after being incubated at 37°C for 24 hours. Adapted from http://www.biotech.univ.gda.pl/odl/biochem/api.html

Table 2-3. API Staph results table

api® Staph 07468H - GB - 2002/10

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS / ENZYMES	RESULT	
				NEGATIVE	POSITIVE
0	No substrate		Negative control	red	—
GLU	D-glucose	1.56	(Positive control) (D-GLUcose)	red *	yellow
FRU	D-fructose	1.4	acidification (D-FRUctose)		
MNE	D-mannose	1.4	acidification (D-ManNosE)		
MAL	D-maltose	1.4	acidification (MALtose)		
LAC	D-lactose (bovine origin)	1.4	acidification (LACtose)		
TRE	D-trehalose	1.32	acidification (D-TREhalose)		
MAN	D-mannitol	1.36	acidification (D-MANnitol)		
XLT	xylitol	1.4	acidification (XyLiTol)		
MEL	D-melibiose	1.32	acidification (D-MELibiose)		
				NIT 1 + NIT 2 / 10 min	
NIT	potassium nitrate	0.08	Reduction of NITrates to nitrites	colorless-light pink	red
				ZYM A + ZYM B / 10 min	
PAL	ß-naphthyl phosphate	0.0244	ALkaline Phosphatase	yellow	violet

Table 2-3. API Staph results table (continued)

				VP 1 + VP 2 / 10 min	
VP	sodium pyruvate	1.904	Acetyl-methyl-carbinol production (Voges Proskauer)	colorless-light pink	violet-pink
RAF	D-raffinose	1.56	acidification (RAFfinose)	red	yellow
XYL	D-xylose	1.4	acidification (XYLose)		
SAC	D-saccharose (sucrose)	1.32	acidification (SACcharose)		
MDG	methyl-αD-glucopyranoside	1.28	acidification (Methyl-αD-Glucopyranoside)		
NAG	N-acetyl-glucosamine	1.28	acidification (N-Acetyl-Glucosamine)		
ADH	L-arginine	1.904	Arginine DiHydrolase	yellow	orange-red
URE	urea	0.76	UREase	yellow	red-violet

The acidification tests should be compared to the negative (0) and positive (GLU) controls.

* When MNE and XLT are preceded or followed by positive tests, then an orange test should be considered negative.

- The quantities indicated may be adjusted depending on the titer of the raw materials used.
- Certain cupules contain products of animal origin, notably peptones.

The table shows different reactions tested giving rise to colours which either give a positive or negative result for each test on API Staph. This table is used to read the reaction colours given by bacteria (S*taphylococcus*) inocula once they have reacted with dehydrated substrate/enzyme in the API Staph strip after being incubated at 37ºC for 24 hours. Taken from http://www.netropica.org/bacteriologia/ApiStaph(1).pdf

Using a fixed volume of 20 µL, discs were impregnated with 4 mg extract /disc. Discs were then left in the laminar flow hood to dry overnight.

The effect of *Momordica* extracts on several bacterial strains was evaluated by the disc diffusion method (Parish and Davidson 1993). A standard bacterial suspension (100 µL in Mueller Hinton broth - MHB) with a cell density of 1.5 x 10^8 cfu/mL (prepared as in Section 2.4) was spread on to the surface of Mueller Hinton agar (MHA) plates using a sterile cotton bud. The spread inoculum was left to dry for about 10 minutes. Discs containing *Momordica* extracts were placed on the surface of the inoculated agar. A positive control was Ampicillin standard discs (10µg/disc). The negative control was 6mm discs loaded with 20 µL of PBS. Each disc was lightly pressed down using sterile forceps to obtain complete contact with the surface of the agar; these were distributed evenly approximately 24 mm apart, centre to centre. Plates were then incubated at 37ºC for 20-24 hours before being examined. Results were expressed as the diameter (in millimetres) of inhibition around each paper disc.

2.6.2 Extract treatment survival assay

The Miles and Misra method (How and Parasakthi 1978) was used to determine viable counts (cfu/mL) of a bacterial suspension in Mueller Hinton broth (MHB) treated with M. *balsamina* and *M. foetida* extracts. Treatments were carried out by adding 155 µL medium (MHB), 25 µL of standard bacterial suspension with a cell density of 1.5 x 10^8 cfu/mL (prepared as in Section 2.4) and 20µL (200 mg/mL) of an extract of *M. balsamina* or *M.*

foetida to make a total of 200 µL. A control was prepared by adding 175 µL MHB and 25 µL bacterial suspension. Treatment time was 2 h at 20°C before diluting the suspension. Using a 96 well microtitre plate, treated samples and controls were serially diluted from 10^{-1} to 10^{-6}. This was done by adding 20 µL treatment or control to 180 µL maximum recovery diluent (MRD). For each sample, 3 x 20 µL of each dilution was carefully dropped onto the surface of dried plates of plate count agar (PCA) agar (Oxoid LTD) and the drops were allowed to spread naturally to give an area of 1.5-2.0 cm. Each plate had 6 dilutions. The plates were left non-inverted on the bench for about 20 minutes to allow the drops to dry before inversion. These were then incubated at 37°C for 18 – 24 h (Figure 2.3 displays typical Miles Misra incubated plates). Counts were made on drops showing between 3 and 30 colonies, and the viable count was calculated using the formula:

$$no.of\ colonies \times 50 \times \text{dilution factor} = no.of\ organisms/mL\ of\ undiluted\ sample$$

2.6.3 Spot tests

The antibacterial activity of crude and fractionated extracts produced by the SPE method (Section 2.3) were evaluated using two different spot test methods: method 1 (Hansen and Jakobsen, 1997) and method 2 (Emerstorfer *et al.*, 2009).

Method 1

One mL of standard bacterial culture suspension in MHB (with cell density of approximately 1.5 x 10^8 cfu/mL) was poured into a Petri dish (90 mm) and mixed with 20 mL, 45°C melted MHA. When the agar had solidified four spots were made on each plate by dropping on 20 µl of each fraction of *Momordica* extract and these were left to dry for about 30 minutes. The plates were then incubated for 24 hours at 37°C. Inhibition of growth (mm) was measured around the spot. Controls were made by use of sterile MHA broth instead of extract.

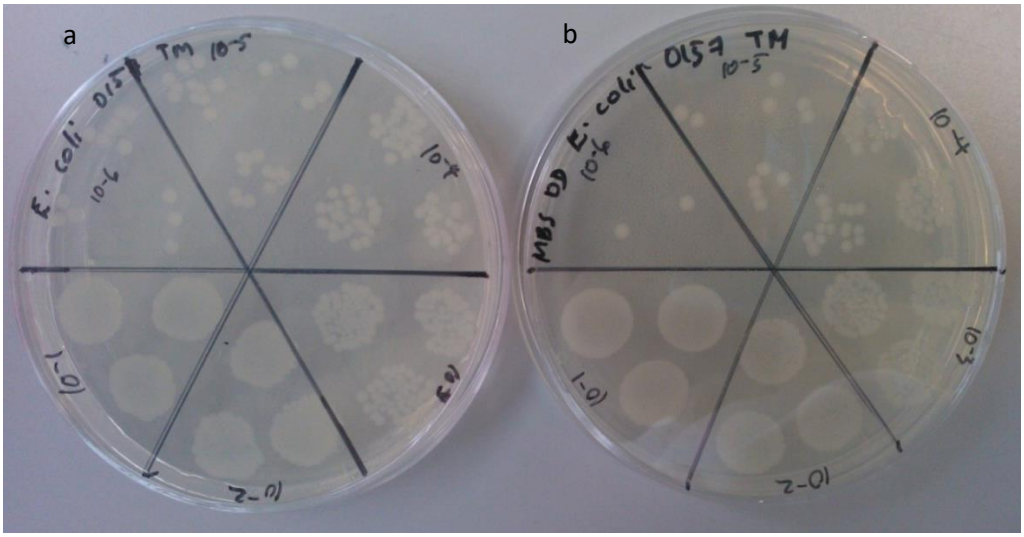


Figure 2-3. Typical Miles and Misra plates showing *E. coli* O157 *stx*$^-$ counts after treatment of the culture with oven-dried *M. balsamina* stem extracts The plates show untreated *E. coli* O157 *stx*$^-$ (a) and treated *E. coli* O157 *stx*$^-$ (b) which had been exposed to oven dried *M. balsamina* stem extracts (2 mg/mL) for 2 hours. Twenty microlitre (x 3) of each treatment was spotted on plate count agar and incubated at 37ºC for 24 hours.

Method 2

After solidification of approximately 20 mL PCA medium in the Petri dish, 20 µL of each extract (fraction) was dotted onto 4 spots on the agar surface and left at room temperature for about 20 minutes to dry. Afterwards, 100 µL of standard bacteria culture suspension in MHB (with cell density of approximately 1.5 x 10^8 cfu/mL) was evenly distributed on the agar surface and left to dry. After incubation for 24 h at 37ºC, the zone of inhibition was measured.

2.6.4 MIC and MBC of extracts

The MIC was determined by a microdilution method using 96 well sterile plates which have a maximum capacity of 200 µL. The extracts were diluted to get a series of concentrations from 0.625 mg/mL to 80 mg/mL in sterile MHB. A single colony from each bacterial strain was cultured in MHB at 37ºC for 18 h and adjusted with MHB to a standard bacterial suspension of 1-5 x 10^5 cfu/mL (Casmiri and Burstein 1998) as determined by a A_{600nm} reading of optical density in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech) read at approximately 0.05. For each well, 100 µL of MHB was added up to row 10. A 100 µL aliquot of the 160mg/mL extract was then pipetted into the first row of wells and mixed. A two-fold dilution was made by withdrawing 100 µL from the first row and adding it to the MHB in the next row. This procedure was repeated until row 8. For the last row after mixing 100 µL was taken and discarded. Five µL of bacterial suspension was added into all broth dilutions in rows 9 to 1 in that order. No bacterium was added to row 10

(sterility control). Plates were incubated at 37ºC over 24h. Colour reaction with P-iodonitrotetrazolium violet (MP Biomedicals, LLC) was used to measure metabolic activity. Twenty µL (0.2mg/mL) of colour reagent was added to each well and incubated for 30 minutes at 37ºC before observing colour changes. Wells with colour indicated metabolic activity; the lowest concentration showing no colour was taken as minimum inhibitory concentration (MIC). Wells with no colour change were then used to determine minimum bactericidal concentrations (MBC).

To establish MBC, 10 µL from each well that did not show any bacterial metabolic activity was taken and spotted on TSA plates, in triplicate. Plates were incubated at 37ºC for 24 hours and these were then evaluated for bacterial growth. The lowest concentration of plant extract showing no growth was taken as the MBC. Values were recorded as mg/mL.

2.6.5 Bacteriostatic and bactericidal effects of extracts against bacterial pathogens

For this study, 5 mL of overnight culture grown in MHB in a 30 mL Universal bottle at 37ºC static conditions was transferred to a 100 mL flask containing 50 mL MHB only, and to another flask containing MHB plus 100 mg extract. A control was prepared using 50 mL MHB and 100 mg extract only. From each flask, 1 mL was taken using a pipette into 10 x 4 x 45 mm cuvettes (Sarstedt) and the absorbance was read at 600 nm using a spectrophotometer; this was then recorded as time 0. The flasks were incubated statically at 37ºC (flasks with extracts were covered with foil to reduce light effects before

incubating) and A_{600nm} readings were then recorded every 30 minutes up to 6 - 10 h and after 24 h. For viable count, samples were plated at each time point using the Miles and Misra method following the procedure given in Section 2.6.2.

2.6.6 Bacteriostatic and bactericidal effects of *Momordica* extracts using mid-log suspension cultures

For this part of the study a culture of *L. monocytogenes* was grown to mid-exponential phase before being treated with extracts of *Momordica balsamina* leaves. A single colony of *L. monocytogenes* was isolated and grown statically overnight in 10 mL BHI broth at 37°C. Five mL of overnight culture was added to 100 mL of fresh BHI and an A_{600nm} reading was taken using the spectrophotometer. The diluted culture was grown at 37°C in a shaker incubator (Gallenkamp) at100 rpm and A_{600nm} readings taken every 30 minutes until mid-exponential phase (A_{600nm} 0.05). From this culture, 5 mL was taken and added to 100 mL of fresh BHI broth and it was grown in a shaker incubator at 37°C until mid-exponential phase. This step was repeated twice before initiating treatment. For treatment, 5 mL of culture grown to mid-exponential phase was added to 50 mL of BHI broth then 100mg/mL of extract was added to the culture. As a control another 5 mL of the culture was added to 50 mL of BHI broth with no additions. To prevent light effects on *M. balsamina* leaf treatment, the flask was completely covered with aluminium paper. The treatment and control were then incubated at 37°C in static conditions. These were then serially diluted and plated in triplicate using the Miles Misra method following the procedure in Section 2.6.2 every hour from

time 0 up to 5 h, then at 24 h. In this case A_{600nm} readings were only taken at times 0, 5 and 24 h.

2.7 Determination of anti-proliferative activity in cell cultures

Human liver hepatocellular carcinoma (HepG2) cell lines were obtained from the Nutritional Science Department, University of Nottingham, United Kingdom. These were originally sourced from the European Collection of Cell Cultures. Stock HepG2 cells were kept in liquid nitrogen. For analysis 1 mL of the cells was defrosted and grown in 10 mL Dulbecco's Modified Eagle Medium (DMEM) high glucose (Sigma D6546), and supplemented with 10% foetal bovine serum (FBS) (Gibco), 1% L-Glutamate (Gibco), 1% Penicillin-Streptomycin (Sigma) and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed every 2 days and cells cultured to approximately 80% confluence. Once cells had reached the desired confluence, they were sub-cultured by removing medium and washing with phosphate buffer saline (PBS) (10mL). Cells were then treated with 2 mL of 0.25% trypsin (Sigma) and 1 mM EDTA for 3 minutes. Trypsinized cells were then re-suspended in 8 mL of fresh DMEM and centrifuged at 200 x g for 5 minutes. Cells were then transferred to a new flask at 1:5 split of the original cell concentration. These were maintained in a humidified atmosphere of 5% CO_2 [generated by supply of CO_2 from a gas cylinder through a valve that is triggered to draw CO_2 whenever the level falls below 5% (Wilson and Walker 2010)] at 37 °C and the medium was changed every 2 days. Cells were then plated in 96-well round-bottomed tissue culture plates (Costar, Corning) for

the cytotoxicity test. Cells were seeded at a density of approximately 80 000 cells/well (100µL). The cell density was counted using a haemocytometer by adding 10 µL of harvested cells and placing on an inverted microscope and using the contrast to distinguish the cells. HepG2 cell lines were maintained for one day at 37°C, to allow cell stabilization. Cells were then treated with different doses of individual extracts (100 µL) dissolved in PBS at concentration ranges of 0.06 to 1 mg/mL for *M. balsamina* and 0.25 to 4 mg/mL for *M. foetida*. Phosphate buffered saline (PBS) was used as a control and each test sample consisted of five replicates. Treated cells were incubated at 37°C and the effects of extracts on the cells were determined at 0, 24 and 48 h.

At each time point, media were carefully removed and the cells washed with 200 µL PBS then 100 µL of Sigmaclean water (Sigma) was added to the wells before incubation at 37°C for 30 minutes. Cells were subjected to three freeze-thaw cycles of 30 minutes at -20°C and room temperature until thawed, respectively to assist in DNA release by rupturing the cell membrane. Cell number in each case was estimated by DNA content using the Hoechst Fluorometric method (Rage *et al.* 1990). 100 µL of Hoechst dye mixture (consisting of 2µg/mL Hoechst 33258 [Sigma] and 2X TNE buffer [20 mM Tris, pH 7.4, 2 mM EDTA and 0.2 M NaCl]) was added to each well and fluorescence was measured at an excitation wavelength of 355 nm and emission of 460 nm (BMG Labtech Fluorstar Optima). Calf thymus DNA (Sigma) was used for the standard curve and was achieved by preparing serial dilutions ranging from 0.6 µg/mL to 40 µg/mL.

2.8 FTIR analysis of extracts

Fourier-transform Infra-Red spectroscopy (FTIR) measurements were performed on crude extracts of *Momordica* leaf and stem using a Bruker Tensor 27 FT-IR spectrometer (Bruker, Coventry, UK). Each extract was applied in powder form. The analysis was carried out in the wavelength range of 500 – 4000 cm^{-1}. For each sample extract three samples under the same conditions were examined. For each extract, 128 scans were averaged with spectra resolution of 4 cm^{-1} and then a final average spectrum was calculated. Peak positions and intensities were obtained using OPUS, Optics Software (Bruker). Principal component analysis (PCA) was used to study any patterns in the characteristics and physio-chemical properties of the extracts together with these of the original dried tissue sample. The Unscrambler 10.1 software (Camo Software AS., Norway) together with Microsoft excel were used for performing PCA.

2.9 Determination of total soluble phenolic content

Total soluble phenolic content (TPC) was determined on crude and SPE extracts using a Folin-Ciocalteu reagent and Gallic acid as standard using the procedures outline below:

10 mg of Gallic acid (Fisher Scientific) was dissolved in 2 mL of 100% ethanol. Additional ethanol was added to make the volume up to 10 mL (this gave a Gallic acid solution of 1000 µg/mL). This was then used to prepare Gallic acid standard concentrations which ranged from 20 to 100 µg/mL

(Table 2-4). A standard curve was then developed using these concentrations (Figure 2-4 presents a typical standard curve using two replicates).

Aliquots (0.5 or 0.1 mL depending on fit within standard curve) of sample (20 mg/mL), standard and control were added to screw-cap test tubes. Using a pipette, 6 mL distilled water and 0.5 mL undiluted Folin-Ciocalteu reagent were added and then after approximately 3 minutes, 1.5 mL saturated sodium carbonate was added to each tube. This was made to a final volume of 10 mL with distilled water, thoroughly mixed by vortexing and then incubated for 2 h at 37°C before measuring the absorbance using a spectrophotometer at 765nm.

Preparation of saturated sodium carbonate

Saturated sodium carbonate was prepared by adding 50 g of sodium carbonate (ACROS) to 200 mL deionised water then mixing and heating until completely dissolved. This mixture was then filtered and stored at room temperature until crystals precipitated.

Table 2-4. Gallic acid standard concentrations (µg/mL) prepared from stock solution

Gallic acid standard (µg/mL)	20	40	60	80	100
Gallic acid solution (mL)	0.2	0.4	0.6	0.8	1.0
Distilled water	0.8	0.6	0.4	0.2	0

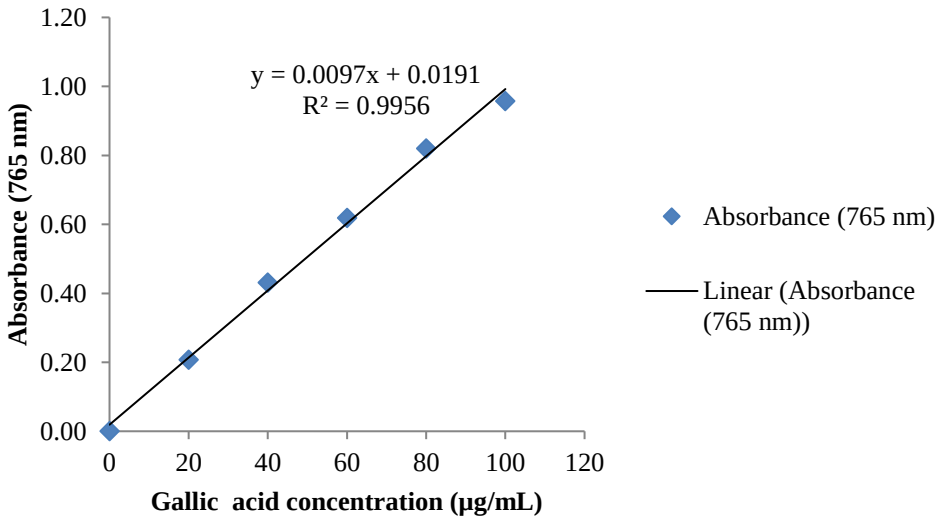


Figure 2-4. A typical gallic acid standard curve

2.10 HPLC profiling of extracts

Four milligrams of each dried methanol-water extract was dissolved in 1 mL of HPLC grade methanol (Fisher Scientific) and the mixture was then either (a) filtered through a 0.45 µm nylon filter (Fisher Scientific) and injected onto the HPLC or (b) extracted through SPE (3.2.2) before being injected onto the HPLC column.

To release conjugated phenolic acids, *Momordica* extracts were saponified. Extracts of *M. balsamina* and *M. foetida* leaf and stem were hydrolysed by suspending 50 mg of extract in 5 mL 0.5 M NaOH for 2 h at room temperature. Each mixture was then adjusted to pH 2 by adding a few drops of 12.1 M HCl (Sigma). Samples were then partitioned by the addition of 10 mL HPLC grade ethyl acetate (Fisher Scientific). This was then centrifuged at 4000 x g for 5 minutes and the ethyl acetate fraction collected. This partitioning was carried out three times. The combined supernatants were

evaporated to dryness under nitrogen then reconstituted to 1 mL with HPLC grade methanol and filtered before injecting onto the HPLC column.

Phenolic profiling of extracts was performed using a Waters Alliance 2695 HPLC system equipped with a pump, plus autosampler and Alliance vacuum degasser. These were coupled with a diode array UV detector and absorbance was monitored between the wavelength range of 220 and 400 nm. Separation of compounds was performed using a Zorbax RP C18 column (150 x 4.6 mm, 5 micron) (Agilent, Germany) and column temperature was kept at 25°C. The mobile phase consisted of solvent A (0.02M phosphate buffer, pH 2.4) and B (100% methanol, HPLC grade). The flow rate was set at 0.5 mL/min. The gradient settings were as follows: equilibrate 90% (A) and 10% (B), then 30 % (A) and 70% (B) for 40 minutes and then changed to 70% (A) and 30% (B) for 5 minutes. Injection volume of sample was 10 µL for the analysis and sample temperature was kept at 4°C. Phenolic compounds present in samples were identified by comparing with the retention time (Rt) and absorbance of standards.

Standards: Caffeic acid, p-coumaric acid, kaempferol, sinapic acid, naringenin, luteolin, epicatechin gallate and catechin were all purchased from Sigma-Aldrich (Gillingham Dorset, UK). Gallic acid, epicatechin and ferulic acid were from MP Biomedicals (Cambridge, UK). Myricetin was from Alfa Aesar (Lancashire, UK). Quercetin was from TOCRIS bioscience, USA). Chlorogenic acid was from Fuorochem LTD (Hatfield, UK). Preparation of standards was performed by dissolving 6 mg of each standard compound in 10 mL of HPLC grade methanol giving a final concentration 600 µg/mL. This

was mixed by votexing, then filtered through a 0.45 µm nylon membrane filter (Fisher Scientific, Leicestershire, UK) and injected onto the HPLC column.

2.11 NMR analysis of extracts

NMR analysis of *M. balsamina* leaf and stem crude extracts and eluted fractions of SPE columns was performed by recording proton (^{1}H) spectra using Bruker MSL 400 Spectrometer operating at 400MHz for 1 h and using 5 mm triple-resonance pulsed field gradient (PFG) probes. Each extract of 4 mg was dissolved in 1 mL deuterated methanol and samples were filtered using 0.45 µm nylon membrane filters. The filtered sample (0.7 mL) was then transferred to NMR tubes (Wilmad Precision NMR tube, 5 mm, LabGlass, Vineland, NJ, USA). The 1D ^{1}H spectra were acquired with a spectral width of 8223.685 Hz, pulse width of 10 seconds, 4 seconds acquisition time and 16 scans.

2.12 LCMS analysis of extracts

LCMS analysis was performed with a Varian quadrupole mass spectrometer (Varian, CA, USA) with an electrospray interface. A mass range of m/z 100 to 700 was used for scanning. Separation was performed using a Phenomenex Evo C18 column (50 x 2.1mm, 5µm) at a flow rate 0.5 mL/min. The separation was performed by gradient elution. The mobile phase consisted of solvent A (0.1% formic acid in water, pH 2-3.5) and B (0.1% acetonitrile) at gradient settings lasting 50 minutes, changing from 30% (A) to 70% (B) for 40

minutes again a combination of 30% - 70% and 80% - 20% for 5 minutes then finally 80% -20% for 5 minutes. Data were acquired using a Varian 310 MS Workstation.

2.13 Statistical analysis

For microbial counts, data were converted to log (cfu/mL) and parametric statistics (means ± standard errors) were used. All the results were expressed as mean ± SD. To determine the statistical differences between treatments, analysis of variance (ANOVA) was performed using GraphPad Prism V6 (GraphPad Software, Inc, CA, USA) statistical package at 95% confidence level. Patterns in the characteristics and physio-chemical properties of the extracts and original dried tissue sample were evaluated by PCA using the Unscrambler 10.1.

CHAPTER 3 PRELIMINARY EVALUATION OF ANTIMICROBIAL ACTIVITY OF *MOMORDICA* EXTRACTED IN DIFFERENT SOLVENTS

3.1 Introduction

There are many solvents available for extracting plant material and these include water, methanol, chloroform, hexane, ethanol and acetone. The solvents vary in their extraction capabilities depending on their chemical properties as well as their chemical structures. In choosing the right solvents for the extraction, it is important to consider certain factors including boiling point, density, viscosity, corrosiveness, flammability, toxicity, stability, compatibility with product, availability and cost (Cowan 1999). The selected solvents also differ in their chemical properties, which may determine the different substances they will extract. Optimisation of extraction conditions is very critical as it could have a significant impact on yield of bioactive compounds from plant materials (Złotek *et al.* 2015).

For this study, it was essential to investigate a range of solvents to evaluate if *Momordica* extracts demonstrated antimicrobial activity. The solvents that were used were water, chloroform-methanol and 70% methanol. Water was among the selected solvents because in the traditional approach it is the only solvent used in extraction, where leaves are boiled to make an infusion or tea. Chloroform-methanol and 70% methanol have been used previously as solvents by other researchers for the extraction of compounds from plants. Alzoreky and Nakahara (2003) screened extracts of some plants from Asia, produced using buffered methanol (80% methanol and 20% PBS) and acetone,

for antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella* Infantis. Their results revealed that both buffered methanol and acetone were good solvents in extracting inhibitory substances from the plants that were tested. Masoko and Eloff (2006) also showed that acetone and methanol extracted more biochemical compounds with antifungal activity from leaves of *Combretum* species compared to the solvents, hexane and dichloromethane. Karaman *et al.* (2003) found that methanol extracts of *Juniperus oxycedrus* showed inhibitory effects against bacteria including *S. aureus*, *S. epidermidis*, *Pseudomonas syringae*, *Pseudomonas putida* and *E. coli* whereas aqueous extracts had no antimicrobial activity against any of the tested bacteria. A study by Parekh *et al.* (2006) also showed that methanol extracts of 12 Indian medicinal plants had better antimicrobial activity against tested bacteria compared to aqueous extracts. The study also revealed that these extracts were more active against Gram-positive bacteria than against Gram-negative species. A study by Duraipandiyan *et al.* (2006) was in agreement with the previous studies showing that some air-dried ethnomedicinal plants from India showed a higher antimicrobial activity when extracted in methanol than in hexane. Parekh and Chanda (2008a) evaluated antibacterial activity of 34 Indian medicinal plants (including *M. charantia*) extracted in water and organic solvents (methanol or ethanol) against *S. aureus*, *S. epidermidis* and *S. subfavia* and demonstrated that methanol extracts had greater inhibition than ethanol with the least activity shown by aqueous extracts. In this study, methanol extracts of one Indian medicinal plant (*Woodfordia fruticose*) showed the greatest inhibition zones (negative control zones subtracted from test zones) against *S. aureus* (15

mm), *S. epidermidis* (9 mm) and *S. subfava* (10 mm). On the other hand, methanol extracts of *M. charantia* aerial parts displayed inhibition zones of 7 mm, 2 mm, and 4 mm against *S. aureus*, *S. epidermidis* and *S. subfavia*, respectively whereas aqueous extracts only showed inhibition zones of 2 mm against S. *epidermidis*. Contrary to other researchers, Madureira *et al.* (2012) revealed that methanol extracts of *M. balsamina* aerial parts gave the lowest antimicrobial activity against *S. aureus*, *Ent. faecalis*, and *P. aeruginosa* compared to extracts of ethanol, dichloromethane, and hexane. Water extracts of a commercial Zulu herbal mixture exhibited the lowest antimicrobial activity compared to extracts made using petroleum ether, dichloromethane and ethanol (Ndhlala *et al.* 2011). In contrast to most of the above results Rojas *et al.* (2006) reported that the water extracts of *Bidens pilosa*, *Jacaranda mimosifolia* D.Don, and *Piper pulchrum* displayed a higher antimicrobial activity against *Bacillus cereus* and *Escherichia coli* than ethanol extracts while the ethanol extracts of most species were active against *Staphylococcus aureus*. Faujda *et al.* (2013) showed that aqueous extracts of *Momordica balsamina* fruit contained more phytochemical compounds (phenolic compounds, flavonoids, amino acids, alkaloids and saponins) compared to 90% methanol extracts which showed phenolic compounds, flavonoids, amino acids but not saponins. In another study, ethanol extracts of *Lawsonia inermis* were shown to have better antimicrobial activity against bacteria such as *S. aureus, B. cereus, E. coli, Salmonella* Typhi compared to petroleum ether and chloroform extracts. For *Mimosa pudica* plants however, antimicrobial activity varied among the different solvent extracts with ethanol extracts exhibiting a higher antimicrobial activity against *S. aureus* and *S.* Typhi; petroleum ether

extracts gave higher antimicrobial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa*; and chloroform extracts had higher activity against *Bacillus cereus* and *Shigella shinga* (BMLRU1013) (Akter *et al.* 2010). Based on the above studies it is clear that antimicrobial activity of a plant may be attributed to the different phytochemical components existing in the crude extracts (Rojas *et al.* 2006; Akter *et al.* 2010) which are dependent on the extraction solvent used.

Antibacterial effects might vary depending on the processing method such as drying and boiling of the tissue, which determines the concentration of active compounds of the plant (Shunying *et al.* 2005). Mayachiew *et al.* (2010) evaluated the effect of drying methods on antimicrobial activity of galangal extracts added to film-forming solutions against *S. aureus*. This study compared ambient drying, hot air drying at 40°C, vacuum drying and low-pressure superheated steam drying within a temperature range of 70 – 90°C at an absolute pressure of 10 kPa. Their results showed that ambient drying, hot air drying and low-pressure superheated steam drying at 70°C led to films with higher antimicrobial activity against *S. aureus*. Ennajar *et al.* (2010) evaluated effects of drying methods comparing sun-drying, shade-drying and oven-drying at 45°C on chemical composition, antioxidant activity and antimicrobial activity of *Juniperus phoenicea* essential oils. This study demonstrated that drying of leaves in the sun and berries in oven-drying were most suitable to obtain higher levels of essential oils. The study recommended the use of oven-drying compared to other drying methods for better antimicrobial activity.

For the current study, effects of oven-drying and air-drying of plant tissues were determined initially in water and chloroform-methanol extracts; for methanol-water extractions, only oven-dried tissue was studied.

Objective of this study: To compare the antimicrobial properties of different solvent extracts of *Momordica* plants.

Hypothesis: The antimicrobial activity of *Momordica* plants extracts will be greatly affected by the solvent used for extraction.

3.2 Results

3.2.1 Bacterial strain confirmation for antimicrobial studies

A broad range of organisms consisting of 2 Gram-negative bacteria, *Escherichia coli* O157 Sakai *stx*- and *Salmonella* Typhimurium, and 4 Gram-positive bacteria, *Listeria monocytogenes* 11944, *Staphylococcus aureus* RN4220, *Staphylococcus aureus* NCTC 10652 and *Staphylococcus aureus* NCTC 10655 were selected to test *Momordica* extracts ability to inhibit their growth. Strains were obtained from the University of Nottingham Division of Food Sciences laboratory culture collection. After recovery from frozen culture, strain identity was checked using a standard set of identification tests (Table 3-1, Figures 3-1).

Table 3-1. Characteristics of test bacteria on different media.

Bacteria	**Medium**	**Bacteria characteristics**
Sal. Typhimurium LT2	TSA	Colonies were colourless/off white, circular, convex and about 3mm diameter
	MacConkey no. 3	Colonies were shiny yellow, irregular circle, convex and about 5 mm diameter
	XLD	Colonies red with black centres, circular, convex and about 2 mm diameter (Figure 4-1)
E. coli O157 Sakai *stx*-	TSA	Colourless, convex, irregular shaped colonies and about 3-4 mm diameter
	MacConkey no. 3	Colonies were pink/red, spindle shaped and about 4-5 mm diameter shape
	Sorbitol/MacConkey	Colourless, round, convex colonies and about 2.5 mm diameter
L. monocytogenes 11944	TSA	Round, colourless, convex colonies and about 1 mm diameter
	L/MOX	Brown round colonies, convex and about 1 mm diameter reaction, surrounded by blackening of the medium

Table 3-1 Characteristics of test bacteria on different media (continued).

S. aureus RN4220	TSA	Cream, round, raised colonies and about 1 – 1.5 mm diameter
	BP Medium	Grey-black shiny, round, convex colonies with a clear halo and about 1 mm diameter
S. aureus NCTC 10652	TSA	Dark cream/ gold round, convex colonies and about 2 – 3 mm diameter
	BP Medium	Grey/black round, convex colonies and about 1.5 mm diameter with halos
S. aureus NCTC 10655	TSA	Light cream/white round, convex colonies and about 1 mm diameter
	BP Medium	Grey/black round, convex colonies and about 1 mm diameter with halos

Bacterial characteristics on different agars. One colony of each bacterium was streaked into the agar following a standard aseptic technique and the four-way streak plate inoculation and this was incubated at 37ºC for 18-24 hours. Results show various reactions depending on bacteria and medium used. TSA – Tryptone Soy Agar, XLD - Xylose lysine deoxycholate agar, L/MOX – Listeria Modified Oxford Agar, BP – Baird-Parker Medium.

Results in Table 3-1 confirm the different characteristics of bacteria on different media as described by Bridson (2006) on bacteria identification systems. These results match with the description of all the bacteria, which confirms the bacteria identification. Figure 3-1 presented a confirmation of *Sal.* Typhimurium on XLD agar

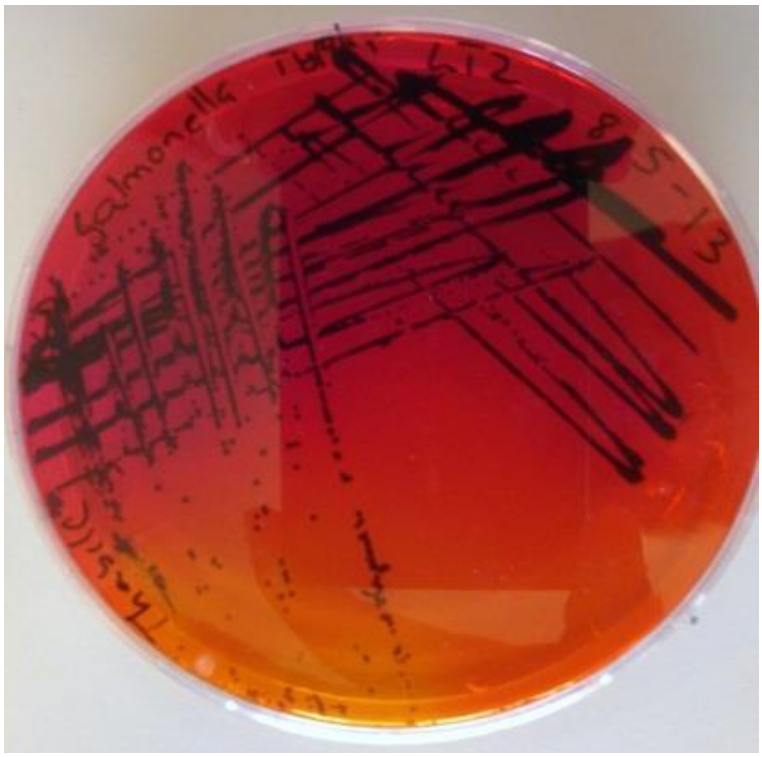


Figure 3-1. *Sal*. Typhimurium grown on XLD agar after 24 hours of incubation. Colonies were red with black centres, circular, convex and about 2 mm diameter that confirmed that bacterium was *Salmonella.*

Salmonella Typhimurium LT2 and *E. coli* O157 Sakai *stx*- were confirmed as Gram negative, catalase positive and oxidase negative and rod-shaped. *Salmonella* polyvalent H and Monovalent specific O antisera tests were both positive confirming that strain was O4 and therefore group B.

Listeria monocytogenes 11944, *S. aureus* RN4220, *S. aureus* NCTC 10652 and *S. aureus* NCTC 10655 were confirmed as Gram positive, catalase positive and oxidase negative. *L. monocytogenes* was also confirmed as a rod-shaped bacterium and *S. aureus* strains were cocci. The coagulase test for all *S. aureus* strains was positive, confirming that strains were indeed *S. aureus*.

3.2.2 Biochemical profiling

API 20 E results gave a good identification of 99.9% for *S.* Typhimurium and 89.6% for *E. coli* O157. API Listeria confirmed a good identification of 98.6% for *Listeria monocytogenes*. API Staph results gave varied confirmation for different *S. aureus* strains. Results for *S. aureus* RN4220 gave a doubtful profile with a lower identification percentage (49.7%) suggesting the possibility that the strain could be *Staphylococcus warneri* or *sciuri*; this was in contradiction to the positive coagulase test as these are coagulase negative species. Results for *S. aureus* NCTC 10652 gave an acceptable identification of 84.4% while *S. aureus* NCTC 10655 gave a good identification (97.4%). Results from the toxin-producing strains of *S. aureus* had an identification that was generally considered good in comparison with the RN4220 strain, which gave an uncertain result. The RN4220 strain was used briefly for antimicrobial

studies but because of its uncertain results in identification, antimicrobial results were unreliable and its use was discontinued.

3.2.3 Effect of extraction solvent and drying method on yield of extract for *Momordica balsamina* and *Momordica foetida*

For the current study, preliminary evaluation of antimicrobial activity employed plant material that had either been air-dried under shade or oven-dried (Section 2.1). For further studies, oven drying was then employed. Air-drying is a method that takes many days for samples to dry due to the ambient temperatures; therefore, it was not a suitable method for this study. Extraction solvents employed were hot water, chloroform-methanol and methanol-water (Section 2.2). These solvents were selected due to their different extracting capabilities based on polarity.

The extracts were dried and the resultant residue weighed to give a yield as g/gDWt. Results for yield for the various hot water, chloroform-methanol and 70% methanol extracts are shown in Figure 3-2. The water extracts of *M. balsamina* and *M. foetida* gave a significantly higher yield ($P < 0.05$) followed by methanol (70%) extracts, whilst chloroform-methanol extracts gave the lowest yield ($P < 0.05$) The results suggest differences were attributable to the different characteristics of the extraction solvents that were used in this study. There was no significant difference ($P > 0.05$) in yield between air-dried and oven-dried extracts.

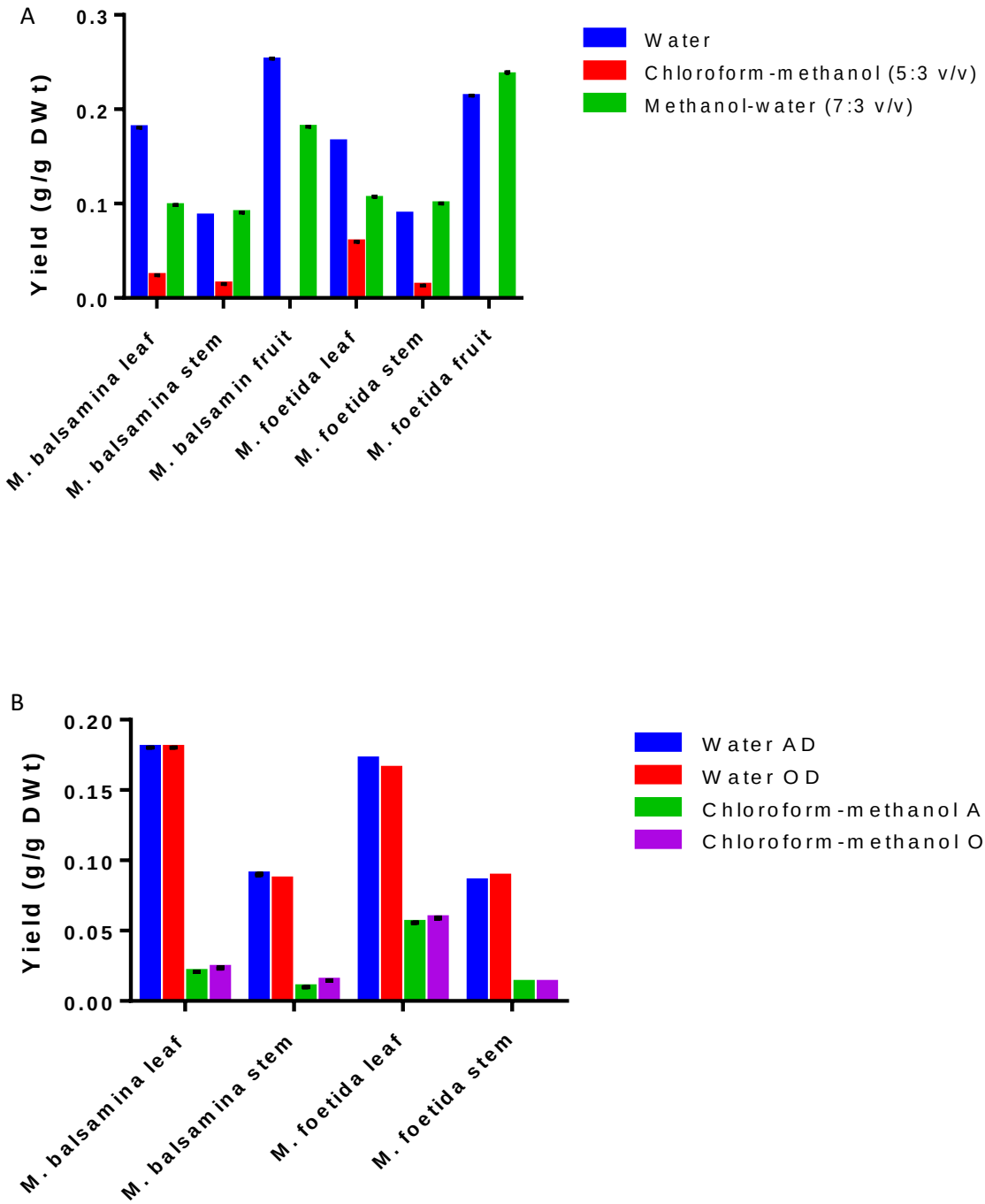


Figure 3-2. Yield (g/g DWt) of water, chloroform-methanol (5:3 v/v) and methanol-water (7:3 v/v) dry extracts of *Momordica* plants. A) Effect of extraction solvent on yield of oven dried extracts. B) Effect of drying method on yield of water and chloroform-methanol extracts. Samples were extracted using a plant tissue: solvent ratio of 1:10 (w/v). AD - Air-dried, OD – Oven-dried. Results are a mean ±SD (n = 3).

3.2.4 Effect of solvent and drying method on antimicrobial activity of *Momordica* extracts

Dried extracts were re-suspended in PBS and used at a concentration of 4mg/disc in a disc assay to assess antimicrobial activity (Section 2.6.1) and the results are shown in Tables 3-2, 3-3 and Figures 3-3, 3-4 and 3-5.

Antimicrobial activity of oven-dried and air-dried *M. balsamina* and *M. foetida* extracted in hot water, chloroform-methanol or methanol-water was determined using the agar disc diffusion method (Section 2.6.1) and results were reported as mean diameter of the zone of inhibition (mm). Table 3-2 presents inhibition zone sizes produced by water and chloroform-methanol extracts while inhibition zones of methanol-water extracts are displayed in Table 3-3. Diameter of inhibition zone sizes (mm) for the positive control (ampicillin 10 µg/disc) are displayed in Figure 3-3.

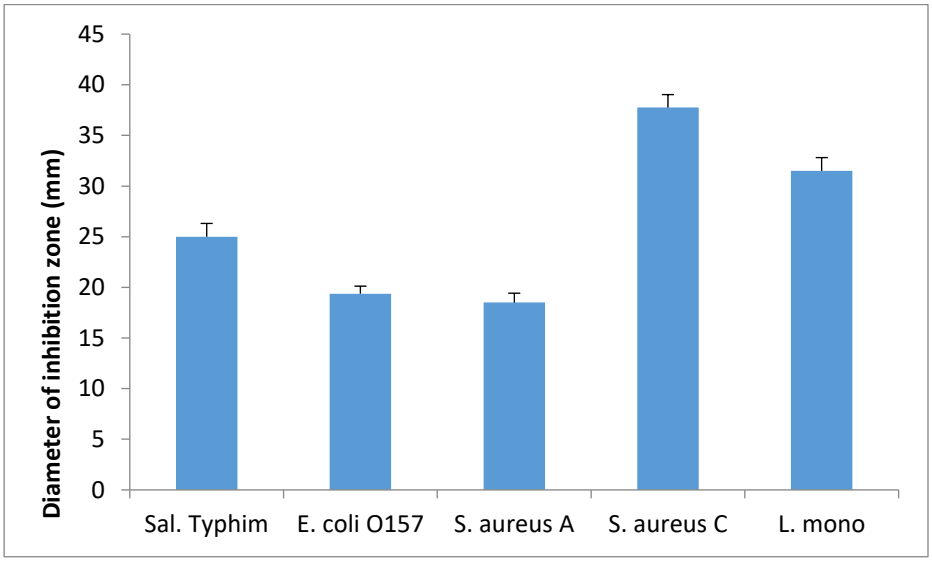


Figure 3-3. Mean diameter of the inhibition zone sizes (mm) of ampicillin (10 µg/disc) against *E. coli* O157, *Sal.* Typhimurium, *S. aureus* and *L. monocytogenes*. *S. aureus* A – NCTC 10652, *S. aureus* C – NCTC 10655.

3.2.4.1 Hot Water extracts

Hot water extracts of air-dried or oven dried *M. balsamina* and *M. foetida* leaves, and stem did not show any antimicrobial activity against any of the bacterial strains tested.

3.2.4.2 Chloroform-methanol extracts

Chloroform-methanol extracts of air-dried *M. balsamina* leaves displayed antimicrobial activity against *E. coli* O157 Sakai *stx-*, *L. monocytogenes*11944, *S. aureus* NCTC 10652, and *S. aureus* NCTC 10655, with inhibition zones of 9.0, 8.3, 8.0 and 8.0 mm, respectively (Figure 3-4 and Table 3.2) while no activity was seen against *Sal*. Typhimurium; interestingly oven-dried leaf extracts did not show any antimicrobial activity. Oven-dried *M. balsamina* stem extracts showed antimicrobial activity against *S. aureus* NCTC 10652, and *S. aureus* NCTC 10655 with inhibition zones of 6.5 and 7 mm, respectively, yet air-dried stem extracts did not show any antimicrobial activity. The results demonstrate that *M. balsamina* leaves and stem extracts have some antimicrobial activity. Negative controls (PBS and chloroform-methanol) did not show any activity therefore antimicrobial activity observed in chloroform-methanol extracts was not a result of residual solvents.

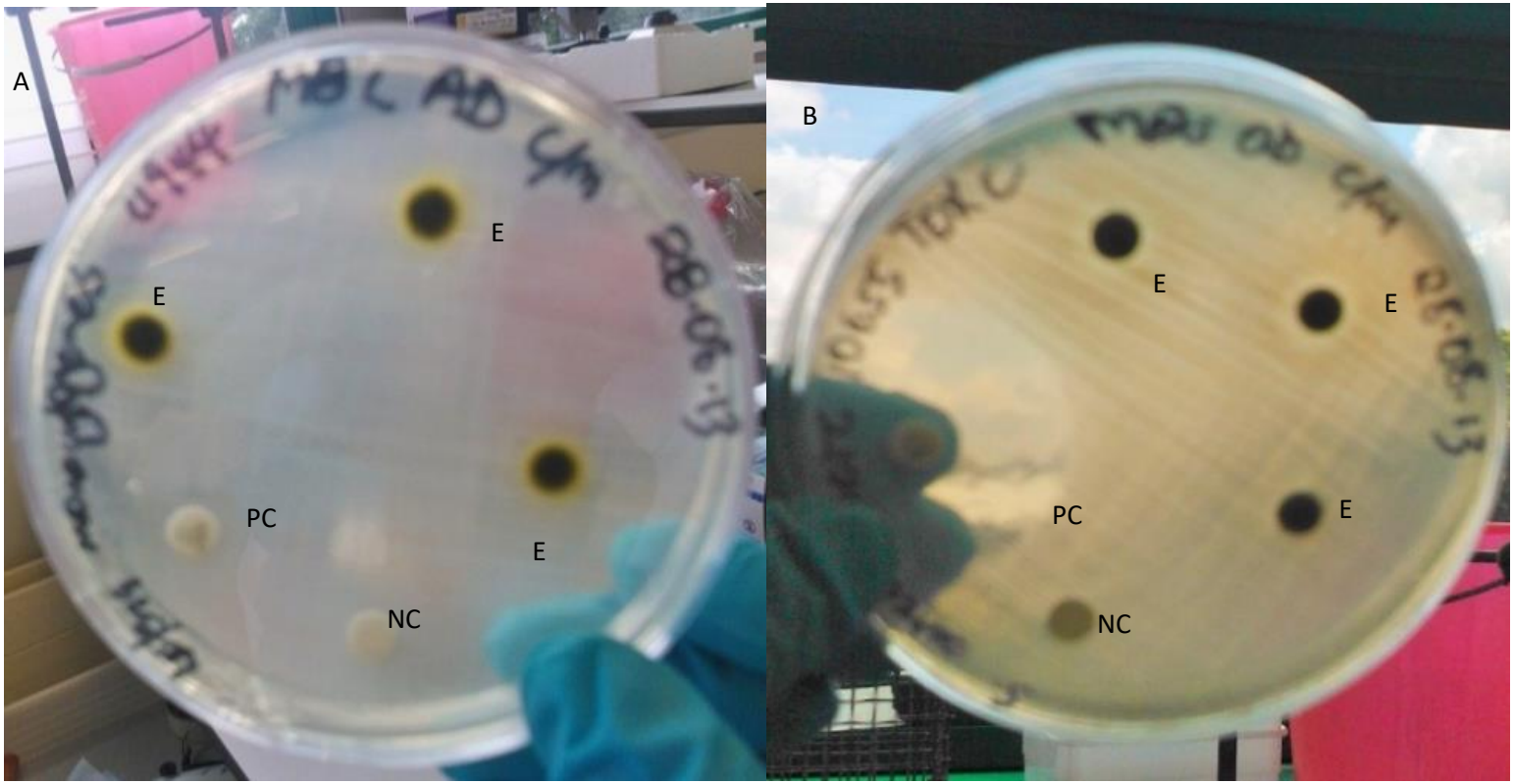


Figure 3-4. Inhibition zone obtained from chloroform-methanol extracts of *Momordica balsamina* leaves and stem
(A) air-dried leaf extract (E) tested against *L. monocytogenes* and (B) oven-dried stem extract tested against *S. aureus* NCTC 10655 both after 24 hours of incubation at 37°C. Negative control (NC) (20 µL PBS) had no inhibition zone while positive control (PC) (ampicillin 10 µg/disc) showed inhibition zone against both strains.

Table 3-2. Antimicrobial activity of *Momordica* water and chloroform-methanol (5:3 v/v) extracts

Extract	Bacterium	Zone of Inhibition (mm)			
		Water extracts (4mg/disc)		Chloro-meth extract (4mg/disc)	
		OD	AD	OD	AD
MBL	*E. coli* O157 Sakai *stx*-	No zone	No zone	No zone	9.0 ± 0.0
	Salmonella Typhimurium	No zone	No zone	No zone	No zone
	Staphylococcus aureus RN4220	No zone	No zone	ND	ND
	Staphylococcus aureus NCTC 10652	ND	ND	No zone	8.0 ± 0.0
	Staphylococcus aureus NCTC 10655	ND	ND	No zone	8.0 ± 0.0
	Listeria monocytogenes 11944	No zone	No zone	No zone	8.3 ± 0.6
MBS	*E. coli* O157 Sakai *stx*-	No zone	No zone	No zone	No zone
	Salmonella Typhimurium	No zone	No zone	No zone	No zone
	Staphylococcus aureus RN4220	No zone	No zone	ND	ND
	Staphylococcus aureus NCTC 10652	ND	ND	6.5 ± 0.0	No zone
	Staphylococcus aureus NCTC 10655	ND	ND	7.0 ± 0.0	No zone
	Listeria monocytogenes 11944	No zone	No zone	No zone	No zone

Table 3-2. Antimicrobial activity of *Momordica* water and chloroform-methanol (5:3 v/v) extracts (continued)

MFL	*E. coli* O157 Sakai *stx*-	No zone	No zone	No zone	No zone
	Salmonella Typhimurium	No zone	No zone	No zone	No zone
	Staphylococcus aureus RN4220	No zone	No zone	ND	ND
	Staphylococcus aureus NCTC 10652	ND	ND	No zone	No zone
	Staphylococcus aureus NCTC 10655	ND	ND	No zone	No zone
	Listeria monocytogenes 11944	No zone	No zone		No zone
MFS	*E. coli* O157 Sakai *stx*-	No zone	No zone	No zone	No zone
	Salmonella Typhimurium	No zone	No zone	No zone	No zone
	Staphylococcus aureus RN4220	ND	ND	ND	ND
	Staphylococcus aureus NCTC 10652	ND	ND	No clear zone	No clear zone
	Staphylococcus aureus NCTC 10655	No zone	No zone	No clear zone	No clear zone
	Listeria monocytogenes 11944	No zone	No zone	No zone	No zone

Mean inhibition zones (mm) produced with water or chloroform-methanol extracts of *Momordica* (4mg/disc). Each value represents 3 replicates. MBL – *M. balsamina* leaves, MBS – *M. balsamina* stem, MFL – *M. foetida* leaves, MFS – *M. foetida* stem, OD – Oven-dried, AD – Air-dried and ND – Not done. No clear zone means reduction of growth around discs was observed.

3.2.4.3 Methanol-water extracts

Methanol -water extracts of oven-dried *M. balsamina* leaves, stem and *M. foetida* fruits displayed antimicrobial activity against *S. aureus* NCTC 10652, *S. aureus* NCTC 10655 and *L. monocytogenes* (Table 3-3 and Figure 3-5). *M. balsamina* leaves extracts displayed the largest inhibition zone, among extracts tested, against *L. monocytogenes* and then *S. aureus* NCTC 10652 and *S. aureus* NCTC 10655 while *M. foetida* fruit and *M. balsamina* stem displayed lower inhibition zones against *L. monocytogenes* and then *S. aureus* NCTC 10652 and *S. aureus* NCTC 10655.

Extracts of *M. balsamina* fruits did not display growth inhibition zones against any test bacteria. *M. balsamina* and *M. foetida* extracts (leaves, stem and fruit) did not exhibit any inhibition zones against the Gram negatives strains *E. coli* O157 Sakai *stx*- and *Sal.* Typhimurium.

Table 3-3. Antimicrobial activity of methanol -water (7:3v/v) extracts of oven-dried *Momordica* samples

Bacterium	Extracts (4mg/disc)			
	MBL	MBS	MBF	MFF
E. coli O157 Sakai *stx*-	No zone	No zone	No zone	No zone
Salmonella Typhimurium	No zone	No zone	No zone	No zone
Staphylococcus aureus NCTC 10652	10.0 ± 0.0	6.5 ± 0.0	No zone	8.3 ± 0.6
Staphylococcus aureus NCTC 10655	9.7 ± 0.6	6.5 ± 0.0	No zone	No zone
Listeria monocytogenes 11944	11.0 ± 0.0	7.8 ± 0.3	No zone	7.0 ± 0.0

Mean inhibition zone (mm) produced by methanol -water extracts (7:3 v/v) of *Momordica* extracts (4mg/disc). Each value represents 3 replicates. MBL – *M. balsamina* leaves, MBS – *M. balsamina* stem, MBF – *M. balsamina* fruit, MFF– *M. foetida* fruit.

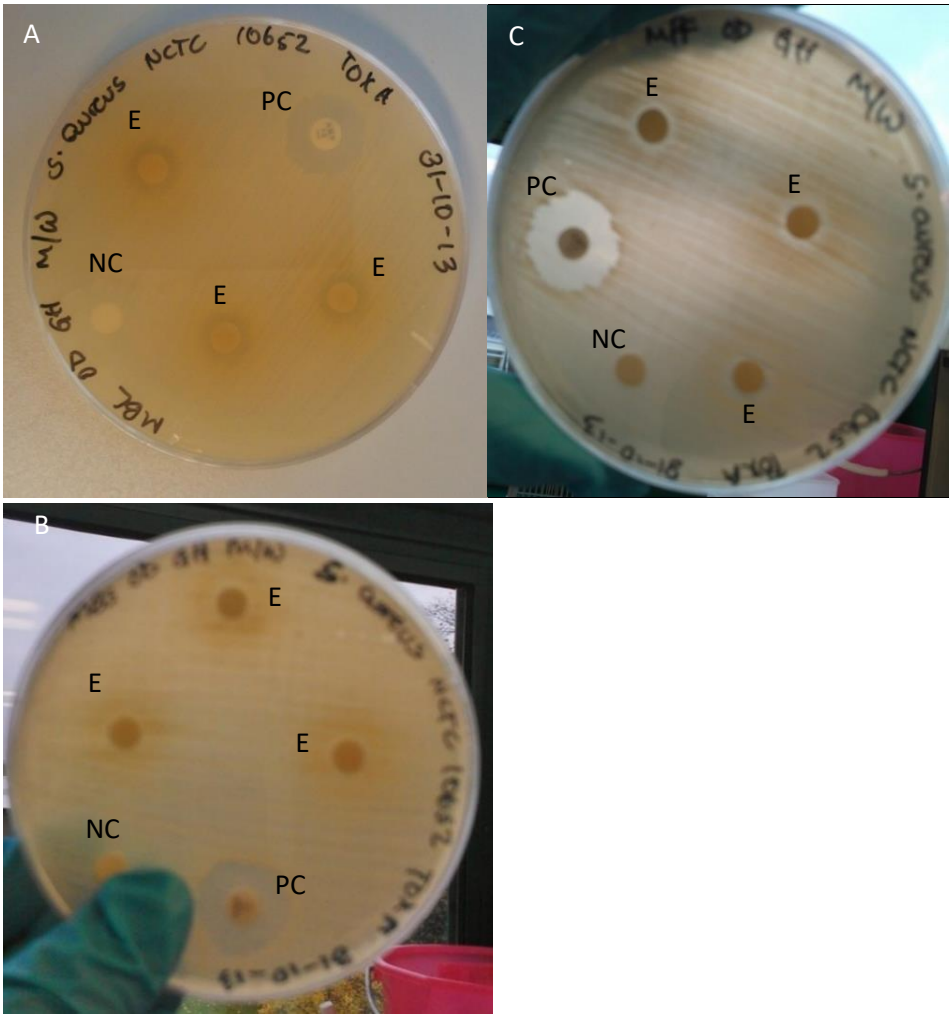


Figure 3-5. Inhibition zones caused by methanol -water extracts (E) (7:3 v/v) of *M. balsamina* leaves (A), *M. balsamina* stem (B) and *M. foetida* fruits (C) against *S. aureus* NCTC 10652 (Tox A) after 24 hours of incubation at 37°C. Negative control (NC) (20 µL PBS) had no inhibition zone while positive control (PC) (ampicillin 10 µg/disc) showed inhibition zones against all strains tested.

3.3 Discussion

3.3.1 Bacterial identification tests

Initially only one *S. aureus* strain (RN4220) was used but from the identification tests, The API Staph result presented a doubtful profile suggesting the possibility that this strain might not be *S. aureus*, although other tests were consistent with this identification. Two other strains (NCTC 10652 and NCTC 10655) were therefore included in the panel of strains and for these the API Staph results (and all other identification tests) gave a good confirmation of strain identity for both strains. These strains were therefore used in the rest of the study. All other strains (*L. monocytogenes*, *E. coli* O157 Sakai *stx*- and *Sal.* Typhimurium) gave the expected test results and so were included for testing the antimicrobial activity of *Momordica* extracts.

3.3.2 Antimicrobial activity of *Momordica* extracts

Extract yield is important when evaluating antimicrobial activity of crude extracts. Screening of antimicrobial activity involves repeating experiments for reproducibility as well as doing different types of tests to determine and confirm the activity. Results from the current study revealed that hot water extracts exhibited a higher yield of dried extract than methanol-water extracts and these in turn gave better yields than chloroform-methanol oven-dried extracts. Drying of *Momordica* tissue by either air or oven did not significantly affect the yield of extracts, however for further studies oven drying was selected because it was a fast and convenient method. Even though water extracts gave the highest yields in both *Momordica* plants, they proved

not to have activity against the test bacteria (Table 3-2). Successful determination of biologically active components from plants is mainly dependant on the type of solvent employed in the extraction procedure (Ncube *et al.* 2008). If antimicrobial activity is from the non-polar compounds, then antimicrobial activity may not be displayed by water extracts of the plants. Results of this study are in agreement with the findings by Karaman *et al.* (2003) who demonstrated that aqueous extracts of *Juniperus oxycedrus* had no antimicrobial activity against all test bacteria. Parekh *et al.* (2006); and Parekh and Chanda (2008b) also confirmed that aqueous extracts of medicinal plants from India had less antimicrobial activity that methanol extracts. Water has also been used by other researchers who indicated that water extracts gave some inhibitory effect against a multi-drug resistant *Salmonella* Typhi (Akinyemi *et al.* 2005) and *Shigella* species (Iwalokun *et al.* 2001) Gram-negative species. Water extracts of *M. balsamina* leaves displayed lower antimicrobial activity against *E. coli* and *S. aureus* strains in another study (Ndhlala *et al.* 2011). Results from the current study are in contrast with findings from these researchers as extracts obtained in the current study displayed activity against Gram-positive bacteria but not against Gram-negative bacteria. This might be attributable to the fact that active components vary depending on many factors such as plant source, species and growth conditions. In this case plants used for this study were sourced from a different area (Swaziland) to those in published studies and thus grown under different conditions. One important factor of solvent effectiveness is the active components of a plant.

Results from chloroform-methanol extracts suggest that there seems to be a varied antimicrobial activity among leaves and stem depending on the drying method used prior to extraction (Table 3-2). This raises a question of whether the drying method does affect antimicrobial activity. An interesting finding is that, for leaves, activity was observed in air-dried plants yet for stems activity was observed in oven-dried plants. This signifies the need for further tests to establish and confirm these results. The other interesting finding is that chloroform-methanol extracts of air-dried *Momordica balsamina* leaves showed antimicrobial activity against *E. coli* O157 Sakai *stx*-, a Gram-negative bacterium but not *Sal.* Typhimurium however this was not a reproducible result as further tests could not confirm antimicrobial activity against *E. coli* O157 Sakai *stx*-. It is important to consider that antimicrobial activity can also be dependent on the type of microorganisms studied. Zaika (1988) reported that microorganisms differ in their resistance to a given plant extract and a certain microorganism differs in its resistance to extracts from many types of plants. Other researchers have used methanol as an extraction solvent and their results showed antimicrobial activity against *S. aureus*, *E. coli* and *Salmonella* Typhi among other bacteria tested (Jigam *et al*. 2004; Otimenyin *et al*. 2008; Madureira *et al.* 2012). Other researchers have studied chloroform extracts of *M. balsamina* against *S. aureus* and *E. coli* among other bacteria tested, and their results displayed antimicrobial activity against *B. subtilis* but not on *S. aureus* and *E. coli* strains (Akbar and Al-Yahya 2011). None of the available studies used chloroform-methanol in combination as an extraction solvent.

Even though results displayed no inhibition from chloroform-methanol extracts of *M. foetida* leaves or stem, the reduction in growth observed around the discs in stem extracts appears to suggest that these extracts may have some antimicrobial activity against the *S. aureus* strains that were tested. Other studies on antimicrobial activity of *M. foetida* suggest that methanolic leaf and stem extracts may have an activity against some bacteria including *S. aureus* (Boily and Van Puyvelde 1986; Geyid *et al.* 2005). Other researchers found that chloroform extracts of *M. foetida* leaf exhibit a higher broad-spectrum antibacterial activity against the bacteria tested which included *E. coli, B. cereus, S. aureus* but not against *Samonella* spp. (Odeleye and Oyedeji 2008).

Results from methanol - water extracts (7:3 v/v) extracts showed antimicrobial activity against the *S. aureus* strains tested and *L. monocytogenes*. Extracts of *M. balsamina* leaves showed the highest antimicrobial activity against these three bacterial strains yet *M. balsamina* stem extracts displayed the least antimicrobial activity. These results are in agreement with a study by Otimenyin *et al.* (2008b), who also used methanolic and water extracts (ratio 70:30) of *M. balsamina* leaves and stem and reported antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Salmonella* Typhi, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Jigam *et al.* (2004) also studied methanolic extracts of the whole *M. balsamina* plant found in Nigeria against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* Typhi and their results showed that whole extracts of *M. balsamina* had antibacterial activity against all test bacteria.

Worth noting is that most of the antimicrobial activity observed in the solvent extracts was against the Gram-positive bacteria. These results are in agreement with Zaika (1988) who reported that plants extracts showed activity mostly against Gram-positive bacteria, in particular *S. aureus* (Hsieh *et al.* 1998) rather than Gram-negative bacteria. Results from these preliminary studies are confirmatory that solvent and type of plant or plant part are all important factors in evaluating antimicrobial activity. This is in agreement with a study by Garvey *et al.* (2011) who evaluated chloroform, methanol and water extracts of some medicinal plants and concluded that varied plants extracted by different solvents displayed varied antimicrobial activities. Their studies reported high antimicrobial activity in *Lithospermum officinale* extracted with chloroform, methanol and water; *Magnolia officinalis* extracted in chloroform and methanol; *Cinnamomum aromaticum* extracted in chloroform; *Pimpinella anisum* extracted in chloroform and *Pulmonaria officinalis* extracted in chloroform and methanol.

3.4 Conclusion

The results of this study displayed that antimicrobial activity was mainly dependant on the extraction solvent and to some extend the drying method. Standardization of the extraction solvent and drying method is therefore important, as they will directly influence the results. The two species of *Momordica* plants gave varied antimicrobial activity based on the plant part studied. Leaf extracts generally displayed higher antimicrobial activity compared to other plant tissues. Methanol-water extracts gave the best

antimicrobial activity. Gram-positive bacteria showed more susceptibility to extracts than Gram-negative bacteria. There were no further studies on chloroform-methanol extracts due to the low yield of the extracts, which contributed to unreliable results. The rest of the studies used oven-dried extracts and methanol-water solvent for extraction of the *Momordica* plants.

CHAPTER 4 ANTIMICROBIAL ACTIVITY OF METHANOL-WATER EXTRACTS OF *MOMORDICA BALSAMINA* AND *MOMORDICA FOETIDA*

4.1 Introduction

Having establishing, from the previous chapter, that methanol-water extracts of *Momordica* plants were the best for further studies, it was necessary to conduct more antimicrobial studies to establish reproducibility of results and for confirmation. There are many different methods employed for the determination of antimicrobial activities. Optimization of screening methods for the identification of antimicrobials from plants is of great significance (Rios *et al.* 1988; Valgas *et al.* 2007). Rios *et al.* (1988) summarized screening methods as diffusion (disc, well and cylinder) designed to screen the amount of antibiotic substances in crude extracts; dilution (liquid and agar dilution) mainly for the determination of MIC; and bioautographic methods (paper chromatography and thin layer chromatography) which enables localization of antimicrobial activity on a chromatogram. Tepe *et al.* (2005) determined antimicrobial activity of essential oils and various extracts of *Salvia tomentosa* Miller using agar well diffusion and disc diffusion before determination of MIC. In their results, the disc diffusion method displayed more activity of extracts compared to the agar well diffusion method, hence MIC was only performed against results obtained from the disc diffusion method. Valgas *et al.* (2007) on the other hand concluded that the well diffusion method was more sensitive than the disc diffusion method in the determination of

antimicrobial activity and also reported that diffusion methods showed similar sensitivity to bioautographic methods.

For the current research, antimicrobial activity of extracts was evaluated using a disc diffusion method, spot test, extract treatment survival assay and liquid dilution methods. The different methods were selected because screening methods are not based on the same principle thus results may be greatly affected by those methods among other factors (Rios *et al.* 1988).

Objectives: to evaluate antimicrobial activity of methanol-water *Momordica* extracts; to compare extracts from plants which were glass-house produced in the UK and those sourced from Swaziland; to establish antimicrobial activity of solid phase extraction (SPE) extracts and to compare activity of the elute fraction of the extracts with activity of crude extracts; and to study the differences in bacterial growth curves in liquid cultures treated with the extracts.

4.2 Summary of methods

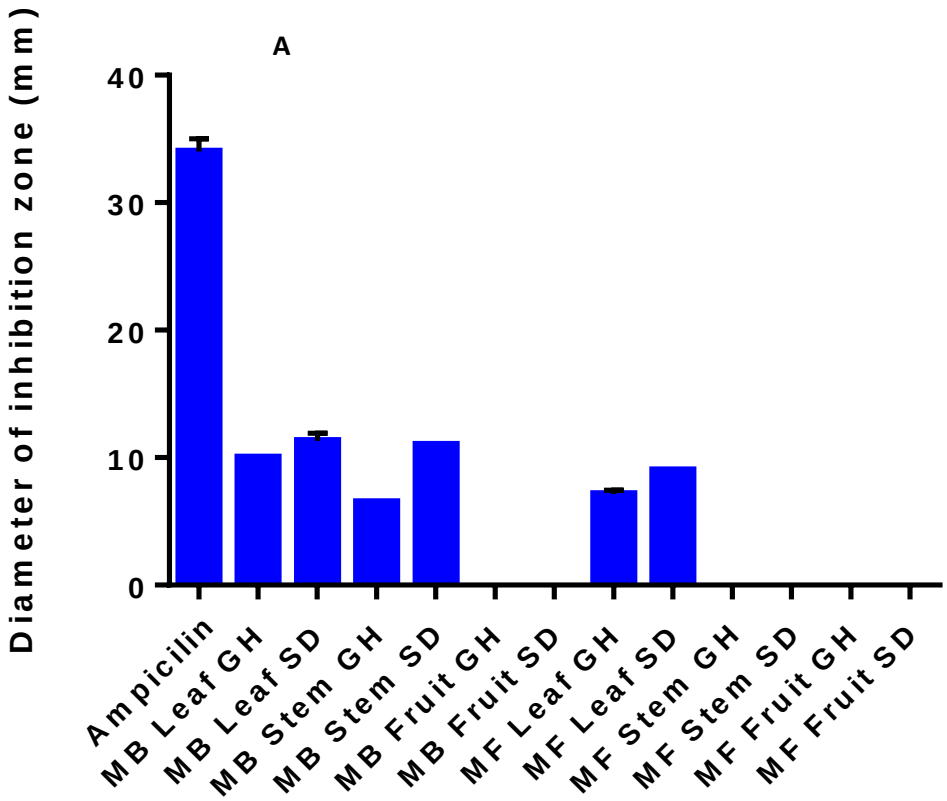
The effect of *Momordic*a crude extracts, and SPE fractions from the crude extract, were tested on several bacteria (*S. aureus* NCTC 10652, *S. aureus* NCTC 10655, *L. monocytogenes* 11944, *Sal.* Typhimurium LT2 and *E. coli* O157:H7 Sakai *stx*-). These were initially assayed by the disc diffusion method or spot test. For crude extracts, further antimicrobial studies including determining the level of microbial sensitivity of a culture mixed with a given concentration of extract over a set exposure time and then plated using the Miles and Misra method (referred to hereafter as extract treatment survival

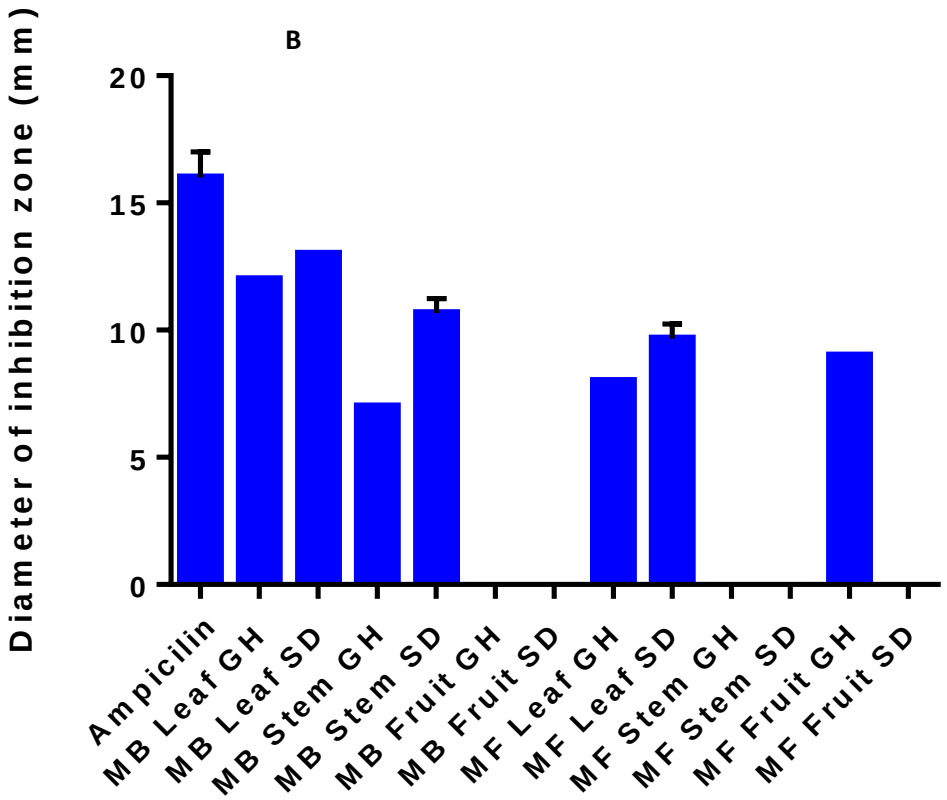
assay), determining the minimum inhibitory concentrations (MICs) using a microdilution method and minimum bactericidal concentrations (MBCs) using a spot test. To determine whether *Momordica* extracts were bacteriostatic or bactericidal, cultures were exposed to the extract and then the growth curves and viable counts (using Miles Misra) were followed. For SPE fractions of the extracts, further antimicrobial studies included determining the MICs and MBCs of the phenolic fraction that had the highest antimicrobial activity in initial screening. All screening methods for antimicrobial activity were described in Section 2.5.

4.3 Results

4.3.1 Antimicrobial activity of *Momordica* extracts: Disc diffusion assay

The antimicrobial activities of methanol-water extracts of *M. balsamina* and *M. foetida* against *E. coli* O157 Sakai *stx*-, *Sal.* Typhimurium LT2, *S. aureus* NCTC 10652 (Tox A), *S. aureus* NCTC 10655 (Tox C) and *L. monocytogenes* 11944 were evaluated using the disc diffusion method. A standard amount of 4 mg of extract was used per disc and phosphate buffer solution (PBS) (20 µL/disc) was applied as the negative control. In all instances the negative controls gave no inhibition zones. The resultant inhibition zone diameters from the extracts are shown in Figure 4-1. *M. balsamina* leaf extracts from the Swazi sample showed a relatively higher inhibitory effect against all Gram positive bacteria tested than similar extracts from the glasshouse grown plants.


A
Diameter of inhibition zone (mm)
40
30
20
10
0
Ampicilin
MB Leaf GH
MB Leaf SD
MB Stem GH
MB Stem SD
MB Fruit GH
MB Fruit SD
MF Leaf GH
MF Leaf SD
MF Stem GH
MF Stem SD
MF Fruit GH
MF Fruit SD


B
Diameter of inhibition zone (mm)
20
15
10
5
0
Ampicilin
MB Leaf GH
MB Leaf SD
MB Stem GH
MB Stem SD
MB Fruit GH
MB Fruit SD
MF Leaf GH
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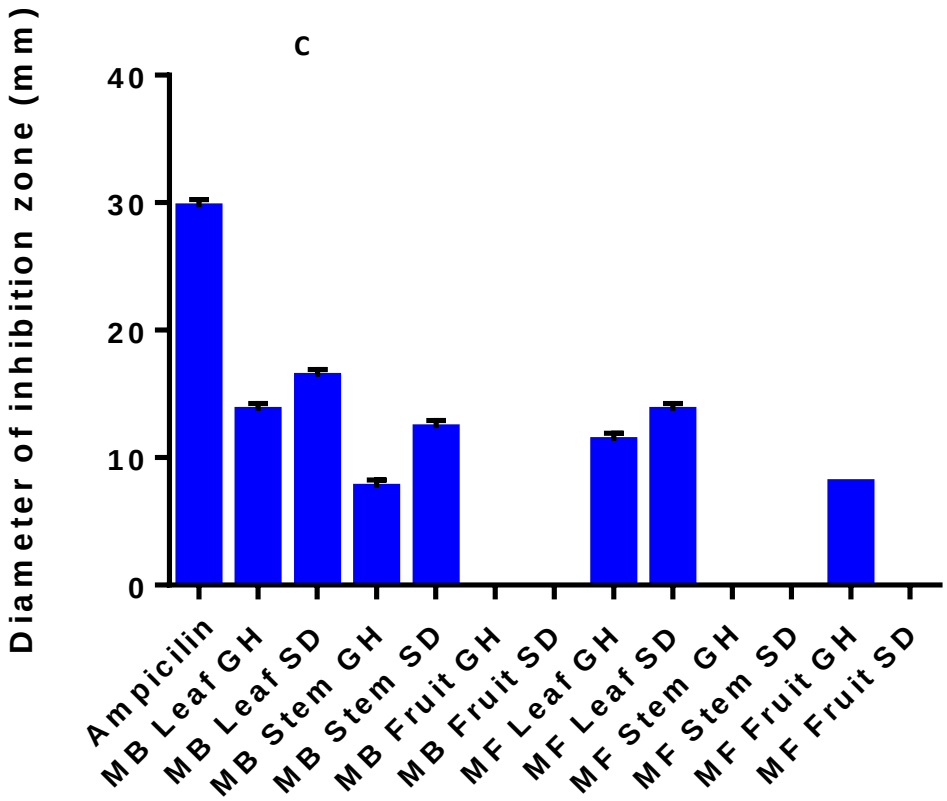
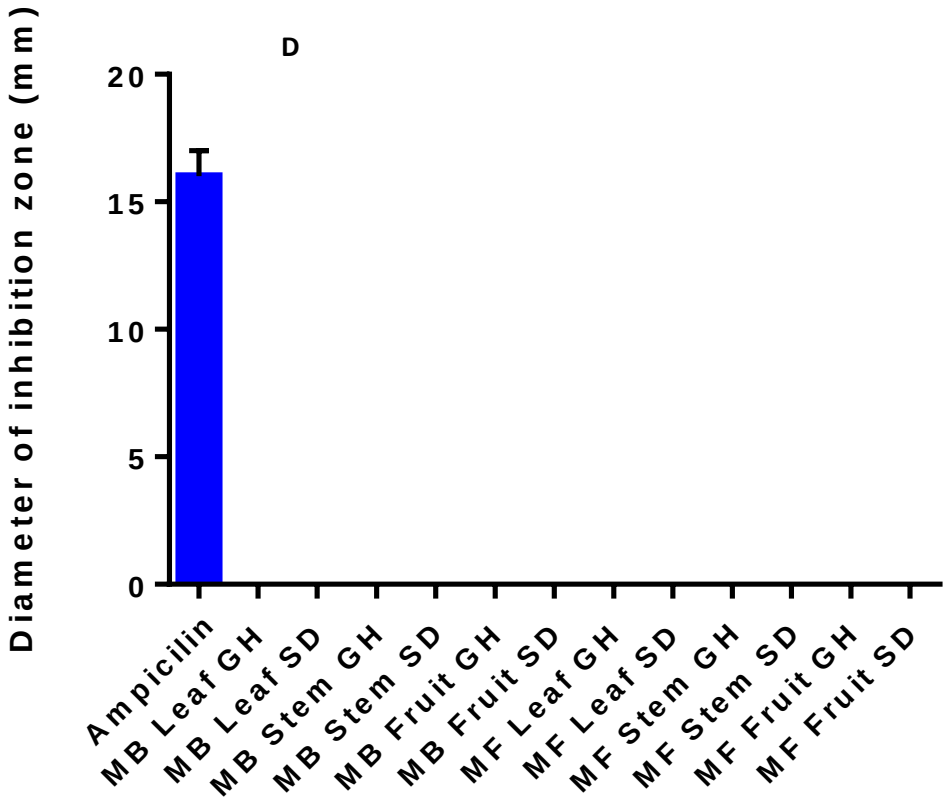
MF Stem GH
MF Stem SD
MF Fruit GH
MF Fruit SD


C
Diameter of inhibition zone (mm)
40
30
20
10
0
Ampicilin
MB Leaf GH
MB Leaf SD
MB Stem GH
MB Stem SD
MB Fruit GH
MB Fruit SD
MF Leaf GH
MF Leaf SD
MF Stem GH
MF Stem SD
MF Fruit GH
MF Fruit SD


D
Diameter of inhibition zone (mm)
20
15
10
5
0
Ampicilin
MB Leaf GH
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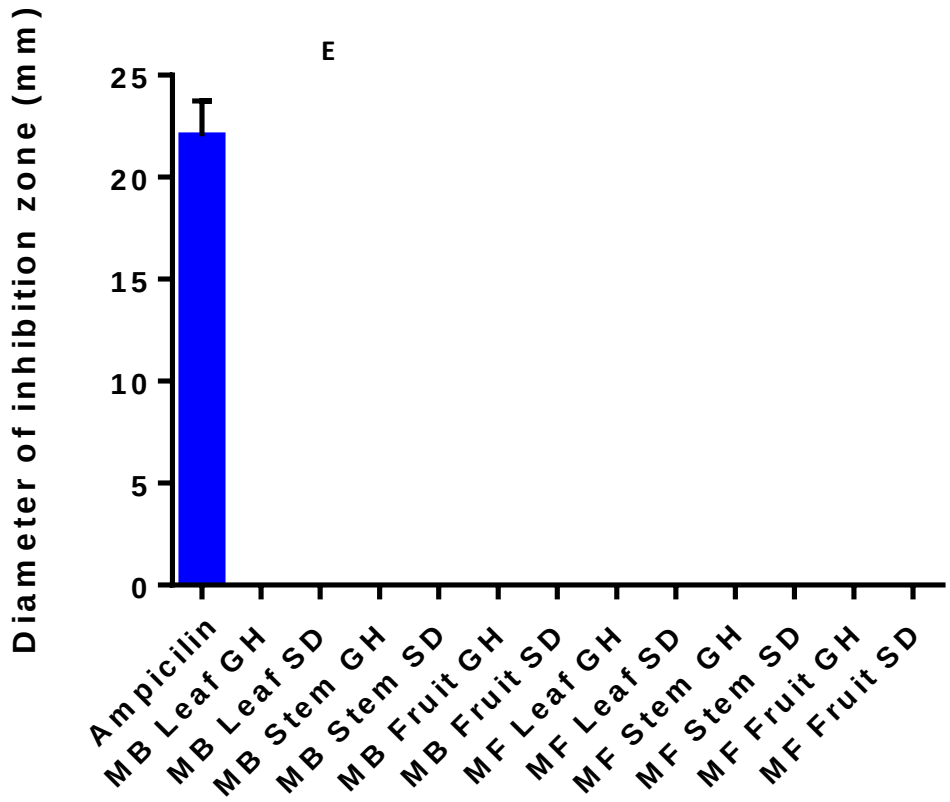


Figure 4-1. Antimicrobial activity measured as diameters of inhibition zone sizes (mm) of *M. balsamina* and *M. foetida* extracts (4mg/disc) against a) *S. aureus* Tox A b) *S. aureus* Tox C c) *L. monocytogenes* d) *E. coli* O157 *stx*- e) *Sal.* Typhimurium. MB – *M. balsamina*, MF – *M. foetida*, GH – UK Glasshouse grown, SD – Swaziland sourced. Positive control was ampicillin (10µg/disc); negative control was PBS (20 µL/disc).

The mean inhibition zone range was 13 to 16.33 mm for *M. balsamina* leaf extracts from Swazi sample and 10 to 13.67 mm for leaf extracts from the glasshouse grown plants. The inhibition zone from both these extracts was however less than that of ampicillin, the positive control which had mean ranges of 15.75 to 34 mm, except for *M. balsamina* leaf extract from the Swazi sample which had an inhibitory effect almost comparable to the positive control against *S. aureus* Tox A. The mean inhibition zone for the positive control was 15.75 mm whilst the mean inhibition zone for *M. balsamina* leaf from the Swazi sample was 13 mm against the *S. aureus* Tox A. *M. balsamina* stem extract from the Swazi sample also displayed a considerable inhibitory effect with zone ranges of 10.67 to 12.33 mm against Gram negative bacteria compared with similar extracts from UK grown plants which showed smaller inhibition zone ranges of 6.5 to 8 mm. In general Swazi sample *M. balsamina* extracts (leaves and stem) gave a significantly higher inhibition zone ($P < 0.0001$) compared to UK glasshouse sample extracts. *M. balsamina* fruit both from glasshouse and Swazi plant sample did not show any antimicrobial activity as no inhibition zone was observed.

M. foetida leaf extracts from the Swazi sample also displayed a significantly higher inhibition zones ($P < 0.0001$) against Gram positive bacteria than glasshouse leaf extracts. The mean inhibition zone range was 9 to 13.67 mm for *M. foetida* leaf extracts from the Swazi sample and 7.17 to 11.33 mm for leaf extracts from the glasshouse grown plants. Contrary to the results of *M. balsamina*, *M. foetida* stem extracts did not show any inhibition against any bacteria. Also *M. foetida* fruit from the Swazi sample showed no inhibition whilst UK glasshouse fruit extract showed antimicrobial activity against *S.*

aureus NCTC 10652 and *L. monocytogenes* but not against *S. aureus* NCTC 10655.

Among the bacteria tested *L. monocytogenes* was the most susceptible to *M. balsamina* and *M. foetida* extracts yet for the positive control *S. aureus* NCTC 10655 was the most susceptible.

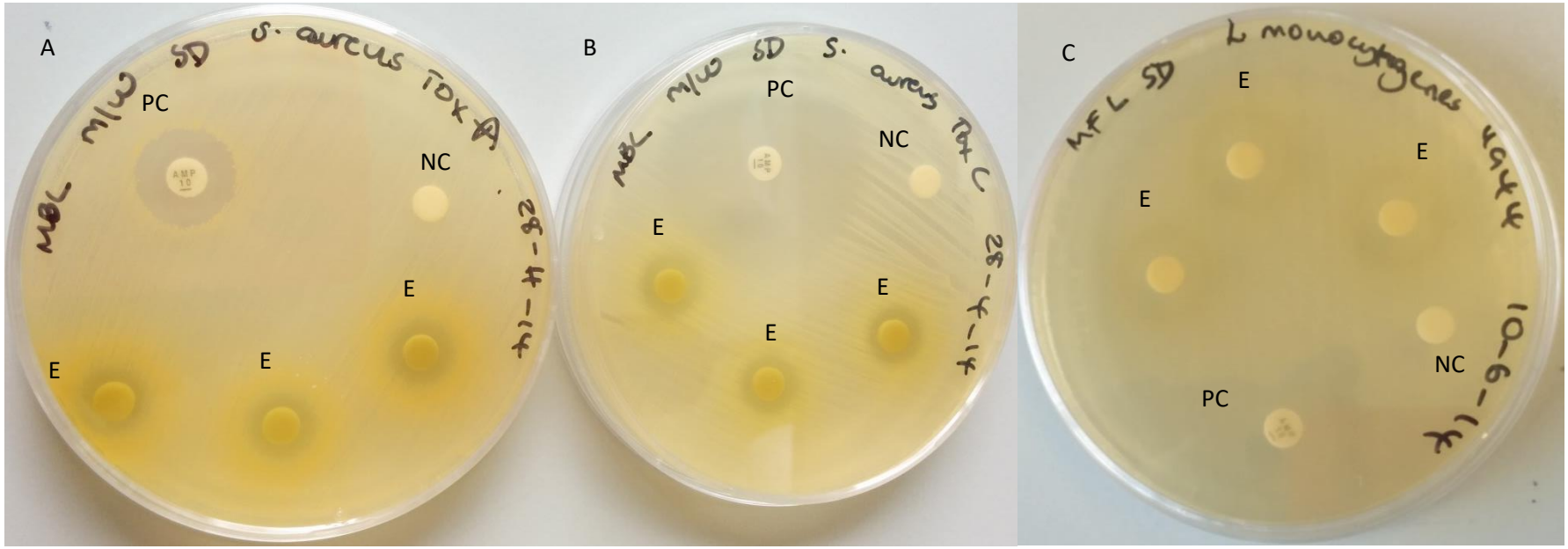
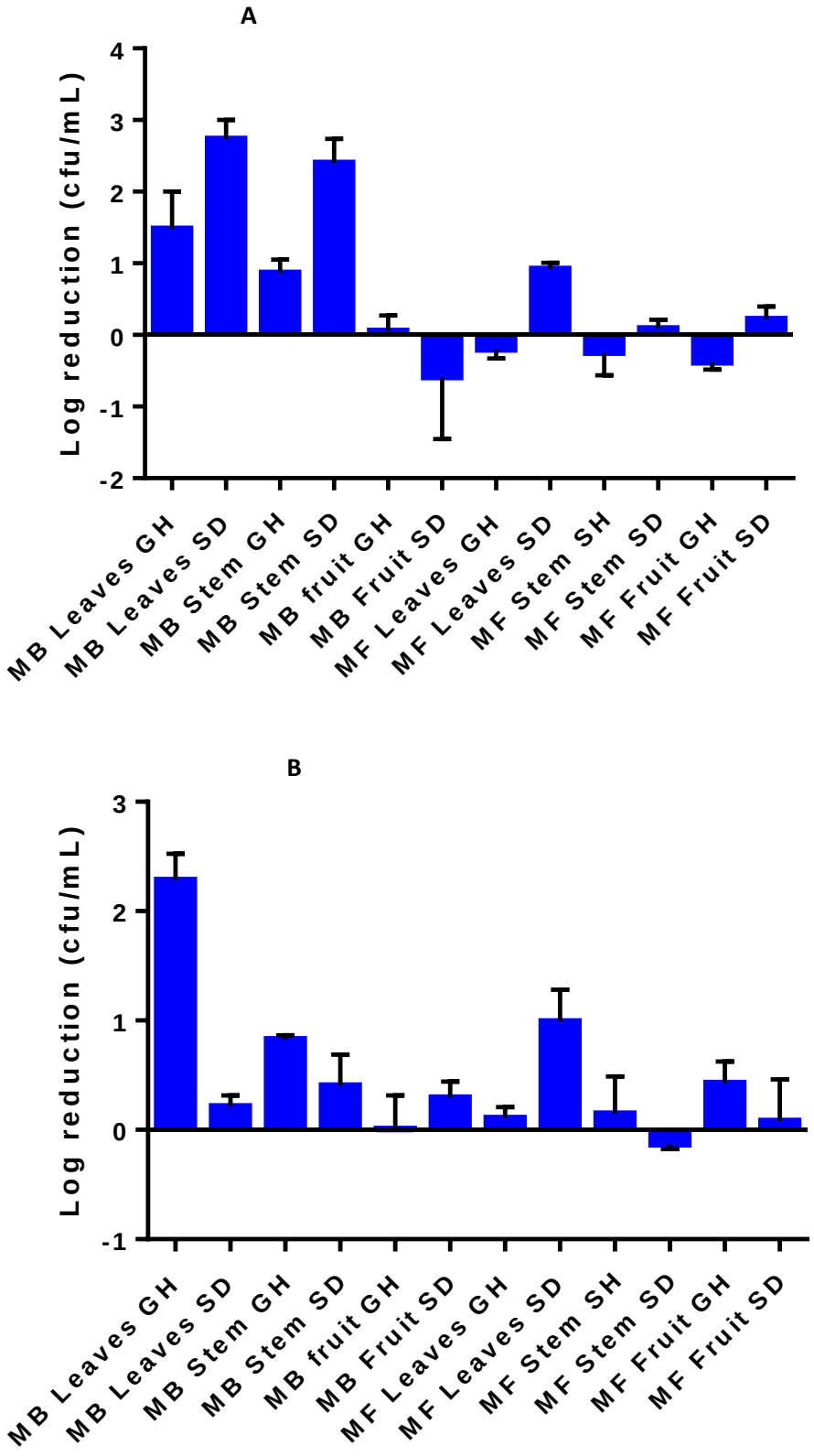


Figure 4-2. Antimicrobial activity shown by inhibition zones of *M. balsamina* leaf extracts against *S. aureus* NCTC 10652 (A), *S. aureus* NCTC 10655 (B) and *M. foetida* leaf extracts (4mg/disc) against *L. monocytogenes* (C) after 24 hours of incubation at 37°C. Negative control (20 µL PBS) had no inhibition zone while positive control (ampicillin 10 µg/disc) showed inhibition zone against all strains tested. MBL – *M. balsamina* leaf, MFL – *M. foetida* leaf, M/W – Methanol-water, SD – Swaziland sourced. E – Extract, NC – Negative control, PC – Positive control.

4.3.2 Microbial sensitivity of bacteria to extracts: Extract treatment survival assay

The antimicrobial activity of the *Momordica* extracts was also assessed using bacterial suspensions challenged with a final concentration of each extract at 2 mg/mL for 2 hours at 20°C. Viable counts on MHA and PCA were initially compared to determine any media effects on counts and the results showed that there was no media effect (data not shown). Survival was then evaluated by viable counts on PCA. The results are shown in Figure 4-3 for *M. balsamina* and *M. foetida* samples. *M. balsamina* leaves and stem extracts sourced in Swaziland showed the highest activity against the three Gram positive bacteria tested. There was very little activity against Gram negative bacteria. *M. balsamina* fruit extract showed little activity against any bacteria. These results demonstrated the same activity as shown in the disc diffusion assay. *M. foetida* extracts displayed a generally weaker antimicrobial activity. In this assay only leaf extracts from the Swazi sample showed a considerable reduction in counts. These results suggest that *L. monocytogenes* was most affected. In general Swazi sample extracts displayed a higher antimicrobial activity than glasshouse sample extracts ($P < 0.0001$). Both Swazi and UK glasshouse extracts displayed little activity against Gram negative bacteria (*Sal.* Typhimurium LT2 and *E. coli* O157:H7 Sakai *stx*-).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Log reduction (cfu/mL)
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0
-1
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MB Leaves GH
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MB Stem GH
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MB Fruit SD
MF Leaves GH
MF Leaves SD
MF Stem SH
MF Stem SD
MF Fruit GH
MF Fruit SD
B
Log reduction (cfu/mL)
3
2
1
0
-1
MB Leaves GH
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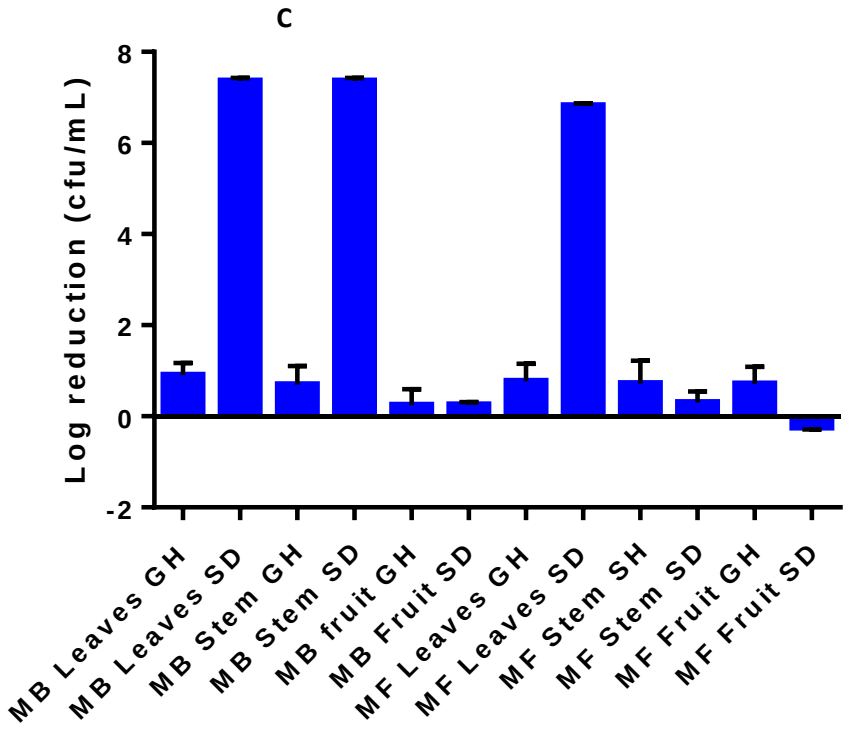
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Log reduction (cfu/mL)
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-2
MB Leaves GH
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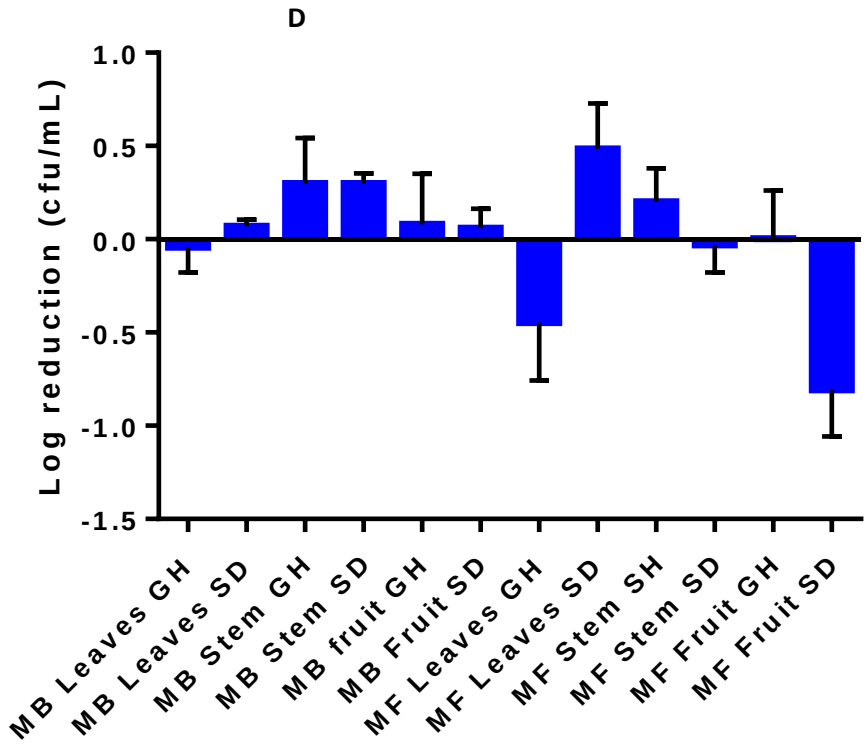
D
Log reduction (cfu/mL)
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0.5
0.0
-0.5
-1.0
-1.5
MB Leaves GH
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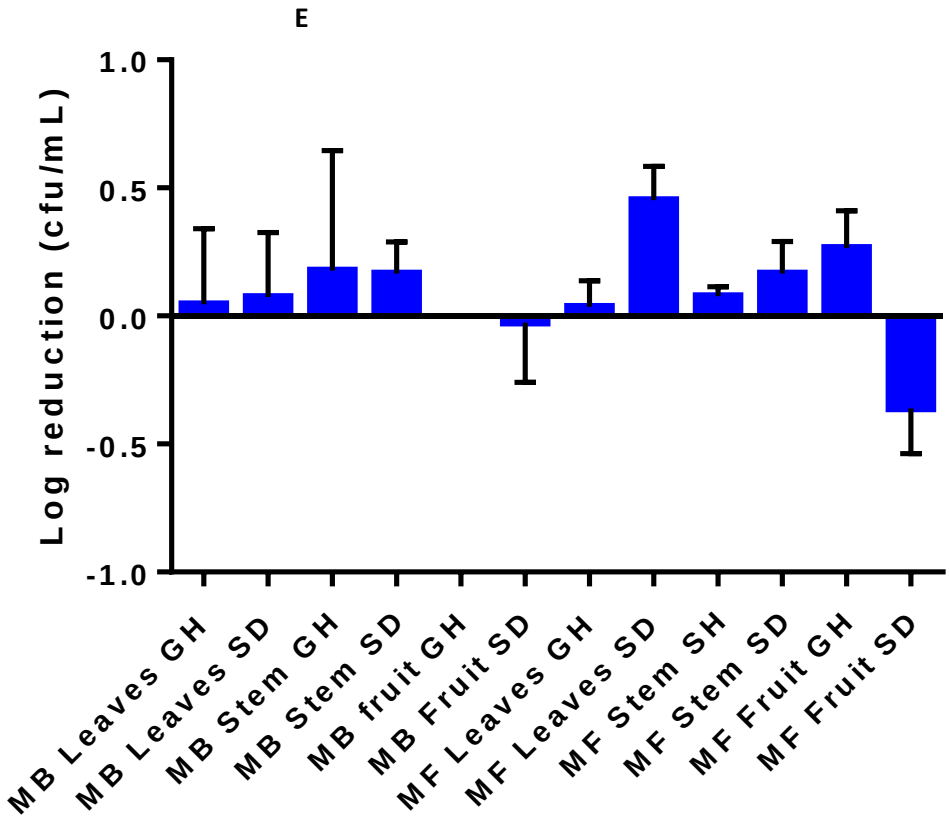


Figure 4-3. Log reduction (cfu/mL) of bacterial viability caused by methanol-water extracts of *M. balsamina* (MB) and *M. foetida* (MF) plants. Log reduction of a) *S. aureus* Tox A b) *S. aureus* Tox C c) *L. monocytogenes* d) *E. coli* O157 *stx*- e) *Sal.* Typhimurium.

4.3.3 Determination of the MIC and MBC values of *Momordica* crude extracts

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was studied only for *L. monocytogenes* using *M. balsamina* and *M. foetida* extracts that showed antimicrobial activity in the disc diffusion assay. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that prevents the growth of a microorganism after a specific incubation time. The MBC is defined as the lowest concentration of an antimicrobial agent needed to kill 99.9% of the initial inoculum. *L. monocytogenes* was chosen for this work because it had been shown to be the most susceptible to the extracts. The MIC and MBC values of *M. balsamina* and *M. foetida* extracts are displayed in Table 4-1. *M. balsamina* leaf extracts from the Swazi sample were the most effective with lowest MIC and MBC. In contrast *M. balsamina* leaf extracts from the glasshouse were much less effective. Likewise, *M. foetida* leaf extracts from the Swazi sample were more effective than leaf extracts from the glasshouse but both were less effective than the equivalent *M. balsamina* tissues. *M. balsamina* stem extracts also showed a similar trend as the leaf extracts where stem extracts from the Swazi sample were more effective than the stem extracts from the glasshouse sample. *M. foetida* fruit extracts from the glasshouse were also less effective as antimicrobial agents.

Results from this assay were in agreement with results obtained in the initial antimicrobial assays: disc diffusion and suspension treatment. Therefore, all these three assays (disc diffusion, suspension treatment and MIC and MBC)

together gave proof that extracts of *M. balsamina* and *M. foetida* were effective as antimicrobial agents against the Gram-positive bacteria tested.

Table 4- 1. The MIC and MBC values of *M. balsamina* and *M. foetida* extracts against *L. monocytogenes* (MIC and MBC in mg/mL).

Extract	**MIC (mg/mL)**	**MBC (mg/mL)**
M. balsamina leaves SD	2.5	2.5
M. balsamina stem SD	10	20
M. balsamina leaves GH	20	20
M. balsamina stem GH	40	80
M. foetida leaves SD	10	20
M. foetida leaves GH	80	>80
M. foetida fruit GH	80	>80

SD, Swazi sample; GH, glasshouse sample

4.3.4 Antimicrobial activity of solid phase extraction (SPE) fractions of plant extracts: Spot test

The methanol-water extracts of *M. balsamina* and *M. foetida* leaf and stem were fractionated using solid phase extraction (SPE) as described in Section 2.3. The SPE Fraction 1 (F1), fraction 2 (F2) and fraction 3 (F3) were used for antimicrobial tests using a spot test assay (Method 1 and 2). In all cases, F1 and F2 did not display any antimicrobial activity (data not shown) against any bacteria tested. However, some of the F3-methanol-eluted-fractions displayed antimicrobial activity against Gram-positive bacteria. Table 4-2 presents data comparing antimicrobial activity of the F3s with those of crude extracts against *S. aureus* NCTC 10652, *S. aureus* NCTC 10655, *L. monocytogenes*

11944, *Sal.* Typhimurium LT2 and *E. coli* O157 Sakai *stx*-. To ensure that similar levels of extract were applied in each case, dried crude extracts or dried SPE F3s were standardized using PBS to 4mg/mL. Four replicates of each volume (20 µL) were spotted at least 24 mm apart ensuring that the spot gave a consistent size of extract treatment and did not spread any further.

Table 4-2. Inhibition zone (mm) of crude and SPE fraction 3 obtained by spot test method1

Plant	Extract	*S. aureus A*	*S. aureus C*	*L. mono*	*E. coli* O157	*Sal.* Typhim
MBL	Crude	12.5 ±0.6	13 ± 1.2	13 ± 0.8	NA	NA
	SPE	11 ± 0.0	11 ± 0.0	11.25 ± 0.5	NA	NA
MBS	Crude	10 ± 0.0	9 ± 0.0	10.5 ± 0.0	NA	NA
	SPE	NA	NA	NA	NA	NA
MFL	Crude	9 ± 0.0	7.75 ± 0.5	NA	NA	NA
	SPE	9 ± 0.0	8 ± 0.0	NA	NA	NA
MFS	Crude	NA	NA	NA	NA	NA
	SPE	NA	NA	NA	NA	NA

MBL – *M. balsamina* leaf, MBS – *M. balsamina* stem, MFL – *M. foetida* leaf, MFS – *M. foetida* stem. NA – Not active. Method 1 for spot test was used to generate data.

SPE extracts of *M balsamina* leaf displayed inhibition against *S. aureus* strains and *L. monocytogenes* with inhibition zone size ranging from11 – 11.25 mm whilst crude extracts had zone ranging from 12.5 to 13 mm. SPE extracts of *M. foetida* leaf displayed inhibition zone ranging from8 - 9 mm only against

the *S. aureus* strains which was comparable to the crude extracts that displayed zone ranges of 7.75 to 9 mm. SPE extracts of *M. balsamina* stem did not display any inhibition, yet their crude counterparts displayed inhibition against the Gram positive bacteria, suggesting the active component was in another fraction. As expected, *M. foetida* stem (crude and SPE) did not yield any inhibition. Similarly, all extracts were not active against *E. coli* O157 and *Sal.* Typhimurium as found previously.

Results for spot test method 1 and 2 (Section 2.6.3) are presented in Figure 4-4 to Figure 4-6. The two methods were used to determine which will show consistency and display reproducible results. Comparison between the two methods was only made for crude extracts and SPE fraction 3 of *M. balsamina* leaf as they displayed a better activity against the Gram positive bacteria tested. Crude extracts displayed inhibition zone ranging from 12.5 to 13.0 mm in method 1 and 12.25 to 14.75 mm in method 2, whilst the phenolic SPE fraction displayed inhibition zone ranging from 11.0 to 11.25 in method 1 and 11.0 to 12.25 mm in method 2. There were no significant differences ($P > 0.05$) in antimicrobial activity of crude extracts and SPE fraction 3 between the two spot test methods even though generally, method 1 plates (as seen in Figure 4-5) were much neater and clearer than method 2 plates (Figure 4-6) after incubation. Slight differences were however observed between crude extracts and SPE fraction 3 where inhibition zone sizes of crude extracts were a little larger than inhibition zones caused by SPE fraction 3 particularly for *M. balsamina* leaf extracts.

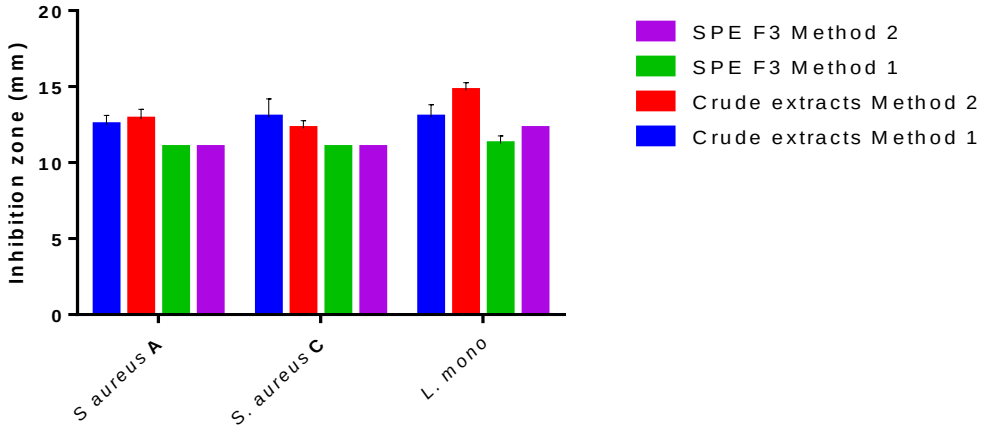


Figure 4-4. Comparison of growth inhibition zone (mm) of crude and SPE fraction 3 (F3) of *M. balsamina* leaf obtained by spot test method 1 and method 2 against *S. aureus* NCTC 10652, *S. aureus* NCTC 10655 and *L. monocytogenes* 11944.

There were no significant differences ($P > 0.05$) in antimicrobial activity of extracts between method 1 and method 2.

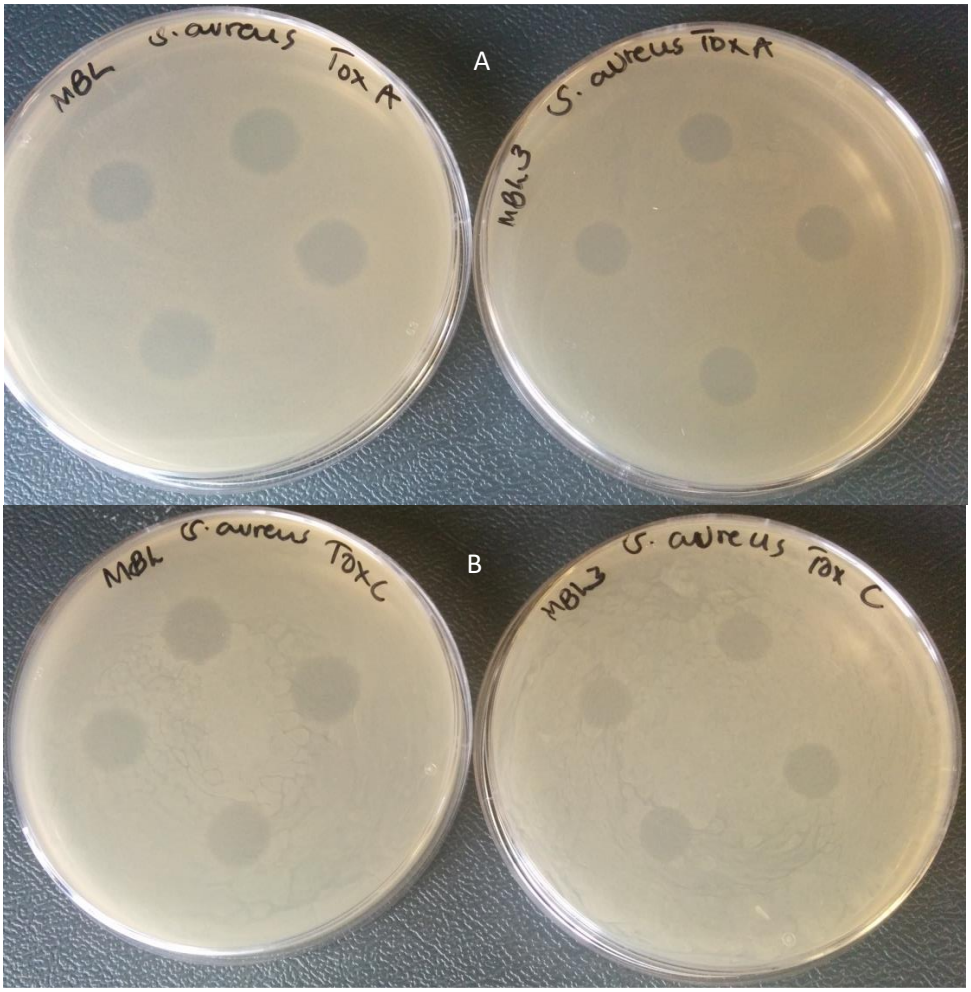


Figure 4-5. Spot test Method 1 – Growth inhibition zones of *M. balsamina* leaf crude extracts (MBL) and SPE fraction 3 (MBL3) (4mg/mL) against *S. aureus* NCTC 10652 (Tox A) (A) and *S. aureus* NCTC 10655 (Tox C) (B) after 24 h of incubation at 37ºC. SPE fraction 1 and 2 did not show any bacterial growth inhibition.

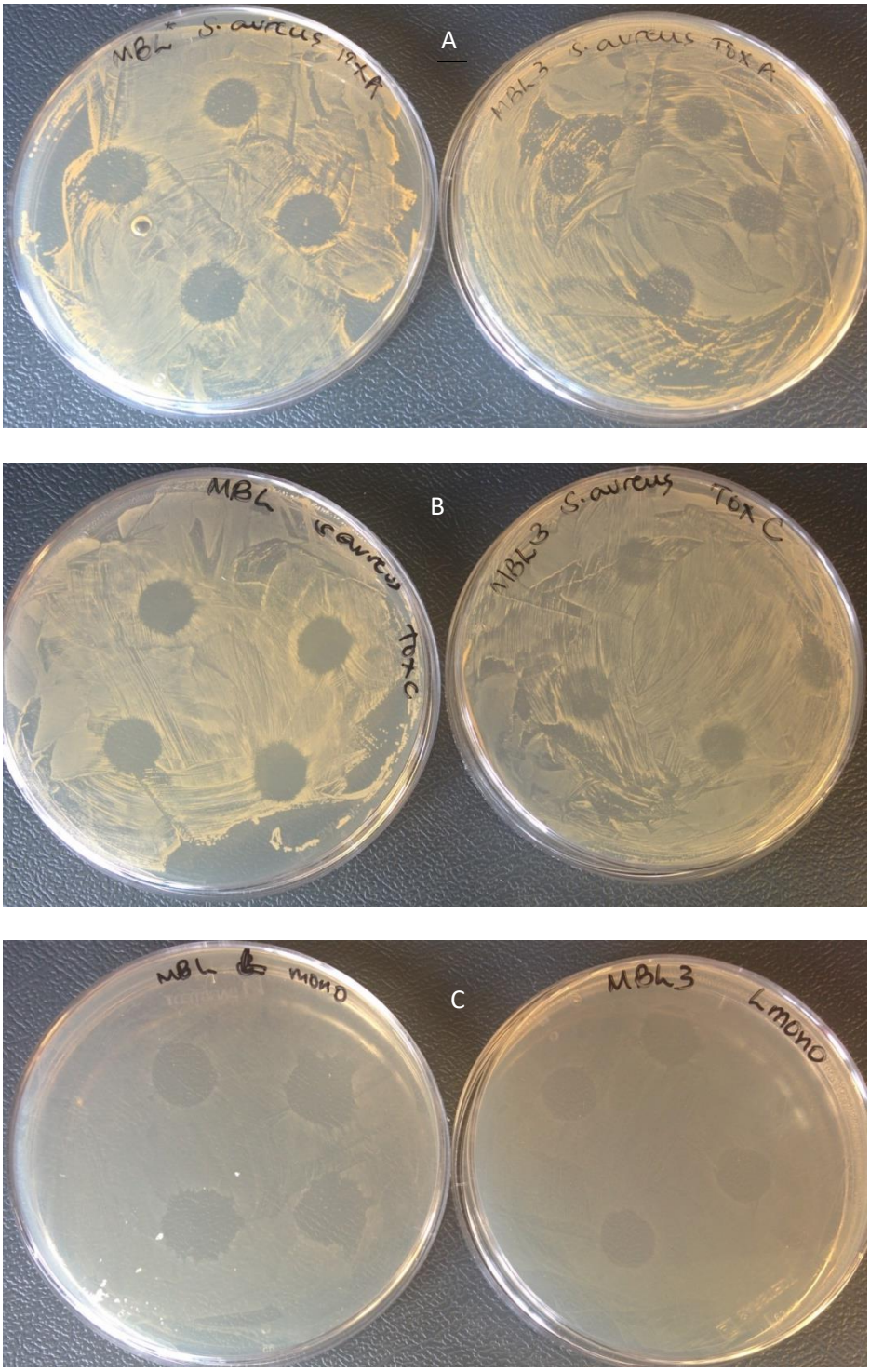


Figure 4-6. Spot test Method 2 – Growth inhibition zones of *M. balsamina* leaf crude extracts (MBL) and SPE fraction 3 (MBL3) (4mg/mL) against *S. aureus* NCTC 10652 (Tox A) (A), *S. aureus* NCTC 10655 (Tox C) (B) and *L. monocytogenes* (C) after 24 h of incubation at 37ºC. SPE fraction 1 and 2 did not show any zone clearing.

4.3.5 Determination of the MIC and MBC values of *M. balsamina* leaf SPE fractions

The MIC and MBC values of extracts were only studied for *S. aureus* NCTC 10652, *S. aureus* NCTC 10655 and *L. monocytogenes* as they showed antimicrobial susceptibility in initial antimicrobial studies. The MIC and MBC values of crude extracts and SPE fraction 3 are displayed in Table 4-3. *L. monocytogenes* was inhibited at lower MIC values for both the methanol-eluted-fraction and the crude extract. The eluted fraction was more bactericidal than crude extracts against *S. aureus* Tox A and *L. monocytogenes*. *S. aureus* Tox C showed more resistance to treatment as it displayed less response to treatments equal or less than 16 mg/mL.

Table 4-3. The MIC and MBC values of *M. balsamina* leaf crude and fraction 3 of SPE extracts against Gram-positive bacteria (MIC and MBC in mg/mL).

Bacteria	Crude extract		Fraction 3	
	MIC	MBC	MIC	MBC
S. aureus NCTC 10652	8	16	8	8
S. aureus NCTC 10655	16	>16	16	>16
L. monocytogenes	4	16	2	8

Mean values of MIC and MBC of *M. balsamina* leaf crude extracts and solid phase extraction (SPE) fraction 3.

4.3.6 Bacterial growth measurements

In trying to understand whether the nature of antimicrobial activity of extracts was bacteriostatic or bactericidal, the effects on bacterial growth in liquid cultures treated with *M. balsamina* leaf extracts were studied through OD measurements and viable counts (Section 2.6.2 extract treatment survival assay).

Initially, a series of tests were performed to establish the effect of medium composition (MHB, TSB and BHI) on the growth of *L. monocytogenes* (Figure 4-7). This was done after obtaining low OD readings when *L. monocytogenes* was inoculated in MHB at 37°C for 24 h at static conditions, thus affecting the ability to show treatment effects on the growth curves. BHI medium showed good growth of *L. monocytogenes* thus treatment effect was also easily displayed. BHI was then selected to study growth.

To determine a suitable method for measuring growth by viable count, the Miles and Misra method was compared with the standard spread plate method. Results for this part presented limitations (data not shown) due to inoculating bacteria in MHB which resulted in poor bacterial growth after 24 h of incubation which also affected growth curves. Despite these limitations, the Miles Misra method was the preferred method because it was faster and more economical as more counts could be made on one plate.

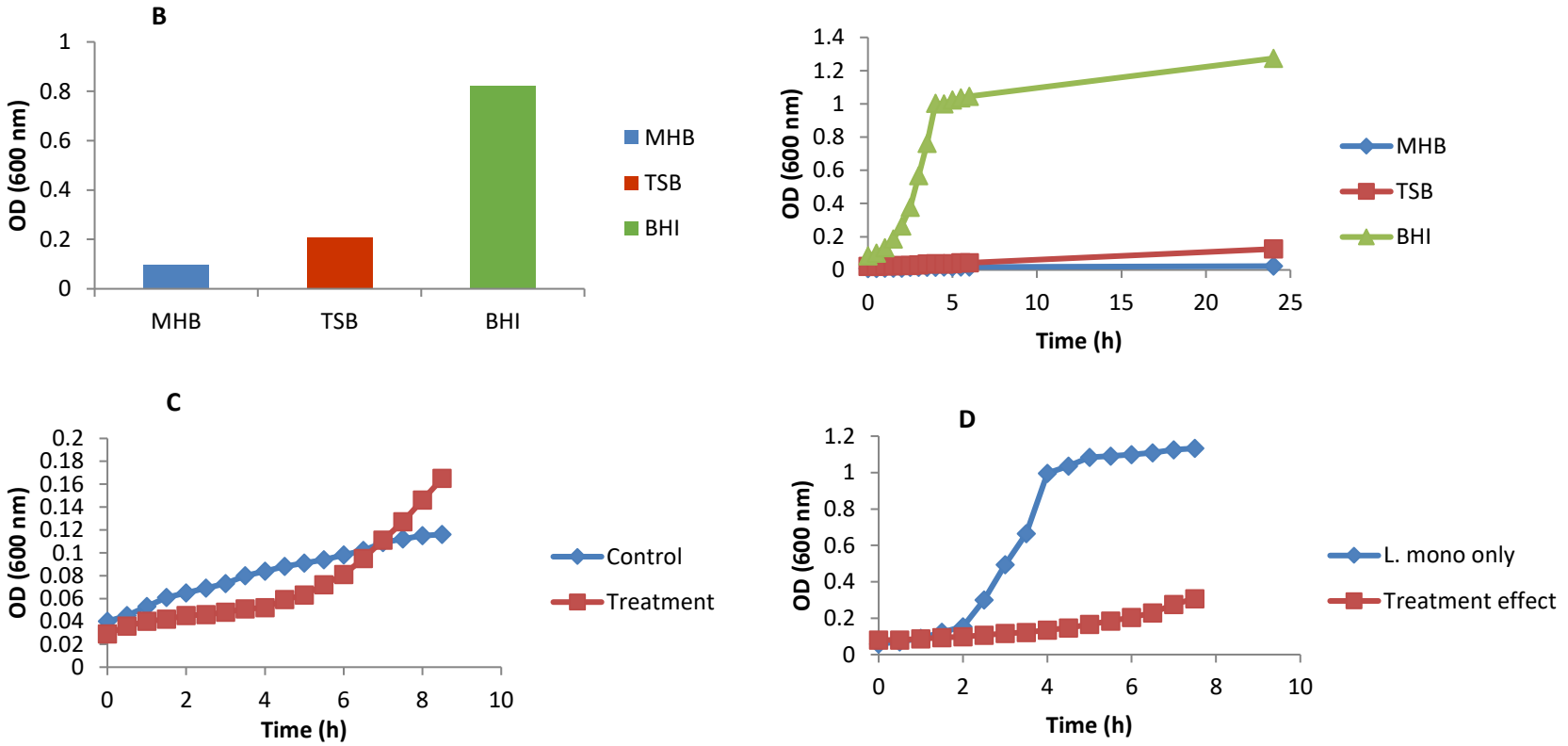


Figure 4-7. Effect of medium (broth) on the growth of *L. monocytogenes* 11944. (A) One colony of *L. monocytogenes* grown on TSA plates was added to 10 mL of each medium (MHB, TSB and BHI) before incubating at 37ºC for 24 h, statically (OD readings at 600 nm after incubation). (B) Inocula in A) (5 mL) were then re-suspended in fresh media (100 mL) before incubating at 37ºC in static conditions then OD readings at 600 nm were recorded every 30 minutes of incubation for 6 h and then at 24 h. Effect of *M. balsamina* extracts (2mg/mL) added at time 0 on *L. monocytogenes* was then determined using growth curves of treatment in Mueller Hinton Broth (C) and in Brain Heart Infusion (D).

M. balsamina leaf extract extended lag time and reduced the viable count of *L. monocytogenes* and *S. aureus* by about 1 to 2 log (cfu/mL) over a period of 24 h (Figure 4-8). The extract was shown to reduce viable counts by 1-2 log cfu/mL on initial contact with both bacteria, and this led to a reduction in counts at all subsequent stages. From the OD readings, there was an obvious lag phase through possible injury and finally an adaptation effect after which recovery is observed throughout the growth period. For *Listeria* the same OD was obtained at 24h as in the untreated sample but for *S. aureus* the final OD was also reduced.

To confirm whether the effect on the growth of *L. monocytogenes* was indeed caused by the *M. balsamina* leaf extract, a treatment effect of an extract that displayed no antimicrobial activity in disc diffusion and the suspension assay was also studied. For this, *M. foetida* stem was chosen as the suitable extract. The assumption was that if *M. foetida* stem showed no treatment effect in growth curves, then this will prove the observed effects in *L. monocytogenes* and *S. aureus* were caused by active components of the *M. balsamina* leaf extracts. Results also displayed in Figure 4-8 clearly showed that the bacterial counts and OD readings of control and culture treated with *M. foetida* stem extract were not different. Therefore *M. foetida* stem extract had no effect on growth characteristics of *L. monocytogenes* in agreement with its failure to produce inhibition zones on plates.

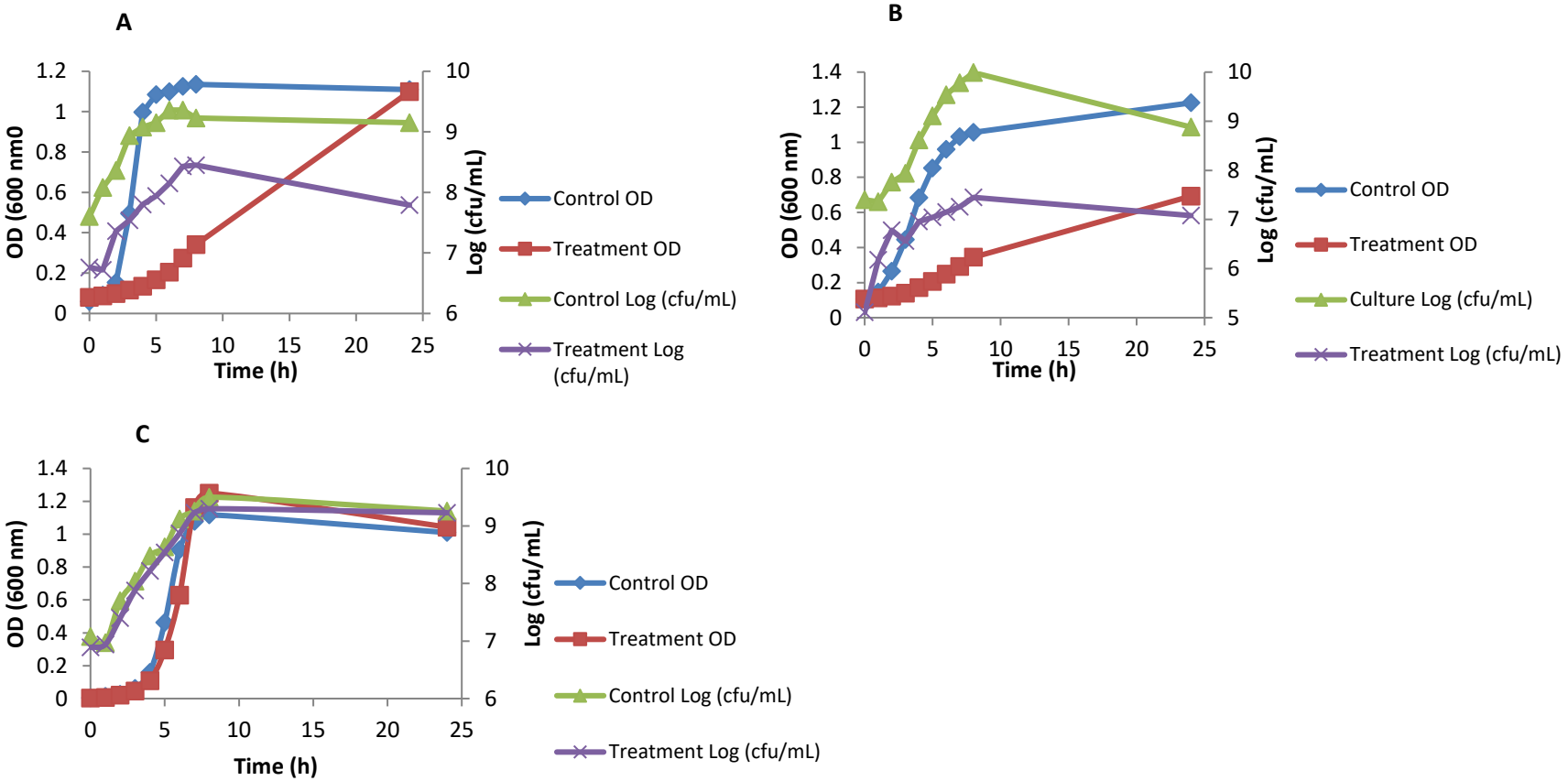


Figure 4-8. Effects of *M. balsamina* leaf extract (2 mg/mL) on the growth characteristics of *L. monocytogenes* (A), and *S. aureus* NCTC 10652(B).
Effects of *M. foetida* stem extract (2 mg/mL) on the growth characteristics of *L. monocytogenes* (C) was used for comparison. OD readings were taken every 30 minutes whilst plating for viable count was performed (in triplicate) on plate count agar every hour for 8 h then at 24 h with incubation at 37°C for 24 h.

Since counts of *L. monocytogenes* treated with *M. balsamina* leaf extract were shown to eventually recover over time when non-selective PCA plates were used for viable counting, confirmation was necessary to determine if the recovery on a selective medium (Listeria Oxford agar) would be similar. Identification of colonies of *L. monocytogenes* is possible using ferric ammonium citrate and aesculin present in the Listeria Oxford agar. The growth (log cfu/mL) of *L. monocytogenes* (control and treated with *M. balsamina* extract) was observed over a period of time at static conditions of 37°C and for viable count these were plated on PCA and selective media. Results in Figure 4-9 displayed a difference of 1 to 2 log cfu/mL between control and treatment on either PCA or selective media. Results show that *L. monocytogenes* treated with *M. balsamina* extract was able to recover on a Listeria Oxford agar and the treatment effect observed on this selective medium was comparable to that displayed on PCA. Thus no significant evidence of injury of cells was evident. Interestingly this time there was no initial decrease in counts between control and treatment samples and both the control and treated samples showed a difference between the PCA and selective medium counts after 24h, which may be an effect of accumulation of toxic metabolic end products in the medium.

It was also important to determine whether bacteria (*L. monocytogenes*) become adapted or resistant after *M. balsamina* leaf extract treatment. Firstly treated and untreated cultures of *L. monocytogenes* inoculated to the same starting OD, were re-inoculated into fresh BHI and monitored for growth (viable count on PCA and OD measurements) for 5 h then 24 h. This was performed to establish whether the treated culture would regrow the same way

as untreated culture. Results in Figure 4-10 suggested that for the treated culture, growth shows a typical exponential growth curve with onset of stationary phase at the same timepoint as the control culture. A difference observed was in the low starting log count for treated culture even though starting OD for both treated and and untreated culture were standardized.

Secondly to ensure that treating *L. monocytogenes* had not created resistance or long-term adaptation, *L. monocytogenes* which had been recovered after treatment with extract in the growth curve experiment was then used to produce cultures to evaluate antimicrobial activity using the spot test, treating again with the same extract. For inoculum preparations, mixed recovered *L. monocytogenes* colonies were taken from the viable count plates and added to 10 mL of BHI then incubated for 24 hours at 37°C before being used in the spot test. Mixed recovered *L. monocytogenes* meant TSA plates were flooded with treated culture (about 1 mL) and left to dry before inverting the plate and incubation of plates for 24 h at 37°C. Results are displayed in Figure 4-11 showing treatment concentration (4 mg/mL) using spot test method 1 and method 2.

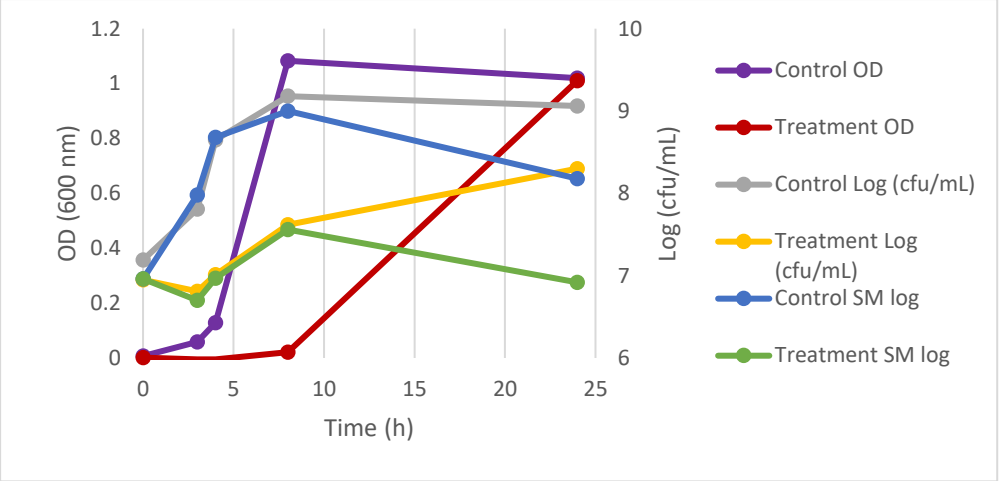


Figure 4-9. Effect of *M. balsamina* leaf extract on *L. monocytogenes* viability determined by viable counts on selective Oxford medium (SM) and non-selective PCA medium. Treatment OD is a result of OD obtained from treated culture minus OD obtained from treatment only (control).

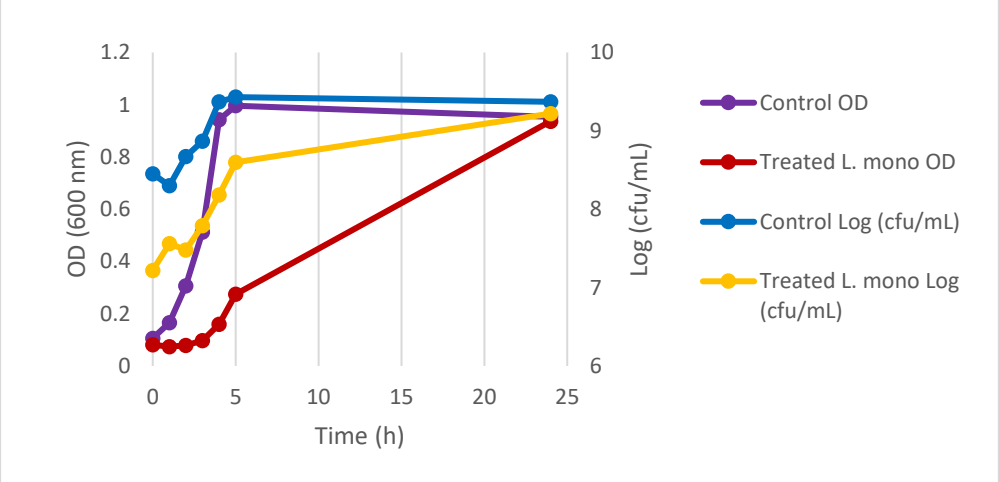


Figure 4-10. Untreated (control) and treated cultures of *L. monocytogenes* re-inoculated into fresh BHI and monitored for growth (both viable count and OD measurements) for 5 h then 24 h.

Results showed that the recovered *L. monocytogenes* did not become adapted (or resistant) to extract treatment. Spot test results showed similar sized zones of clearing in treated cultures as it did in untreated cultures (Figure 4-11). For method 1 the inhibition zone range was 11.5-12mm for untreated culture and 11-12 mm for treated culture. For method 2 inhibition zone range was 15.5-16 mm for untreated culture and 15-16 mm for treated culture. Method 2 gave much clearer and bigger inhibition zones with the same extract concentration.

Stationary phase cells are known to show greater resistance to many stresses compared to exponentially growing cells. In a standard growth culture, cells can be in a mixture of growth phases and potentially a resistant subpopulation could be selected from the population because of this asynchrony (Booth 2002). To ascertain that the observed effect of treatment on the bacteria was not a result of growth phase, a mid log-suspension culture (Section 2.6.6) was treated with the same extract before observing for growth.

Results displayed in Figure 4-12 from mid log-suspension culture culture looked similar to those displayed in a standard growth culture suggesting that there was no growth phase effect.

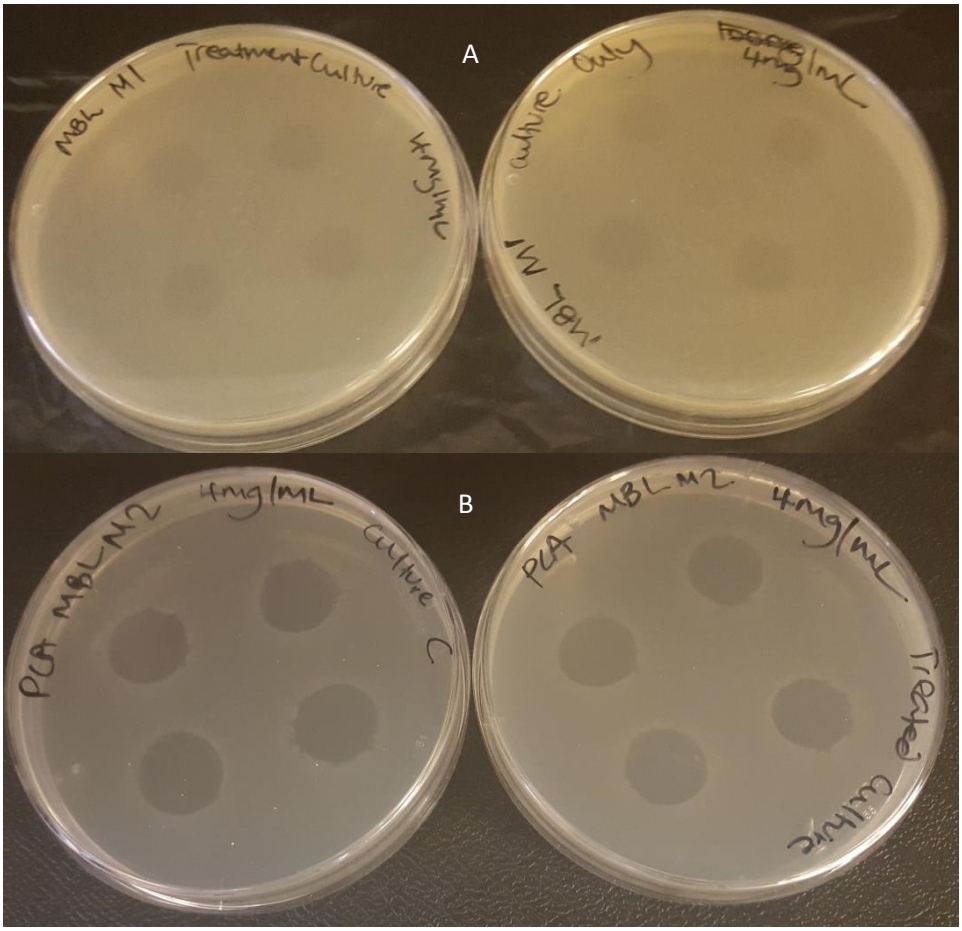


Figure 4-11. Growth inhibition zone of *M. balsamina* leaf crude extracts (MBL) (4mg/mL) against *L. monocytogenes* using spot test methods 1 and 2 after 24 h of incubation at 37°C.

Method 1 (A) -1 mL standardised culture, 20 mL molten MHA [45°C], 20 µL extract (4mg/mL). Method 2 (B) - Solid PCA, 20 µL extract (4 mg/mL), 1 mL standardised culture.

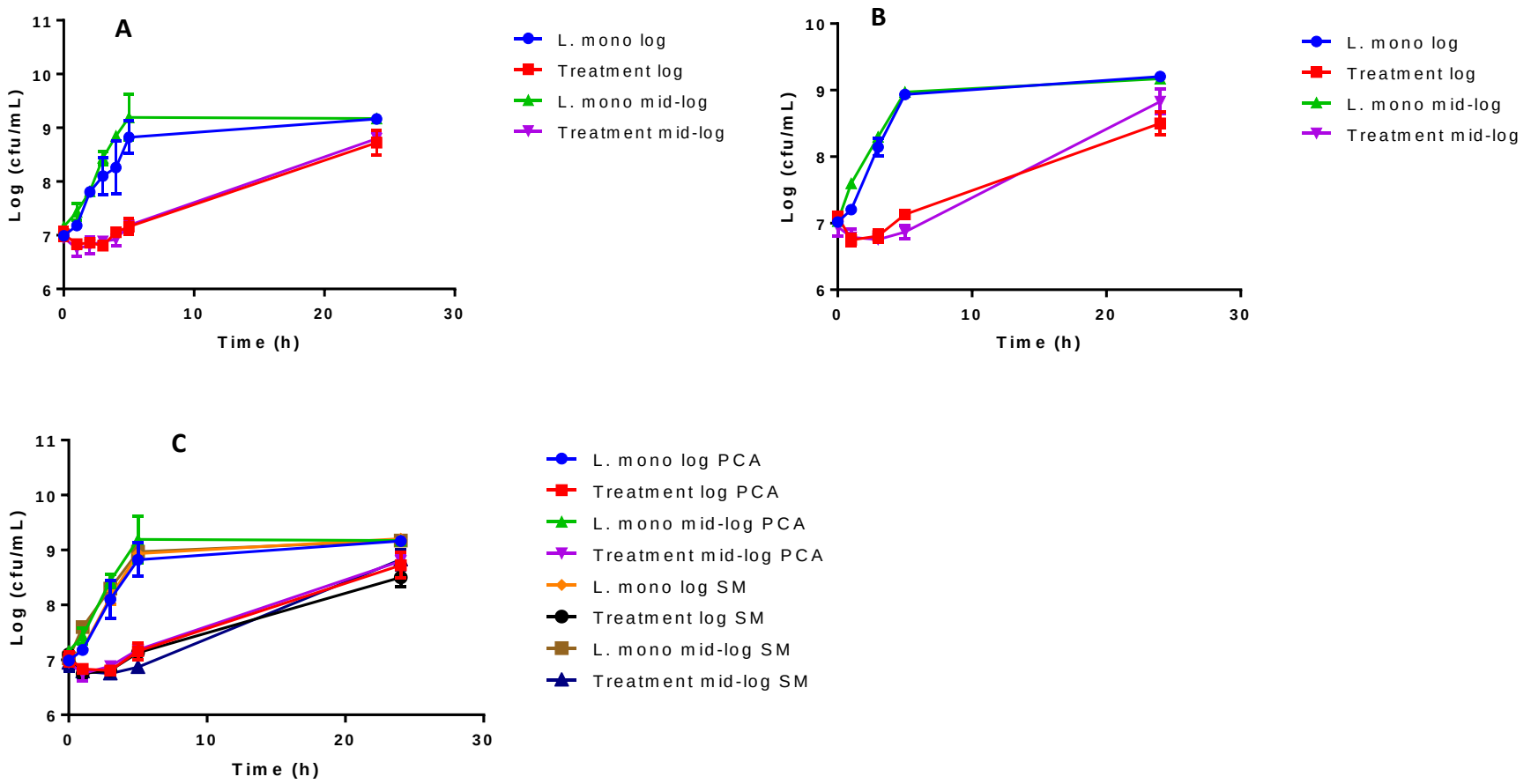


Figure 4-12. The effect of *M. balsamina* leaf extract treatment on survival of *L. monocytogenes* from a mid-log culture plated on to plate count agar (PCA) (A), Listeria Oxford agar (SM) (B) and the combination of PCA and SM results (C).

4.4 Discussion

The methanol and water (7:3 v/v) extracts showed antimicrobial activity against the two *S. aureus* strains and *L. monocytogenes* (Gram positive bacteria) but were much less effective against the Gram negative bacteria tested. These results are in agreement with previous reports by Zaika (1988), Consentino *et al.* (1999), and Karaman *et al*. (2003) who also demonstrated that generally Gram positive bacteria are more sensitive to plant extracts. Gram negative bacteria showed resistance towards the *Momordica* extracts. Diverse degrees of sensitivity of the test bacteria may be attributable to both the intrinsic tolerance of microorganisms and the nature and combinations of phytochemical compounds present in the extracts of the plants. The outer cell membrane of Gram negative organisms has several lipid compounds that protect cells from antimicrobial agents (Shelef *et al.* 1980; Russel 1991) and thus, protect them from the action of the extracts. These results are not in total agreement with studies conducted by other authors that showed antimicrobial activity of *Momordica* extracts against both Gram positive and Gram negative bacteria. Otimenyin *et al.* (2008) studied methanolic and water extracts (ratio 70:30) of *M. balsamina* leaves and stem found in Nigeria and reported antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Salmonella* Typhi, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Jigam *et al.* (2004) also studied the antimicrobial activity of methanolic extracts of whole *M. balsamina* found in Nigeria against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* Typhi and their results showed that extracts of *M. balsamina* had antibacterial activity against all the bacteria tested. Boily and

Van Puyvelde (1986) studied methanol extracts of *M. foetida* leaves in Rwanda and the results showed activity against *Bacillus subtilis*, *Candida albicans*, *Mycobacterium smegmatis* and *Staphylococcus aureus*.

Extracts of *M. balsamina* leaves showed the highest antimicrobial activity against the three bacterial strains with the lowest MIC and MBC followed by *M. balsamina* stem and *M. foetida* leaves, all from Swazi samples. *M. foetida* stem did not show any antimicrobial activity which is in agreement with Boily and Van Puyvelde (1986) who reported that *M. foetida* stem from Rwanda had an activity against *Bacillus subtilis* and *Mycobacterium smegmatis* but not against *S. aureus*. Methanol extracts of aerial parts of *M. foetida* from Ethopia were also studied for antimicrobial activity and results showed that extracts had activity against *Bacillus cereus* and *Neisseria gonorrhoea* only (Geyid *et al.* 2005).

Among the extracts tested in this study *M. balsamina* fruit and *M. foetida* fruit extracts from Swazi samples did not show antimicrobial activity against any of the test bacteria. These results are in agreement with one study that displayed no antimicrobial activity of methanol extracts of *Momordica charantia* fruit (Nanasombat and Teckcheun 2009). Surprisingly, *M. foetida* fruit extract from the glasshouse sample showed some antimicrobial activity against one strain of *S. aureus* and *L. monocytogenes*. The differences seen in antimicrobial activity of plant parts could be attributable to the difference in active components of the extracts as affected by environment (Geidam *et al.* 2007). In general, results from this study showed that Swazi sample extracts displayed a higher antimicrobial activity than UK glasshouse sample extracts suggesting that growth conditions are critical to the level of antimicrobial

activity in the plants. The extent to which plant extracts inhibit microbial growth depends on their source (Zaika 1988). Antimicrobial properties change according to the environment in which the plant is grown. Climate and soil play an important role in the concentration of the plant's active ingredients and medicinal properties (Geidam *et al.* 2007). These results suggest that extracts of *M. balsamina* leaves and stem as well as those from *M. foetida* leaves could be used to control *S. aureus* and *L. monocytogenes* but not *E. coli* O157:H7 Sakai *stx*- or *Sal.* Typhimurium.

The SPE methanol elute fraction (fraction 3) from the extracts proved to be as effective as an antimicrobial agent as the crude extracts. Similar to the crude extracts, high antimicrobial activity was observed in *M. balsamina* leaf SPE fraction 3 only against the Gram positive bacteria tested. The only other SPE fraction that displayed antimicrobial activity was the *M. foetida* leaf against the *S. aureus* strains and not against *L. monocytogenes.* This was not so surprising as crude extracts of *M. foetida* leaf also displayed antimicrobial activity against *S. aureus* strains when the spot test was used. SPE fractions of *M. balsamina* stem did not yield any activity. This may mean that, for the stem, antimicrobial activity displayed in crude extracts might not be from the fraction 3 but may be from a combination of compounds that were separated during fractionation. There may be some active compounds other than phenolics in plant extracts that are responsible for antimicrobial activity (Rauha *et al.* 2000). MIC and MBC results of SPE F3s displayed that *M. balsamina* leaf was most active against *L. monocytogenes* followed by *S. aureus* NCTC 10652 and least active against *S. aureus* NCTC 10655. *L. monocytogenes* 11944 and *S. aureus* NCTC 10652 are both food isolates yet *S.*

aureus NCTC 10655 is a clinical isolate which might explain the differences in their susceptibility.

In the growth curves studies, *M. balsamina* leaf extracts strongly inhibited the growth of *L. monocytogenes* and *S. aureus* NCTC 10652. The level of growth in the control cultures was higher than that of treated cultures. The extract effect was generally observed within an hour of exposure. These results were in agreement with those results obtained in the disc diffusion, and spot tests as well as MIC and MBC tests which showed strongest activity of *M. balsamina* leaf extracts against the two strains. These results further confirm the potential of *M. balsamina* leaf extracts as potent antimicrobial agents to control pathogenic bacteria such as *L. monocytogenes* and *S. aureus*.

4.5 Conclusion

The antimicrobial activity of *M. balsamina* and *M. foetida* varied depending on source, plant species and plant part. Generally, plants sourced from Swaziland displayed a greater antimicrobial activity than the glasshouse grown plants. Also *M. balsamina* extracts gave a higher antimicrobial activity compared to *M. foetida*. In general, leaves, especially of *M. balsamina*, showed a higher antimicrobial activity. Gram positive bacteria were mostly susceptible to the extracts and *L. monocytogenes* showed a higher sensitivity towards the extracts treatment. The methods used for screening of antimicrobial activity in this study proved that using only one method may not precisely reproduce the comparative efficacy of the test compounds, thus using diffusion methods together with liquid culture assays was necessary.

Fractionation by SPE showed that the fraction 3 did have a comparative antimicrobial activity particularly in *M. balsamina* and *M. foetida* leaf extracts but not in *M. balsamina* stem. This could suggest that the high antimicrobial activity of extracts of *Momordica* may be related to the phenolic compounds present in the extracts. However, this remains to be confirmed since whilst the SPE fraction 3 contains the bulk of the phenolics from the original extract these are by no means likely to be the only compounds present in this fraction. These results could indicate the potential use of *M. balsamina* leaves as antimicrobial agents and also provide the basis for isolation and identification of the biologically active substances in these extracts. Studying the effects of extracts to host cells to determine safety and toxicity is also necessary.

CHAPTER 5: ANTI-PROLIFERATIVE ACTIVITY OF METHANOL-WATER EXTRACTS OF *M. BALSAMINA* AND *M. FOETIDA* LEAF

5.1 Introduction

Since plants have long been considered to have a wide variety of biologically active compounds, it is important to explore their possible medicinal applications (Talib *et al.* 2010). Cancer is a disease that is a result of many factors including environment, physical, metabolic and genetic factors. There is evidence suggesting that consumption of fruits and vegetables rich in antioxidants help reduce the risk of many cancers. In the past years a large part of cancer research has focused on identification and development of these antioxidants. Phenolics are a diverse group of plant metabolites many of which exhibit antioxidant and other biological functions that may contribute to the modulation of carcinogenesis (Dai and Mumper 2010). These facts were supported by Kim *et al.* (2010) who found high correlation between anti-proliferative activity and phenolic as well as flavonoid content indicating that the anti-proliferative effects of mango extracts may be due to a combination of polyphenols and flavonoids present in the extracts. Sun *et al.* (2002), however, concluded that even though total phenolic content and antioxidant activity were highly correlated ($R^2 = 0.979$), there was a weak correlation of total phenolic content and anti-proliferative activity of HepG2 cells ($R^2 = 0.415$) as well as antioxidant activity and anti-proliferative activity of the fruits tested ($R^2 = 0.369$).

Plants from tropical areas are considered to be one possible source for the evaluation of anti-cancer agents (Manosroi *et al.* 2006). As already discussed,

M. balsamina (*inkakha*) and *M. foetida* (*inshubaba*) are used in several countries, including Swaziland, both for food and as a traditional medicine to treat several diseases. These plants have been used in Swaziland traditional medicine for patients suffering from diabetes and hypertension (Masarirambi *et al.* 2012), are often recommended to patients with HIV/AIDS to strengthen their immune system and for the treatment of boils and other skin diseases. Furthermore, from the results presented in this thesis, the Swazi species of the *Momordica* plants have demonstrated a high antimicrobial activity against Gram positive bacteria. Exploring these indigenous vegetables for other potential bioactivities is important for their potential applications. Currently, there is limited knowledge of the potential anti-proliferative activity of *M. balsamina* and *M. foetida* extracts. Most available literature reports anti-proliferative activity of *M. charantia*. Jilka *et al.* (1983) reported that crude extracts of *M. charantia* fruits inhibited tumour formation in DBA/2 mice. Hsiao *et al.* (2013) reported that isolated cucurbitanes from *M. charantia* fruits displayed cytotoxic activity on MCF-7 (human breast adenocarcinoma), Doay (human medulloblastoma), Hep-2 (human laryngeal carcinoma) and WiDr (human colon adenocarcinoma) tumour cell lines. An iridoid lactone (plumericin) isolated from *M. charantia* vine which displayed antibacterial activity against *Enterococcus faecalis* and *Bacillus subtilis* was also found to inhibit proliferation of two leukemic cancer cell lines namely NB4 (acute promyelocytic leukemia (APL)) and K562 (human chronic myeloid leukemia) (Saengsai *et al.* 2015).

Anti-proliferative activity has been assayed from other plants by many researchers using methods such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5

diphenyl tetrazolium bromide), MTS-based (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), ATP-based (adenosine triphosphate) and DNA (deoxynucleic acid) based assays. The cell viability and proliferation assay used may significantly influence the results (Wang *et al.* 2010). When using the MTT- assay, it is not possible to differentiate between inhibition of cell growth and increase in cell death (García-Alonso *et al.* 2006). In other research comparing anti-proliferative activity assays, MTT and MTS- based methods resulted in underestimation of the anti-proliferative effects of EGCG ((-)-epigallocatechin-3-gallate), yet ATP- and DNA- based methods were highly correlated (r = 0.95) and both consistently demonstrated anti-proliferative activity of EGCG (Wang *et al.* 2010).

For the current study, the ability of *M. balsamina* and *M. foetida* leaf extracts to inhibit the proliferation of human liver hepatocellular carcinoma (HepG2) cells was determined by estimating cell number by DNA content using the Hoechst Fluorimetric method. The HepG2 cell line has been widely used in biochemical and nutritional studies as it is considered an experimental model that closely resembles the human hepatocyte in culture thus also permits the study of anti-proliferative activity for liver cancer research (Ramos *et al.* 2005; García-Alonso *et al.* 2006). The DNA-based method is based on the measurement of cellular DNA to show the relative cell number as cellular DNA content is highly regulated (Jones *et al.* 2001). In the DNA-based assay, the fluorescent dye binds to cellular nucleic acid and displays strong fluorescence with an excitation wavelength at 355 nm.

5.2 Results

Anti-proliferative activity determination was performed on crude extracts of *M. balsamina* and *M. foetida* leaf. These were chosen based on their activity in antimicrobial studies. This part of the study was performed to determine if the leaf extracts have the ability to reduce or stop multiplication of human liver hepatocellular carcinoma (HepG2) cells. The methods employed were described in Section 2.6. The extracts were tested for anti-proliferative activity at a concentration range of 0.6 to 1 mg/mL for *M. balsamina* and 0.25 to 2 mg/mL for *M. foetida*. These concentration ranges were selected following an initial screening using a wider range and determining the range that impacted on proliferation.

A concentration of *M. balsamina* extract above 1 mg/mL was found to result in cell death (data not shown) whilst for *M. foetida* extracts cell death was noticed at concentrations above 2 mg/mL.

5.2.1 Morphology of untreated and treated HepG2 cells

The morphological images of untreated HepG2 cells and those that had been treated with crude methanol-water extracts of 0.5 mg/mL for *M. balsamina* leaf and 1 mg/mL for *M. foetida* leaf, either sourced from Swaziland or UK glasshouse grown, after 24 h of incubation are presented in Figures 5-1 to 5-4.

In all instances, untreated HepG2 cells exhibited normal structure. The HepG2 cell line treated with *M. balsamina* for 24 h displayed a remarkable decrease in the number of cells (Figure 5-1 and Figure 5-2). Also the treated cells looked different from untreated cells, particularly in those cells treated with extracts of *M. balsamina* leaf sourced from Swaziland, where cells seemed to have lost

some of their neuronal-like features and were slightly smaller in size (Figure 5.1). The treated cells also seemed to have separated from each other with less clumping.

The HepG2 cell line treated with *M. foetida* extract for 24 h displayed a slight decrease in the number of cells (Figure 5-3 and Figure 5-4). Unlike the morphological images of cells treated with *M. balsamina* extracts, the *M. foetida* treated cells did not look very different from the normal cells. They did however show signs of separation from each other with less clumping. The differences were particularly evident with the cells treated with extracts from UK glasshouse grown *M. foetida* leaf (Figure 5.4).

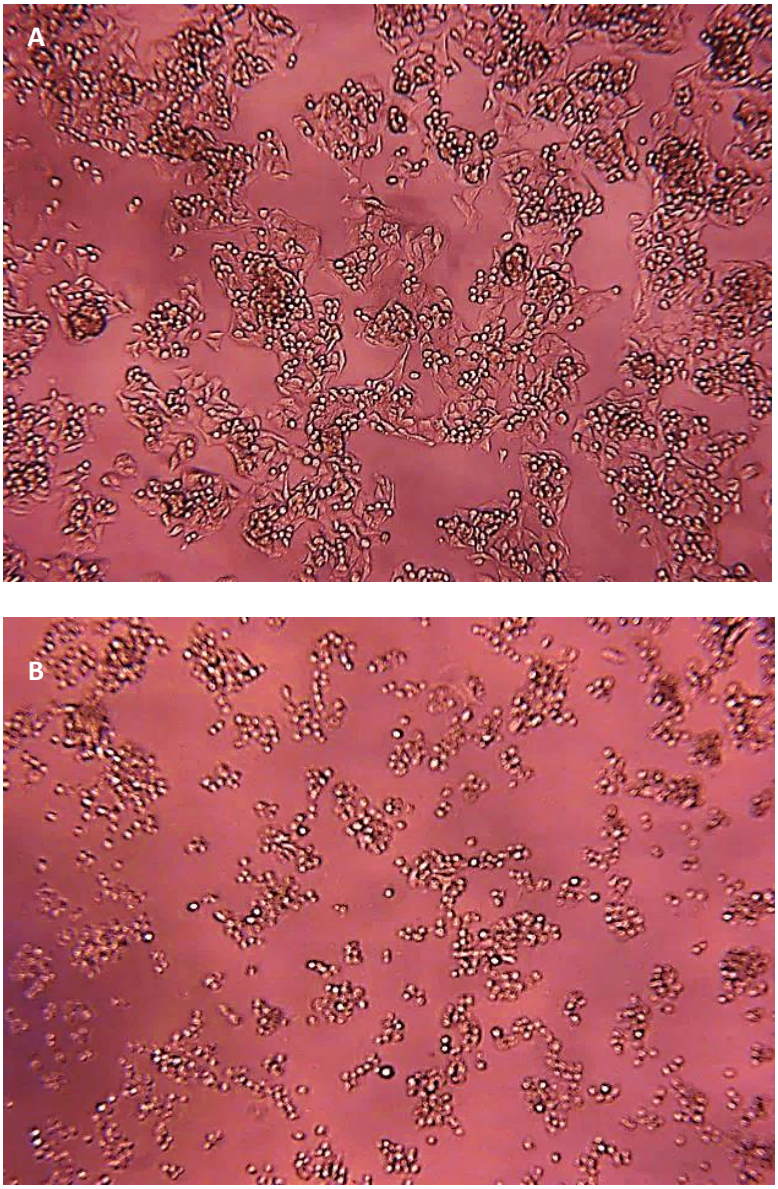


Figure 5-1. Effect of Swaziland sourced *M. balsamina* leaf extract on HepG2 cell morphology.

HepG2 cell lines were treated with 0.5 mg/mL of crude methanol-water extracts of *M. balsamina* leaf sourced from Swaziland for 24 h. The figure shows confocal images at 100x total magnification, showing morphology of untreated (A) and treated (B) cells. Pictures were taken by a Leitz inverted microscope with camera (Qcapture).

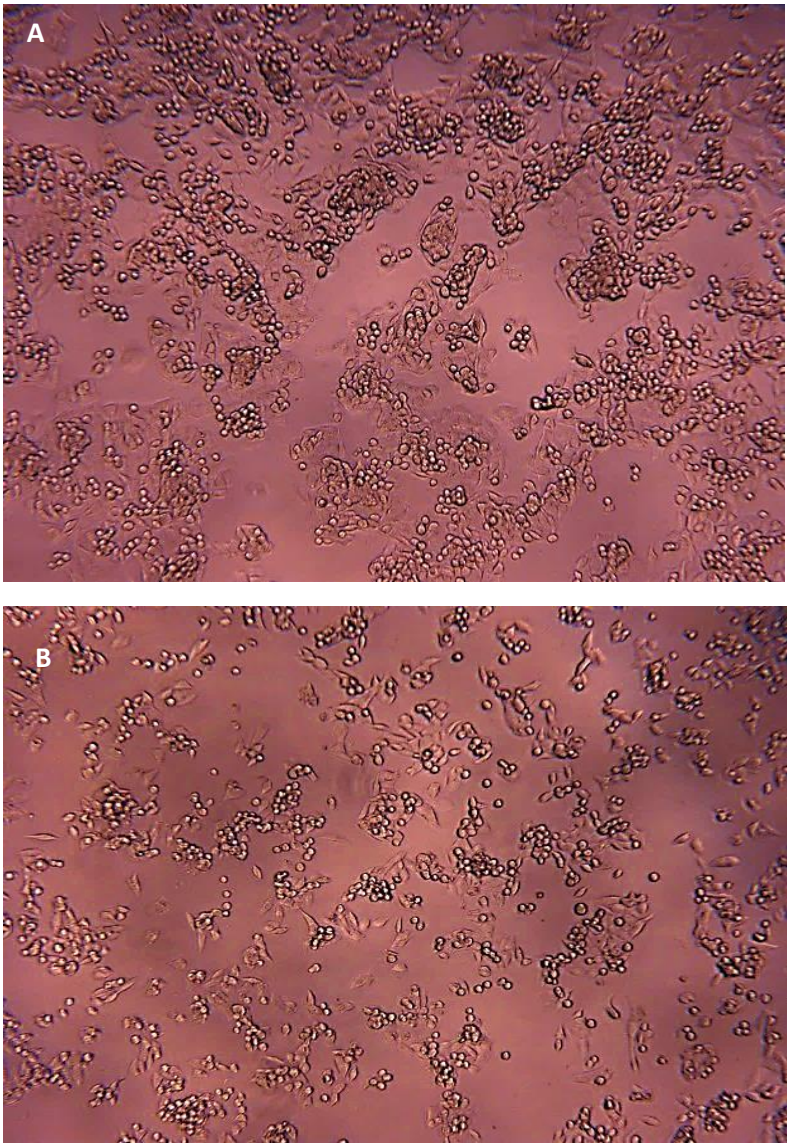


Figure 5-2. Effect of UK glasshouse grown *M. balsamina* leaf extract on HepG2 cell morphology.

HepG2 cell lines were treated with 0.5 mg/mL of crude methanol-water extracts of *M. balsamina* leaf grown in the UK glasshouse for 24 h. The figure shows confocal images at 100x total magnification, showing morphology of untreated (A) and treated (B) cells. Pictures were taken by a Leitz inverted microscope with camera (Qcapture).

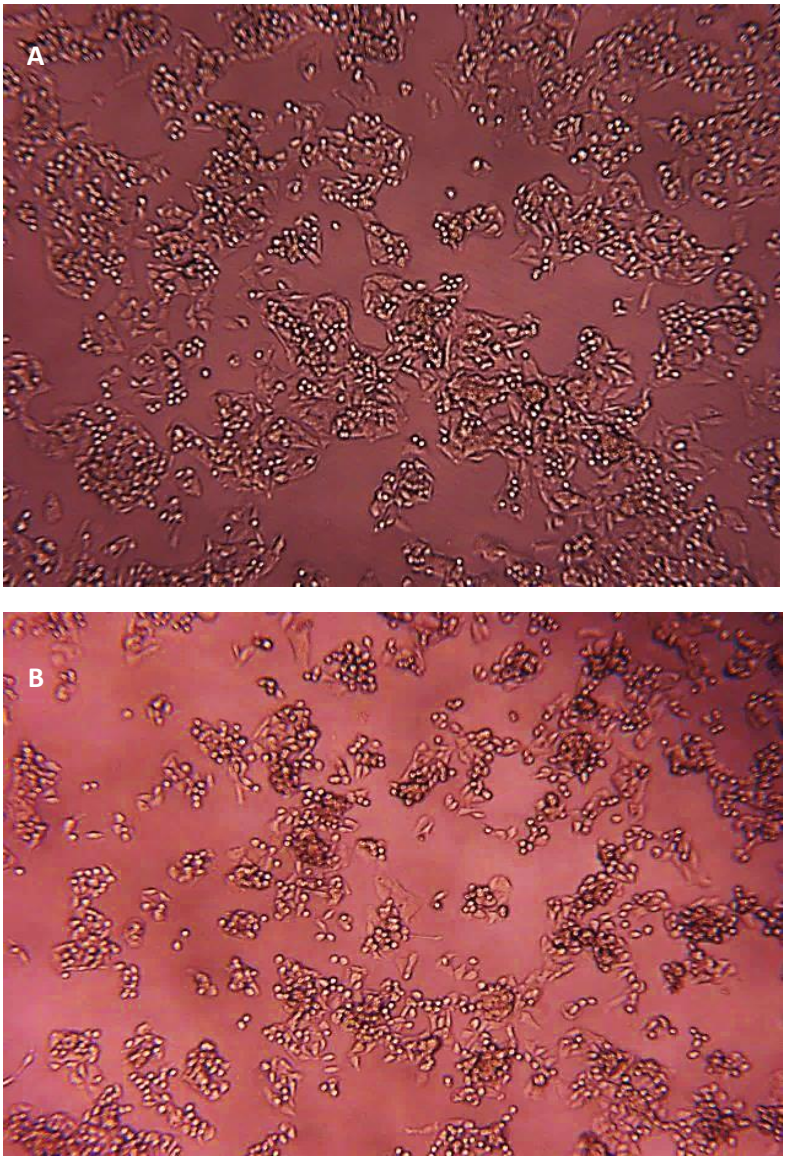


Figure 5-3. Effect of Swaziland sourced *M. foetida* leaf extract on HepG2 cell morphology.

HepG2 cell lines were treated with 1 mg/mL of crude methanol-water extracts of *M. foetida* leaf sourced from Swaziland for 24 h. The figure shows confocal images at 100x total magnification, showing morphology of untreated (A) and treated (B) cells. Pictures were taken by a Leitz inverted microscope with camera (Qcapture).

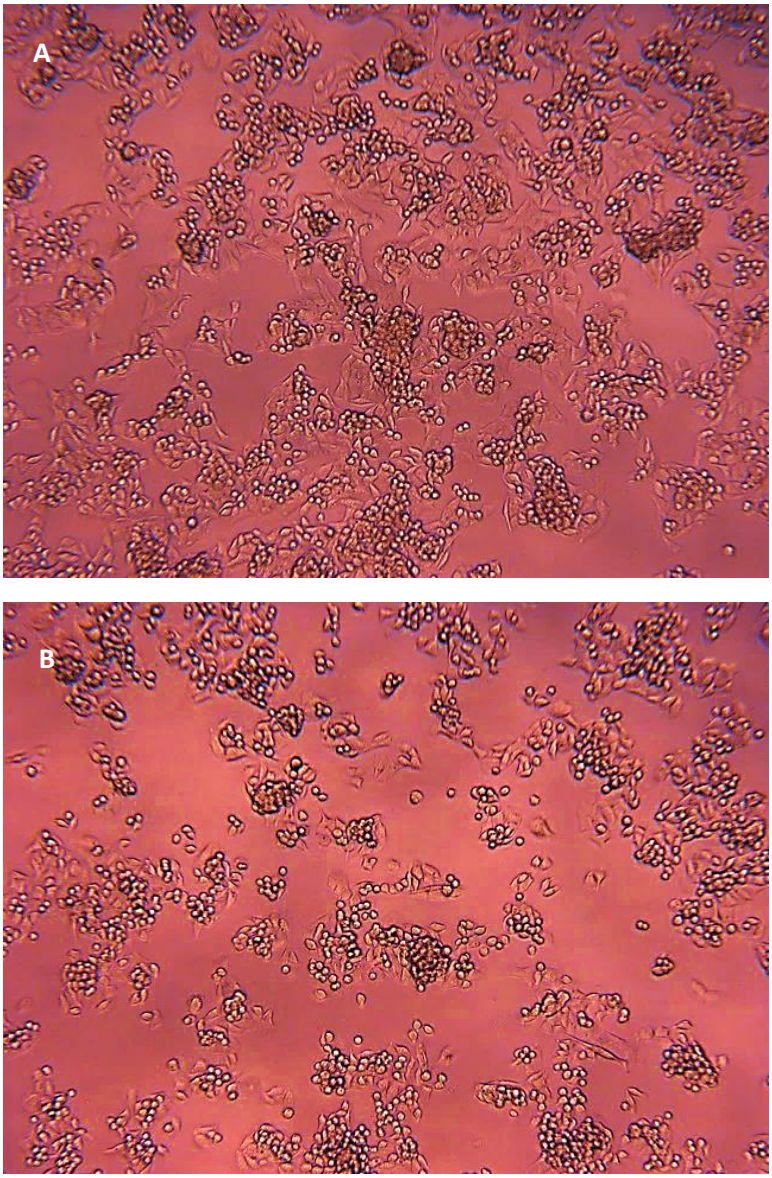


Figure 5-4. Effect of UK glasshouse grown *M. foetida* leaf extract on HepG2 cell morphology.

HepG2 cell lines were treated with 1 mg/mL of crude methanol-water extracts of *M. foetida* leaf grown in the UK glasshouse for 24 h. The figure shows confocal images at 100x total magnification, showing morphology of untreated (A) and treated (B) cells. Pictures were taken by a Leitz inverted microscope with camera (Qcapture).

5.2.2 Anti-proliferative activity of *M. balsamina* and *M. foetida* leaf extracts

The results for the anti-proliferation assays are displayed in Figures 5-5 to 5.7. All the controls showed a clear increase in DNA concentration over the 48 h of incubation. This demonstrated that cells were properly proliferating. Anti-proliferative activity was observed in both *M. balsamina* and *M. foetida* extracts but at different concentrations in each case. At 0 h DNA concentrations of untreated and treated cells were not significantly different ($P>0.05$) however, significant differences ($P<0.05$) were observed in DNA concentrations of HepG2 cells at 24 and 48 h. The treatment results displayed a time-dependant dose response, where even after 24 h of incubation cytotoxic activity was observed.

The highest anti-proliferative activity was observed in cells treated with extracts of *M. balsamina* leaf. Anti-proliferative activity was noticed at 0.25 mg/mL after both 24 and 48 h of incubation of cells treated with *M. balsamina* extracts sourced from Swaziland after which cytotoxic activity was observed. For the cells treated with UK glasshouse grown extracts of *M. balsamina* leaf, anti-proliferative activity was noticed at 0.5 mg/mL and cytotoxic effects were observed at 0.75 mg/mL after 24 h of incubation and at 0.5 mg/mL after 48 h of incubation.

Cells treated with extracts of *M. foetida* leaf displayed lower anti-proliferative activity. Inhibition of proliferation was observed at higher concentrations compared to *M. balsamina* leaf extracts. Initially anti-proliferative activity was observed at 1 mg/mL after 48 h of incubation for both Swaziland sourced and

UK glasshouse grown *M. foetida* extracts while cytotoxic effects were displayed at 2 mg/mL for these extracts.

Passage number seemed to have an effect on the anti-proliferative activity of *M. foetida* extracts. Results showed that with the higher passage cell line (passage 47) of HepG2, anti-proliferative activity of extracts may be lowered compared to cells treated at lower passage (passage 33). In this regard, anti-proliferative of *M. foetida* extracts were observed at even higher concentrations of 1.5 to 2 mg/mL and there were no cytotoxic effects displayed in this instance.

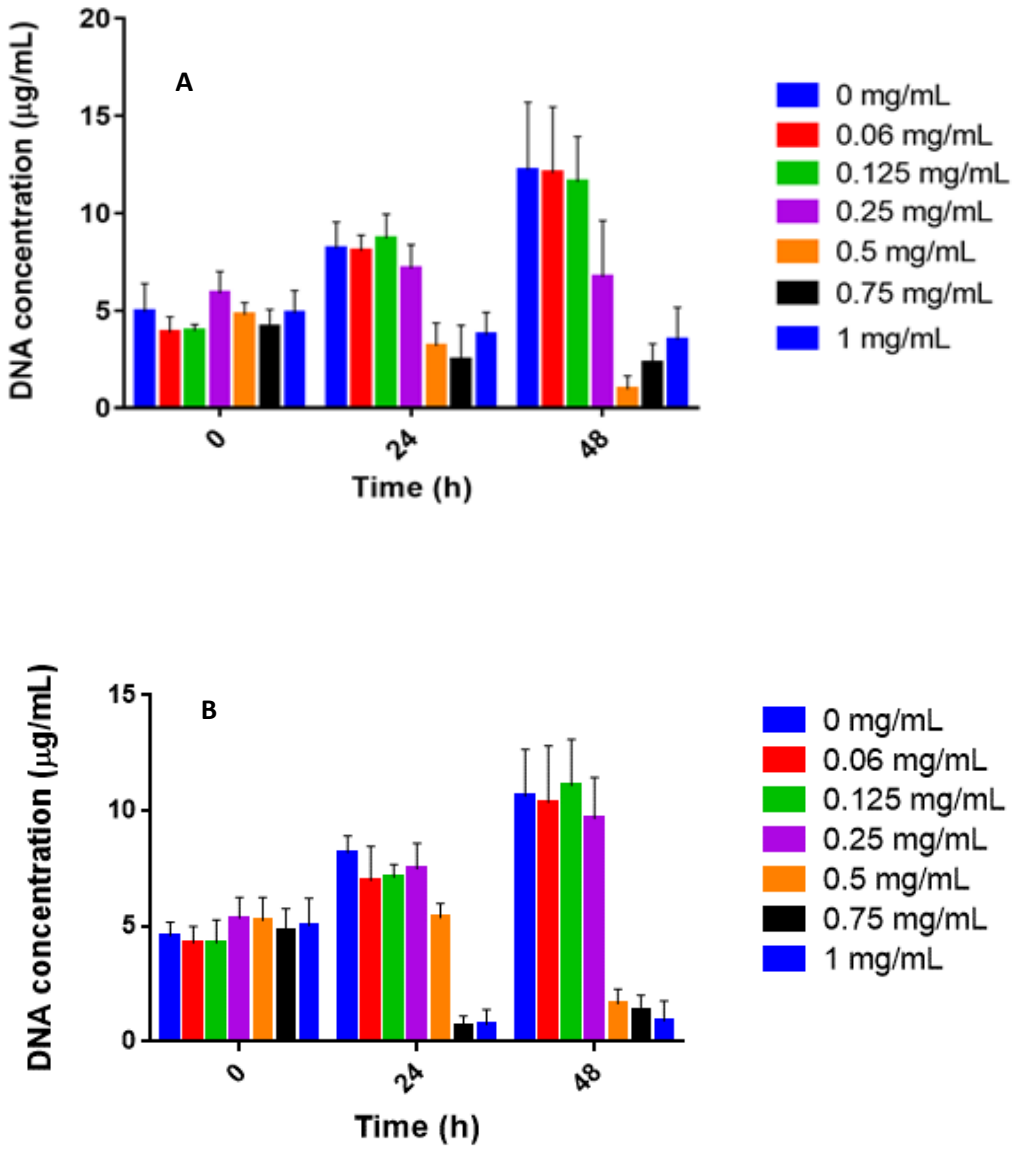


Figure 5-5. Effect of extracts of *M. balsamina* leaf on the proliferation of HepG2 cells. Extracts from *M. balsamina* leaf either (A) sourced from Swaziland or (B) UK glasshouse grown were applied to HepG2 cells (Cell passage 44) at concentrations of 0.06 to 1 mg/mL and DNA concentration monitored over 48 h of incubation. Results are the mean ± SD (n=6)

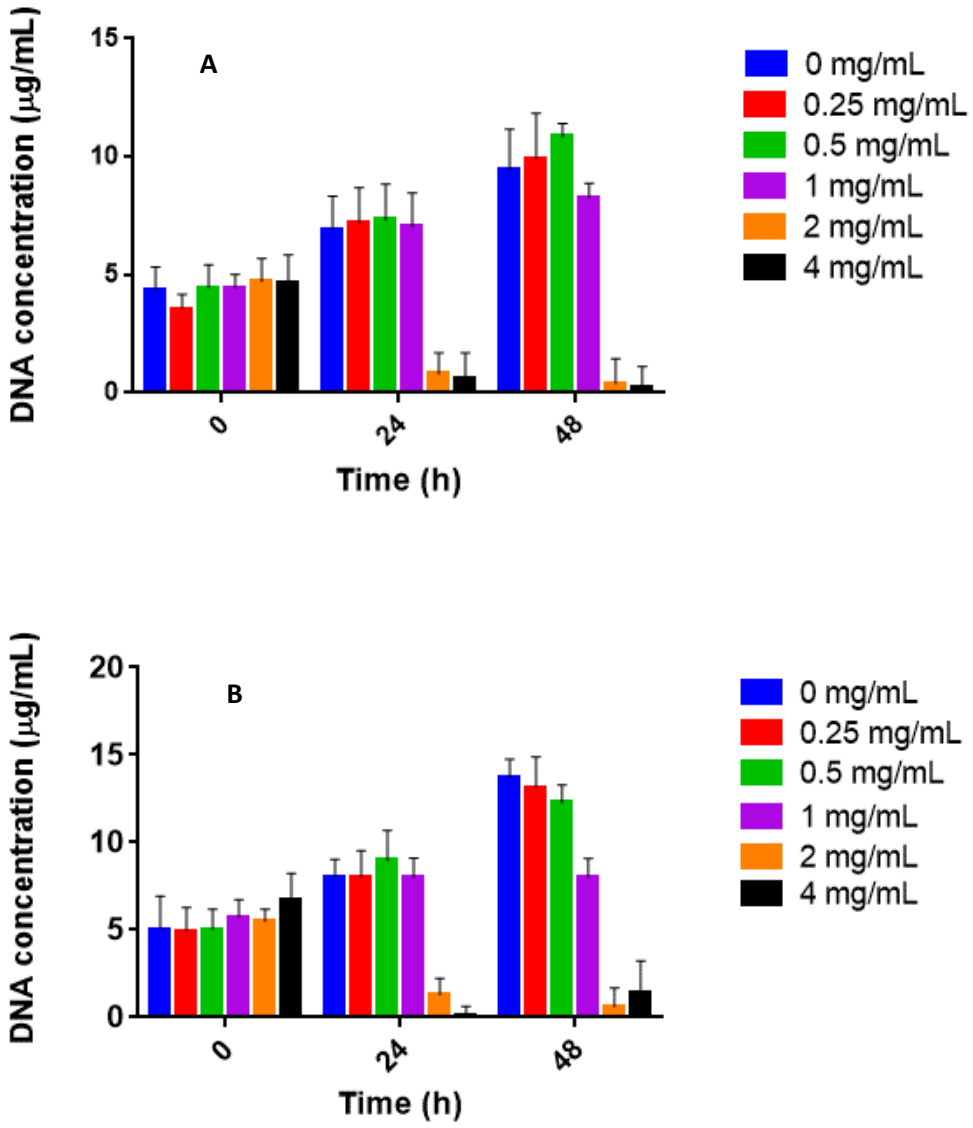


Figure 5-6. Effect of extracts of *M. foetida* leaf on the proliferation of HepG2 cells. Extracts from *M. foetida* leaf either (A) sourced from Swaziland or (B) UK glasshouse grown were applied to HepG2 cells (Cell passage 33) at concentrations of 0.25 to 4 mg/mL and DNA concentration monitored over 48 h of incubation. Results are the mean ± SD (n=6)

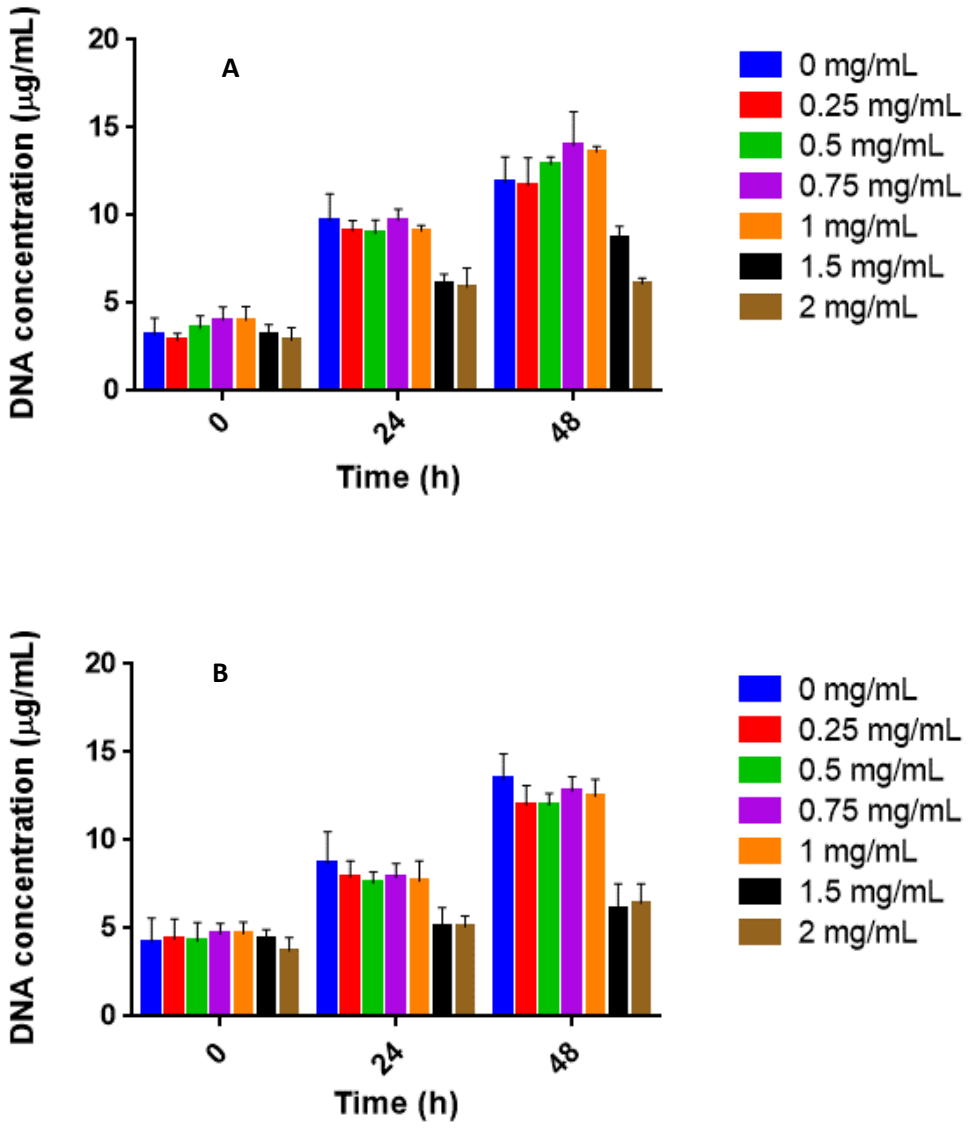


Figure 5-7. Effect of extracts of *M. foetida* leaf on the proliferation of HepG2 cells. Extracts from *M. foetida* leaf either (A) sourced from Swaziland or (B) UK glasshouse grown were applied to HepG2 cells (Cell passage 47) at concentrations of 0.25 to 2 mg/mL and DNA concentration monitored over 48 h of incubation. Results are the mean ± SD (n=6)

5.2.2 Relationship of antimicrobial activity and anti-proliferative activity of *Momordica* extracts

The previous chapter displayed, through disc diffusion, spot test, sensitivity tests and growth curves, that antimicrobial activity of *M. balsamina* leaves especially those sourced from Swaziland was higher than *M. foetida* leaves. Establishing whether anti-proliferative activity of the two plants would be related to the antimicrobial activity was necessary to determine whether there was any connection in their potency. Antimicrobial activity results chosen for this analysis were from the disc diffusion assay and inhibitions zones (mm) against *S. aureus* NCTC 10652, *S. aureus* NCTC 10655 and *L. monocytogenes* were used. This assay was selected because it is a well standardized method and the selected bacteria were used because they gave inhibition zones compared to other bacteria used in this study. Results for this analysis are presented in Figure 5-8.

Even though *M. balsamina* and *M. foetida* leaf extracts displayed both antimicrobial activity and anti-proliferative activity, there was only a weak correlation ($R^2 = 0.19$) between these two activities.

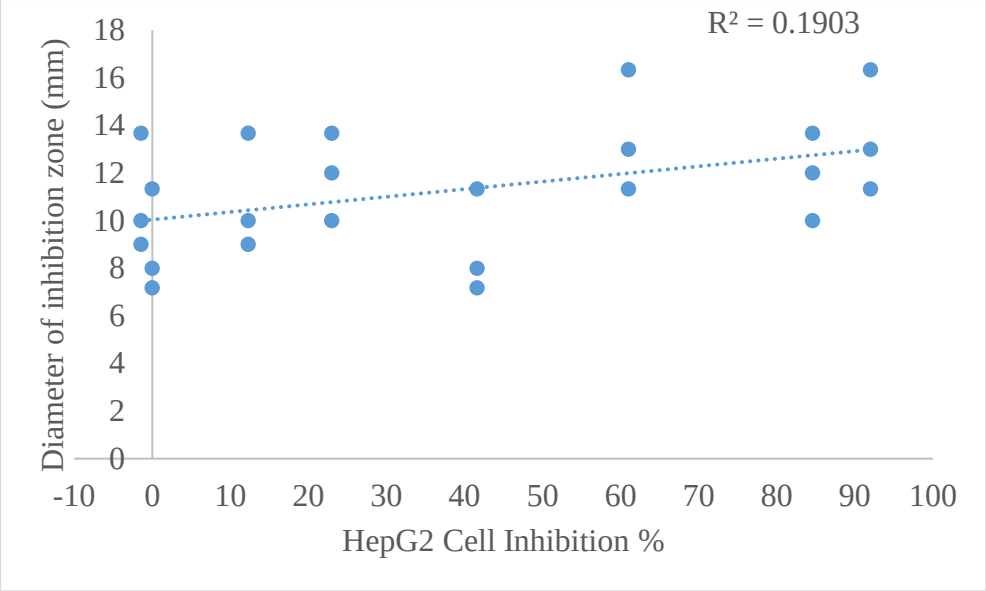


Figure 5-8. Correlation between antimicrobial and anti-proliferative activities of *Momordica* extracts.

Relationship of HepG2 inhibition (%) by *Momordica* extracts after 24 h and 48 h of incubation with inhibition zone (mm) of extracts (4 mg/mL) against *S. aureus* and *L. monocytogenes* strains.

5.3 Discussion

Leaves of plants have been used for food flavouring, food preservation and medicinal purposes since ancient times. Today, strategies to improve health are one of the driving forces for market growth in the food and beverage industry and this is one of the reasons for the increased interest in the use of natural products (Kaefer and Milner 2008). In Swaziland about 85% of the population is dependant mainly on medicinal plants to meet primary health care needs (Amusan 2009; Nguta *et al*. 2011). This study investigated the potential anti-proliferative activity, against a HepG2 cell line, of methanol-water extracts of *M. balsamina* and *M. foetida* leaves either sourced directly from Swaziland or UK glasshouse grown. Neither of these *Momordic*a plants had previously been used traditionally for the treatment of cancer in Swaziland. This study revealed three main findings.

First, anti-proliferative activity of *Momordica* plants varied depending on species and growth conditions. Effect of the extracts was also shown to be time and dose dependant with maximum effect observed mostly after 48 h of treatment. Extract of *M. balsamina* leaf sourced from Swaziland was the most potent and showed anti-proliferative activity at the lowest dose of 0.25 mg/mL and cytotoxicity at 0.5 mg/mL. Extract of *M. foetida* leaf was the least potent and showed anti-proliferative activity at a dose of 1 mg/mL and cytotoxicity at 2 mg/mL. The morphological data of this study clearly displayed some changes in structure of the HepG2 cell line when comparing treated with untreated cells after 24 h. This set of results has shown that methanol-water extracts of *Momordica* leaves initiate visible changes to the structure of the cells indicating either cell death or a decrease in cell viability. The results are

in agreement with findings of a study which showed that methanol extracts of *M. charantia* had a higher anti-proliferative effect against HepG2 cells compared to HCT116 (human colorectal cancer cells) and MCF-7 cell lines, showing an IC50 of *M. charantia* in HepG2 of 0.77 µg/mL while IC50 for HCT116 and MCF-7 were 0.81µg/mL and 135 µg/mL, respectively (Alshehri 2016). It is worth noting that the study displayed anti-proliferative activity of extracts at much lower concentrations compared to this thesis and this may be due to the different species studied or cell passage number. Contrary to this, another study reported that methanol extracts of *M. charantia* did not display any effect on all cell lines studied except for a human astrocytoma (1321N) cell line where, at high concentrations, a small decrease in cell viability was observed (Manoharan 2011). This may mean that methanol extraction in the current study does not denature the active compound responsible for the activity.

The second observation was that cell passage may have an effect on anti-proliferative activity of extracts. This was particularly evident in cells treated with extracts of *M. foetida* leaves where at much higher passage number (P47) of the HepG2 cells, growth inhibition took place without any cytotoxicity, even at higher concentrations of the extract, compared to results with lower passage (P33) cells which displayed cytotoxicity. This may imply that when working with HepG2 cells it may be necessary to work within a less variability range for continuous cell lines. The differences may indicate altered genetic expression of the cells, affecting their susceptibility. O'Driscoll *et al.* (2006) compared low passage (P18) and high passage (P40) MIN-6 cells (insulinoma cell line) and found significant differences in the expression of

several mRNAs involved in regulated secretion, adhesion and proliferation. Another study compared LNCap (human prostate cancer cells) at P25 and P60 and demonstrated that the regulation of androgen receptor activity by P13K/Akt (phosphoinositide 3-kinase / serine/threonine kinase) pathway was dependant on the passage number and this may affect the various stages of prostate cancer (Lin *et al.* 2003).

Lastly, the current study observed a weak correlation between HepG2 cell inhibition and antimicrobial activity. The weak connection suggested that the different activities caused by the extracts could be from different compounds. This current study was supported by a study which reported little connection of antimicrobial activity and anticancer activity on some Yemeni medicinal plants (Mothana *et al.* 2009). The results are in contrast to a report that suggested a strong connection between plants known from indigenous cultures to have medicinal properties compared to empirically determined cytotoxicity (Booth *et al.* 2012) however, this would rather depend on the particular medicinal properties claimed and might not mean antimicrobial activity. Mackeen *et al.* (2000) also reported that *G. atroviridis* extracts displayed antimicrobial, anti-tumour-promoting and antioxidant activities which rendered them appropriate as potential therapeutics.

5.4 Conclusion

The results in this thesis demonstrated that whilst anti-proliferative activity of *M. balsamina* and *M. foetida* leaf extracts was displayed, this activity was not particular strong compared to other studies. This study observed a dose and time dependant anti-proliferative activity on HepG2 cells after incubation with several concentrations of the *Momordica* extracts. In general *M. balsamina* leaf extracts were cytotoxic at lower doses than *M. foetida* extracts. It was also demonstrated that passage number may have an effect on anti-proliferative activity especially for *M. foetida* extracts. Lastly, similarly to antimicrobial activity, *M. balsamina* leaf extracts resulted in a greater reduction in HepG2 cell proliferation than *M. foetida* even though there was less relationship established between these activities. These results confirm the potential beneficial uses of *M. balsamina* and *M. foetida* in cancer prevention treatments. The preliminary anti-proliferative activity of the *Momordica* leaf extracts observed for the first time in this study against HepG2 cells requires further investigation to determine compounds that may be responsible for this activity and their potential mechanism(s). Similar to antimicrobial activity studies, studying the effects of these extracts on normal cell line is necessary to ascertain selectivity of anti-proliferative and cytotoxic activity to cancer cells.

CHAPTER 6: BIOCHEMICAL ANALYSIS OF *MOMORDICA* EXTRACTS

6.1 Introduction

Biochemical analysis of plants encompasses a wide variety of organic substances that are accumulated by plants and is concerned with chemical structures of these substances, their biosynthesis, metabolism, natural distribution and biological function (Harbone 1998). Sasidharan *et al.* (2011) summarized the main steps to investigate biologically active compounds from plants as extraction, pharmacological screening, isolation, characterization of bioactive compounds, toxicological evaluation and clinical evaluation. In all the above there are different methods required to separate, purify, and identify the variety of components present in the plants (Harbone 1998).

The medicinal value of plants is influenced by chemical substances that exert a certain physiological action on the human body and the most important bioactive constituents include tannins, alkaloids, flavonoids and phenolic compounds (Edeoga *et al.* 2005). Flavonoids are hydroxylated phenolic components that are synthesized by plants, often as a response to infection by microorganisms, and these have been reported to have antimicrobial activity against a variety of microorganisms in some studies (Yadav and Agarwala 2011). Phenolic compounds are abundant in plants and they enable the plants to adapt to changing biotic and abiotic environments and also provide colour, taste and health-promoting benefits to plant products (Mradu *et al.* 2012). Phenolics are formed by three biosynthetic pathways: the shikimate pathway, which produces the phenyl propanoid derivatives (C_6–C_3); the

acetate/malonate pathway, which produces the side-chain-elongated phenyl propanoids, including the large group of flavonoids (C_6–C_3–C_6) and some quinones; and lastly, the acetate/mevalonate pathway, which produces the aromatic terpenoids, mostly monoterpenes, by dehydrogenation reactions (Bhattacharya *et al*. 2010).

Recently, there has been an increase in studies focusing on the characterization of health-promoting properties of many phenolic compounds that exhibit antioxidant properties (Mradu *et al.* 2012). There are some suggestions that crude extracts from plants are more biologically active than isolated compounds due to their synergistic effects (Vaghasiya *et al.* 2011). Knowledge of chemical substances of plants is desirable because such information will be valuable in the synthesis of complex chemical components (Parekh and Chanda 2007; 2008; Mojab *et al.* 2010; Yadav and Agarwala 2011). Plants used in traditional medicine are more likely to produce pharmacologically active compounds (Hostettmann and Wolfender 1997).

The chemical nature of phenolics differs from simple to highly polymerized substances and they may also occur in complexes with carbohydrates, proteins as well as other plant components (Naczk and Shahidi 2004). Solid phase extraction (SPE) is one of the commonly used methods to fractionate crude extracts (Robbins 2003). The SPE technique is fast, cheap and sensitive and can now be automated (Dai and Mumper 2010). The release of phenolic compounds from conjugated molecules such as sugars can be achieved through acidification or saponification of samples. Saponification involves treating the sample with a solution of sodium hydroxide at concentrations from 1 to 4 M at room temperature for between 15 minutes to overnight

(Robbins 2003; Stalikas 2007). There are many techniques that can be employed in chemical analysis and these include thin layer chromatography (TLC), paper chromatography (PC), gas chromatography (GC), liquid chromatography (LC), gas chromatography-mass spectrometry (GCMS), liquid chromatography-mass spectrometry (LCMS), Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) (Harborne 1998). For this study biochemical analysis of methanol-water extracts of *M. balsamina* and *M. foetida* were carried out through FTIR, high performance liquid chromatography (HPLC), NMR and LCMS analyses. These were selected based on their ability to provide information on a broader range of substances and molecules. FTIR is a valued tool for characterization of compounds or functional groups existing in an unknown mixture (Sasidharan *et al.* 2011). HPLC is a versatile, vigorous and broadly used technique for isolation of natural products (Sasidharan *et al.* 2011) and some of its uses in phytochemistry include piloting the preparative isolation of natural products and optimization of the experimental conditions. It is the most suitable technique for an efficient separation of crude plant extracts and can be used together with different spectroscopic detection methods (Hostettmann and Wolfender 1997). LCMS is a powerful technique for the analysis of complex extracts (Sasidharan *et al.* 2011). MS is one of the most sensitive methods of molecular analysis and has the potential to produce information on the molecular mass as well as on the structure of the analyte (Hostettmann and Wolfender 1997). Liquid chromatography (LC) coupled with MS detection is an emerging field and seems to be a promising technique in addressing complexity of plant extracts (Robbins 2003). Proton NMR spectroscopy

provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms (Harborne 1998). NMR detection is more of a structure confirmation than a quantification method (Dai and Mumper 2010).

6.2 Results

6.2 1 FTIR-ATR analysis

FTIR measurements were performed on crude extracts of *Momordica* leaf and stem as described in Section 2.8. FTIR spectra were collected over the range of 4000 – 500 cm^{-1}. For each material, three samples were studied under the same conditions.

In the first instance FTIR spectra from crude extracts were obtained from both un-extracted dried tissues and crude extracts from both species of *Momordica*. Figure 6-1 displays the spectra of the investigated *Momordica* crude extracts over the whole spectral range. Similar spectra were obtained for the un extracted samples (data not shown). It was not possible to detect any major differences, by visual analysis of the whole spectra, between either tissues or extracts.

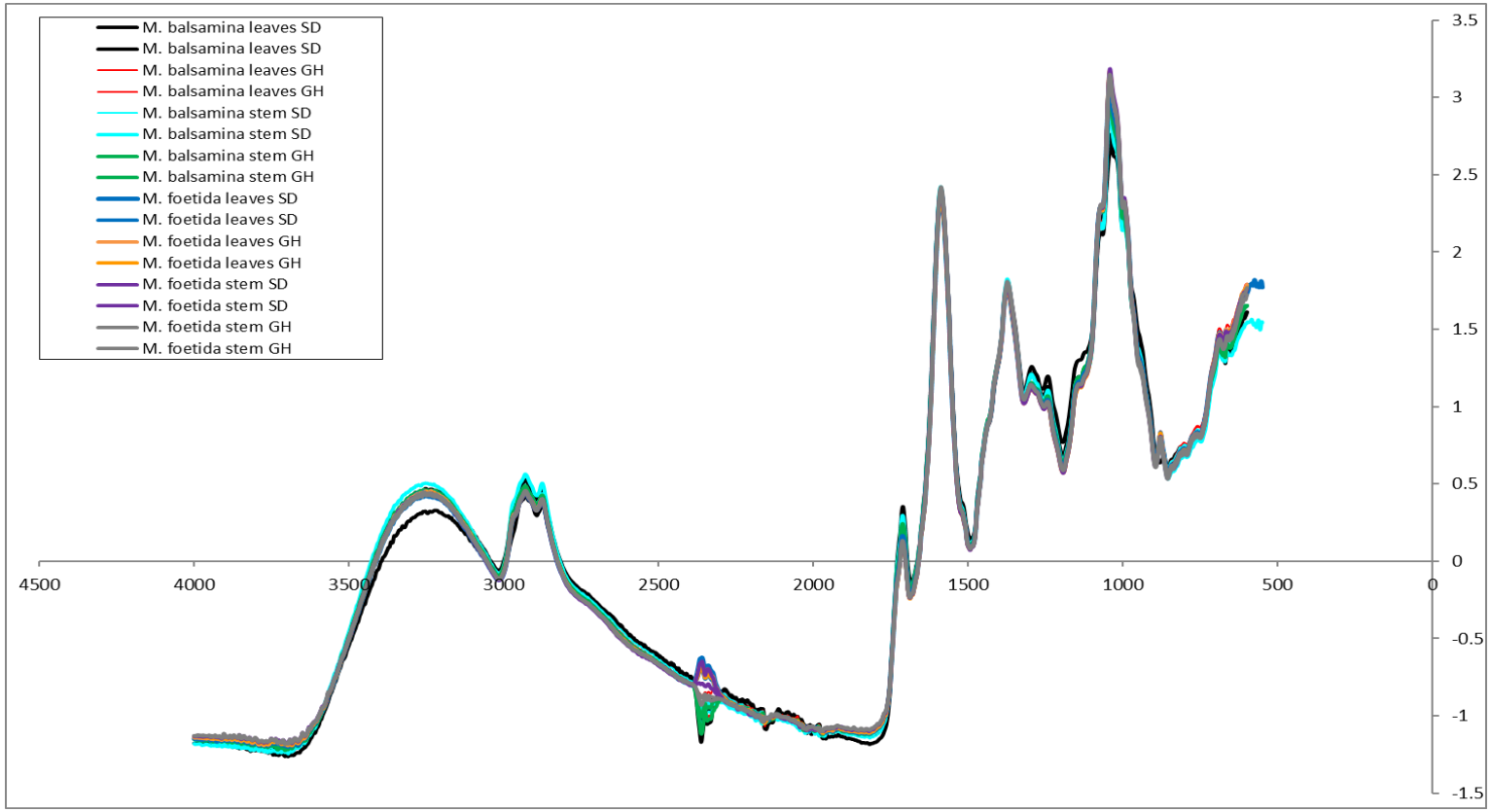


Figure 6-1 FTIR spectra of *Momordica* methanol-water extracts in the range of A) 4000 to 500 cm^{-1}. SD - Swaziland sourced, GH - UK glasshouse sourced.

The FTIR data were thus subjected to PCA and the results are displayed in Figure 6-2. PCA results for the dried un-extracted tissue of *M. balsamina* and *M. foetida* leaf and stem displayed differences according to plant part and growth conditions but not between species. UK glasshouse grown tissues were all grouped according to either stem or leaf. Similarly, the same grouping was observed for Swaziland sourced tissue.

Interestingly, for the crude extracts PCA results showed a different pattern of grouping. Results displayed a nice clustering of extracts based on the plant species and the plant tissue extracted thus revealing differences between both species and plant parts for the studied extracts.

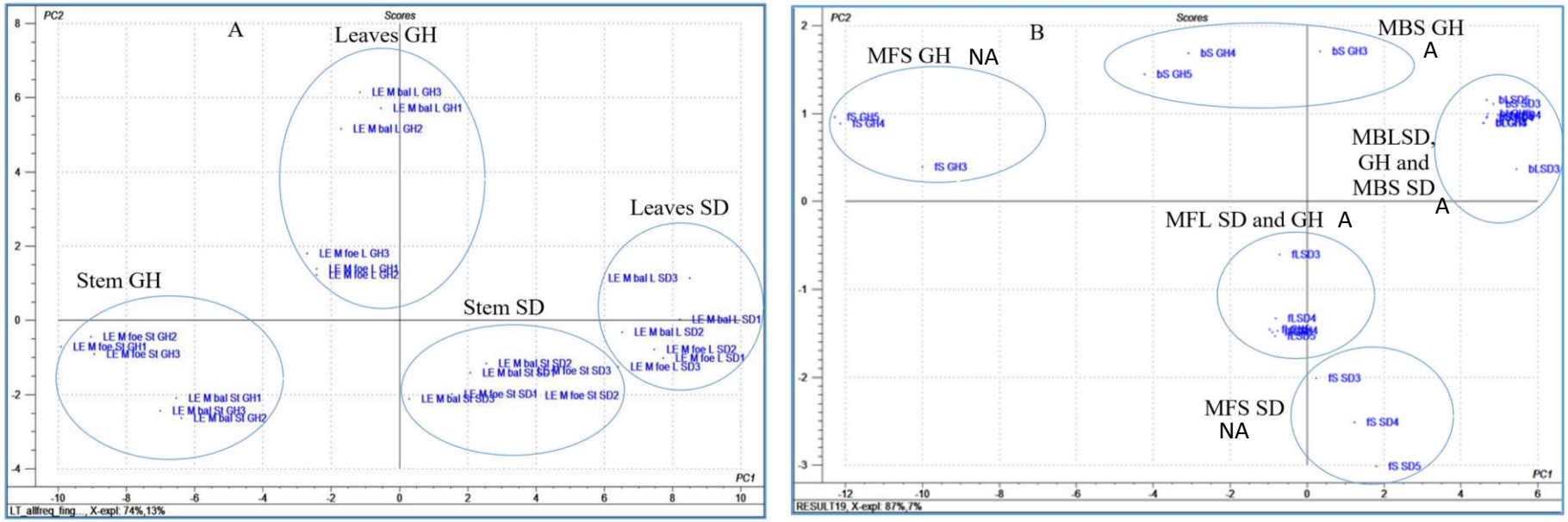


Figure 6-2 PCA scores scatter plots derived from FT-IR spectra in the fingerprint region 1800 to 900 cm^{-1} of *M. balsamina* and *M. foetida* leaf and stem (A) dried tissue and (B) methanol-water extracts of tissues from UK glasshouse (GH) and Swaziland (SD) sourced plants. For each extract, 3 replicates were recorderd which are replicates displayed in the PCA plots. For plot A M. bal indicates *M. balsamina*; M. foe indicates *M. foetida*; L = leaf, St = stem. For plot B MBL – *M. balsamina* leaf, MBS – *M. balsamina* stem, MFL – *M. foetida* leaf and MFS – *M. foetida* stem, NA – No antimicrobial activity, A – Antimicrobial activity.

6.2.2 Total soluble phenolic content

Total soluble phenolic content was determined, following the procedure described in Section 2.9, and the results expressed as mg GAE/g of dry extract. Total soluble phenolic content was initially determined on crude extracts of *M. balsamina* and *M. foetida* leaf, stem and fruit sourced from Swaziland (SD) and UK glasshouse grown (GH). Fractionation of extracts using solid phase extraction (SPE) was then performed on *M. balsamina* and *M. foetida* leaf and stem sourced from Swaziland. This selection was based on their relative antimicrobial activities. *Momordica* crude extracts reconstituted in PBS (2 mg/mL) were fractionated using Oasis SPE columns to give three fractions: F1, F2 and F3 (Section 2.3). These fractions together with the crude extracts were assessed for total soluble phenolic content.

6.2.2.1 Total soluble phenolic content of crude extracts

Results for total soluble phenolic contents of extracts of *M. balsamina* and *M. foetida* leaf, stem and fruit sourced from Swaziland and UK glasshouse grown are presented in Figure 6-3. In general leaf extracts yielded a higher phenolic content followed by stem. Leaf extracts of *M. foetida* sourced from Swaziland displayed significantly higher ($P < 0.05$) phenolic content compared to all the extracts studied. In all instances the fruit extracts displayed the lowest phenolic content with a range of 3.84 to 12.11 mg GAE/g.

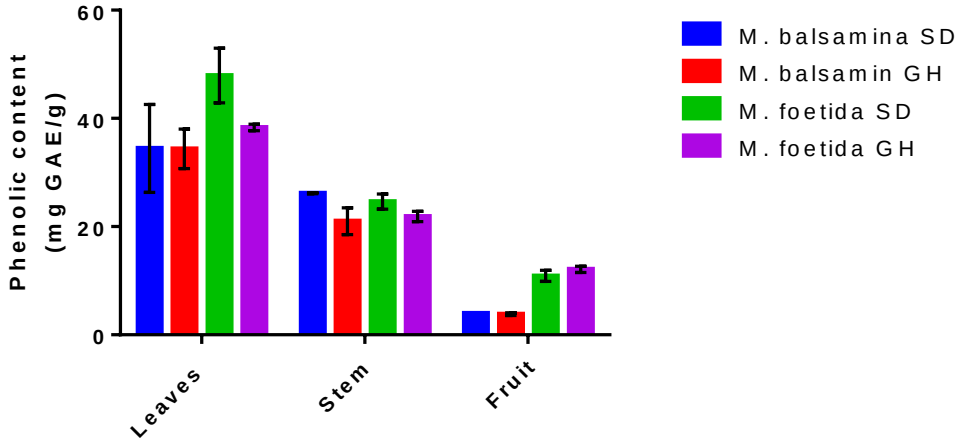


Figure 6-3 Total soluble phenolic contents (mg GAE/g) of extracts of *M. balsamina* and *M. foetida* leaf, stem and fruit sourced from Swaziland (SD) and UK glasshouse grown (GH). Values are expressed as means ± standard deviation (n = 2). *M. foetida* leaves SD had significantly higher ($P < 0.05$) phenolic content than all extracts.

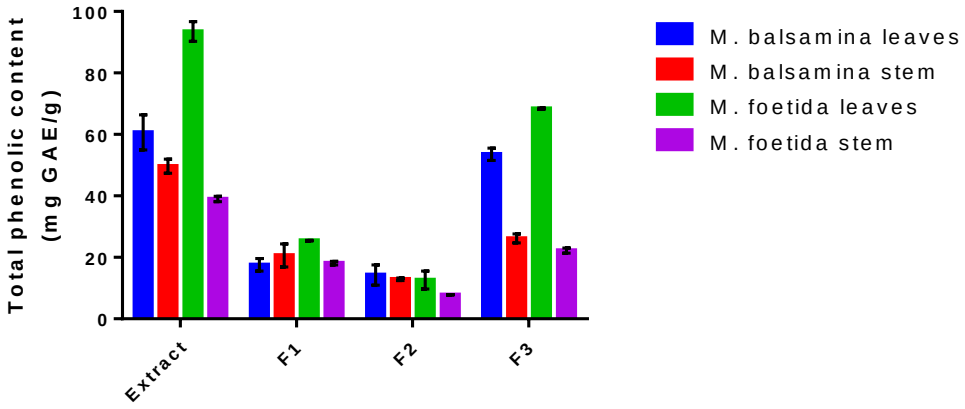


Figure 6-4 Total soluble phenolic contents (mg GAE/g) of crude extracts and their SPE fractions (F1, F2 and F3) of *Momordica* leaf and stem sourced from Swaziland. Values are expressed as means ± standard deviation (n = 2). Crude extracts and SPE fraction 3 were all significantly different ($P < 0.05$) except for *M. balsamina* leaves.

6.2.2.2 Total soluble phenolic content of SPE fractions

Results for the total phenolic content of the SPE fractions are shown in Figure 6-4. Results showed that, as expected, the majority of the phenolics were in fraction 3 for all the SPE extracts. In all instances recovery of total phenolic content for fraction 3 was shown to be 53% or higher. Fraction 3 of *M. balsamina* leaf displayed the highest recovery of 88%. The lowest recovery (52.7%) was observed in the fraction 3 of *M. balsamina* stem which showed a total phenolic content of 26.16 mg GAE/g compared to their original extracts which displayed a total phenolic content of 49.66 mg GAE/g.

The mass balance for the recovery of total phenolic content in SPE extracts is shown in Table 6-1. The percentage recovery in each case was between 113.84 and 140.77%.

6.2.2.3 Relationship of antimicrobial activity and total soluble phenolic content of crude extracts

A comparison of antimicrobial activity (inhibition zone against *L. monocytogenes* using disc diffusion method) and total phenolic content was carried out for crude extracts of *M. balsamina* and *M. foetida* (Figure 6-5). To ensure that similar levels of extract were applied in each case, dried crude extracts were standardized to 4mg/disc. This displayed a linear relationship between antimicrobial activity and total phenolic content of *Momordica* extracts with a correlation coefficient of $R^2 = 0.579$.

Table 6-1 Mass balance for the recovery of total soluble phenolic content (mg GAE/g) in SPE extracts

Extract	Original extract	Sum of SPE extracts	Recovery (%) of SPE extracts
MBL	60.66	85.39	140.77
MBS	49.66	59.68	120.19
MFL	93.49	106.43	113.84
MFS	38.99	48.14	123.47

MBL – *M. balsamina* leaf, MBS – *M. balsamina* stem, MFL – *M. foetida* leaf and MFS – *M. foetida* stem.

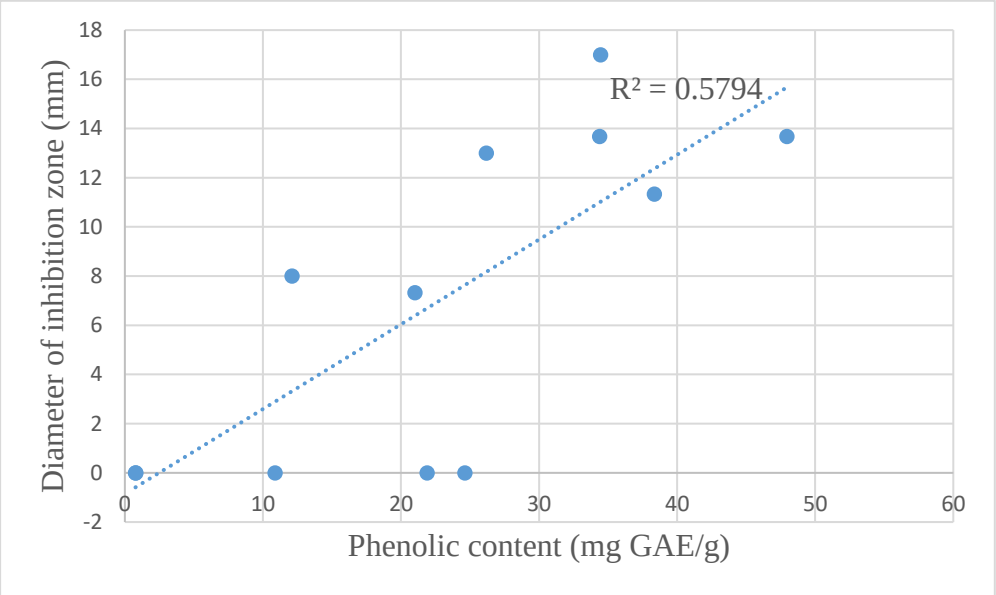


Figure 6-5 Correlation between total soluble phenolic content (mg GAE/g) and inhibition zone (mm) of extracts of *M. balsamina* and *M. foetida* against *L. monocytogenes*.

6.2.3 HPLC analysis

Crude extracts and SPE F3 samples of *M. balsamina* and *M. foetida* leaves and stem were profiled by HPLC as described in Section 2.10. The phenolic compounds present in *Momordica* samples were identified by comparison with retention times and spectra of known standards. The HPLC profiles of 11standard phenolics are presented in Figure 6-6. The standard compounds were clearly eluted at different retention times.

6.2.3.1 Crude and SPE extracts

Figure 6.7 shows some typical HPLC elution profiles. There were only a few peaks in *Momordica* extracts that corresponded to phenolic standards and the rest were unidentified. The putative phenolic compounds identified (Table 6-2) in qualitative evaluation of *Momordica* crude extracts and SPE F3s included gallic acid, ferulic acid, sinapic acid, myricetin, kaempferol, epigallocatechin gallate, *p*-coumaric acid, and epicatechin (some chemical structures of common phenolic compounds present in plants are presented in the Appendix). Gallic acid was the only phenolic compound which was identified in all the *Momordica* crude and SPE fractions. Based on the phenolic standards used, there were more phenolic acids identified in *M. balsamina* leaf and stem compared to their counterparts, *M. foetida* leaf and stem.

Crude extracts of *M. balsamina* leaf and stem (presented in Appendix 6) displayed clear differences with a few common peaks detected, while extracts of *M. foetida* leaf and stem displayed minimal differences (Figure 6.7). Figure 6-8 presents a typical HPLC chromatogram comparing crude extracts of *M.*

balsamina leaf and *M. foetida* leaf. This comparison between the two species revealed that there were clear differences observed in peaks but with only one or two common peaks identified.

Figure 6-9 shows a typical HPLC chromatogram comparison of *M. foetida* leaf crude extract and SPE fraction 3. Results for this part displayed that SPE F3s had lost some of the peaks, suspected to be *p*-coumaric acid (RT, 22.87 minutes) and sinapic acid (RT, 23.59 minutes) that were observed in the crude extract and was showing one new peak previously not clearly showing in the crude extract, suspected to be kaempferol (RT, 39.16 minutes). Also the intensity of the peaks had decreased and gallic acid (RT, 4.09 minutes) was among the suspected phenolic compounds that reduced in intensity for SPE fraction 3. Also SPE F3 fractions for extracts of both *M. foetida* leaf and stem displayed a similar pattern of peaks (presented in Appendix 6) just as observed in their crude extracts.

It was also observed that some peaks in the chromatograms were not clearly showing individual components. To further explore these components, samples were saponified to release conjugated phenolics. Figure 6-10 displays typical HPLC chromatograms comparing crude and saponified extracts of *M. foetida* leaf. The rest of the saponified extracts are presented in Appendix 6. HPLC chromatograms for saponified extracts generally confirmed that *Momordica* extracts are variable in their composition. These chromatograms revealed that there were some peaks which appeared to be degraded by the two-hour hydrolysis, while some peaks after hydrolysis may have merged into one peak resulting in larger peaks. An increased intensity of one particular merged, unidentified peak (RT, 30.94 minutes), was observed in saponified

extract of *M. foetida* leaf compared to the crude extract which displayed two low intensity peaks at RT, 31.36 and 32.57 minutes all showing the same absorption bands. In some cases, such as extracts of *M. balsamina* leaf (also shown in Appendix 6), saponification resulted in decreased intensity of peaks.

6.2.4 NMR analysis

NMR analysis was performed on *M. balsamina* leaf and stem crude extracts and their SPE F3 fractions using proton (^{1}H) spectra following the procedure as described in Section 2.11. Results showing the whole spectra and aromatic sections are presented in Figure 6-11. Visual analysis of the whole NMR spectra displayed that aromatics (6.0 – 8.5 ppm) were present at lower concentrations compared to either sugars (3.4 – 6.0 ppm) or compounds in the aliphatic region (0.0 – 3.4 ppm). Results in the aromatic region showed significant similarities but also exhibited some differences between the extracts.

NMR results confirmed total phenolic content data showing that aromatics were being preferentially isolated in the methanol eluted fraction 3 from the SPE. Results also displayed that there are some aromatics that are being lost into the other SPE eluted fractions. These results also show that some sugars are being reduced in the SPE F3s (Figure 6-11 A) thus showing reduced intensities of peaks in the carbohydrate region.

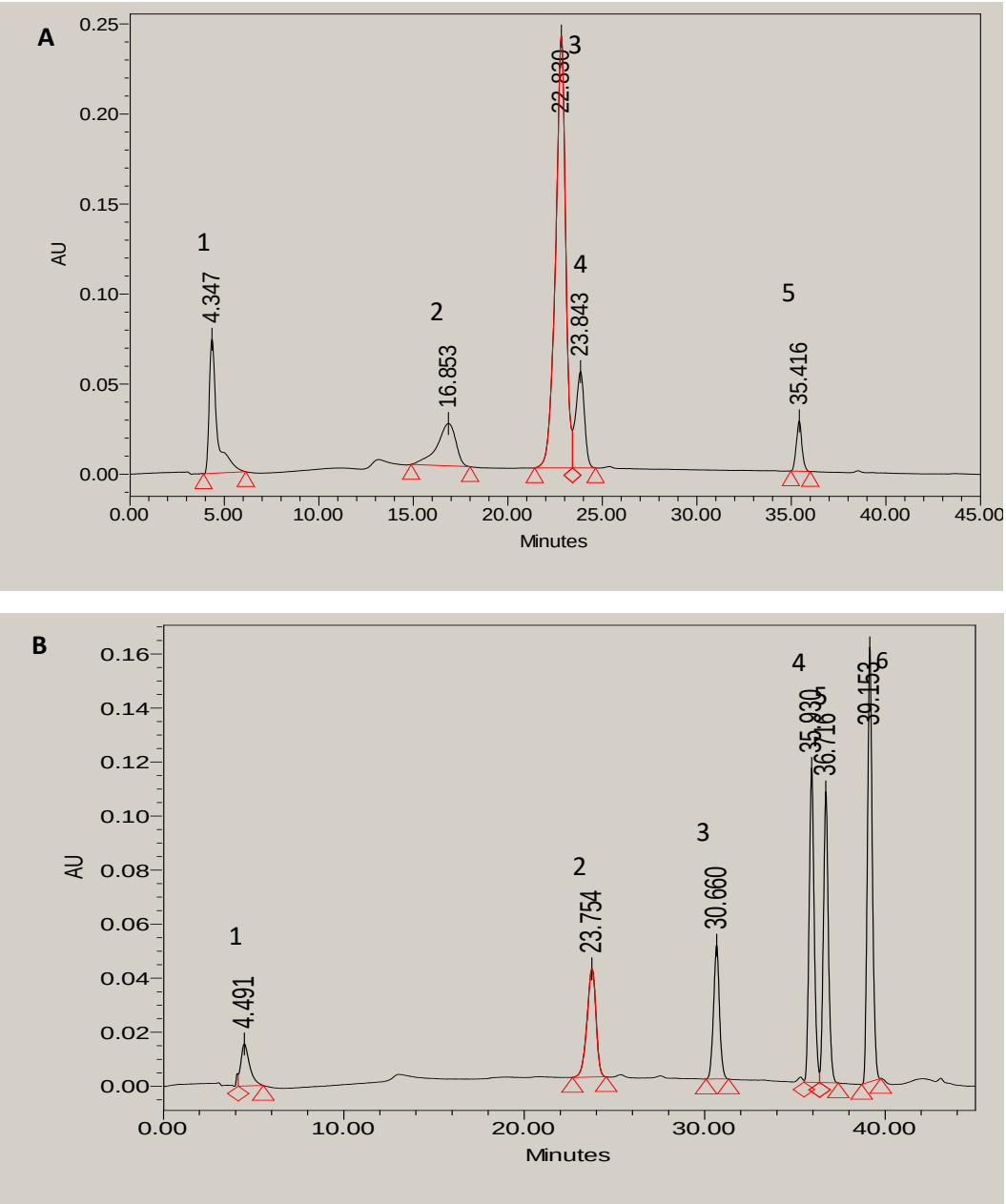


Figure 6-6 HPLC chromatogram of phenolic standard mixture A and B. Mixture A: 1 – Gallic acid, 2 – Caffeic acid, 3 – *P*-coumaric acid, 4 – Ferulic acid and 5 – Quercetin. Mixture B: 1 - Epigallocatechin gallate, 2 – Sinapic acid, 3 – Myricetin, 4 – Naringenin, 5 – Luteolin and 6 – Kaempferol. HPLC conditions are described in Section 2.10.

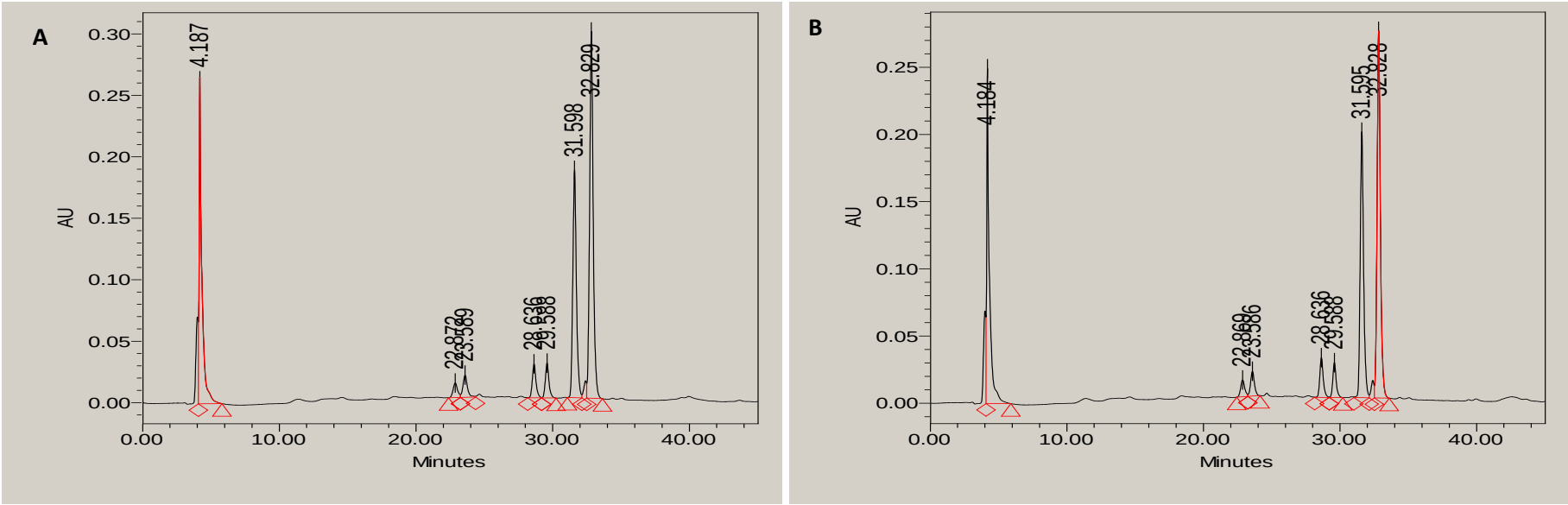


Figure 6-7 Typical HPLC chromatogram of crude extracts of A) *M. foetida* leaf and B) *M. foetida* stem. HPLC conditions are described in Section 2.10.

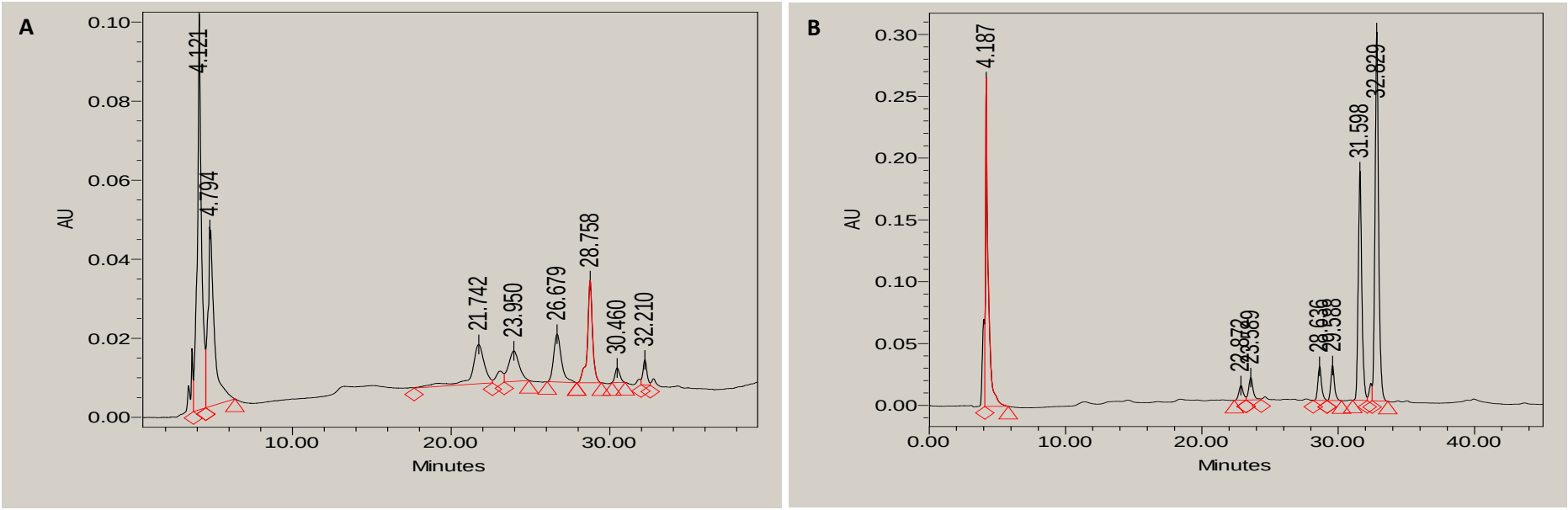


Figure 6-8 Typical HPLC chromatogram of crude extracts of A) *M. balsamina* leaf and B) *M. foetida* leaf. HPLC conditions are described in Section 2.10.

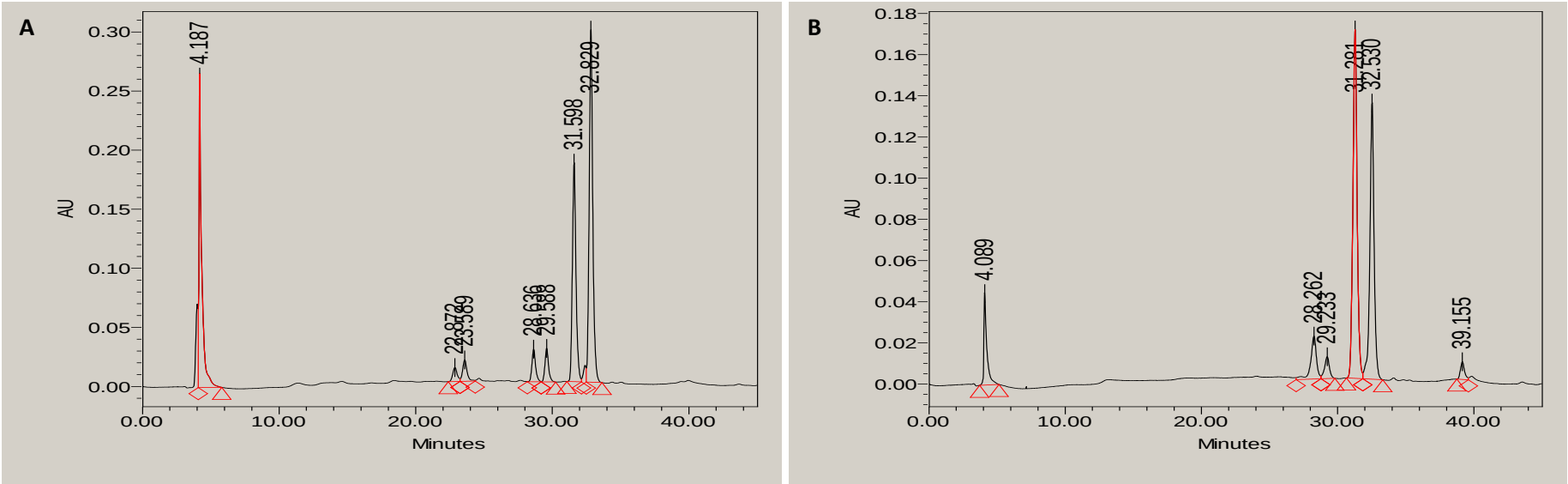


Figure 6-9 Typical HPLC chromatogram of *M. foetida* leaf A) crude extract and B) SPE fraction 3. HPLC conditions are described in Section 2.10.

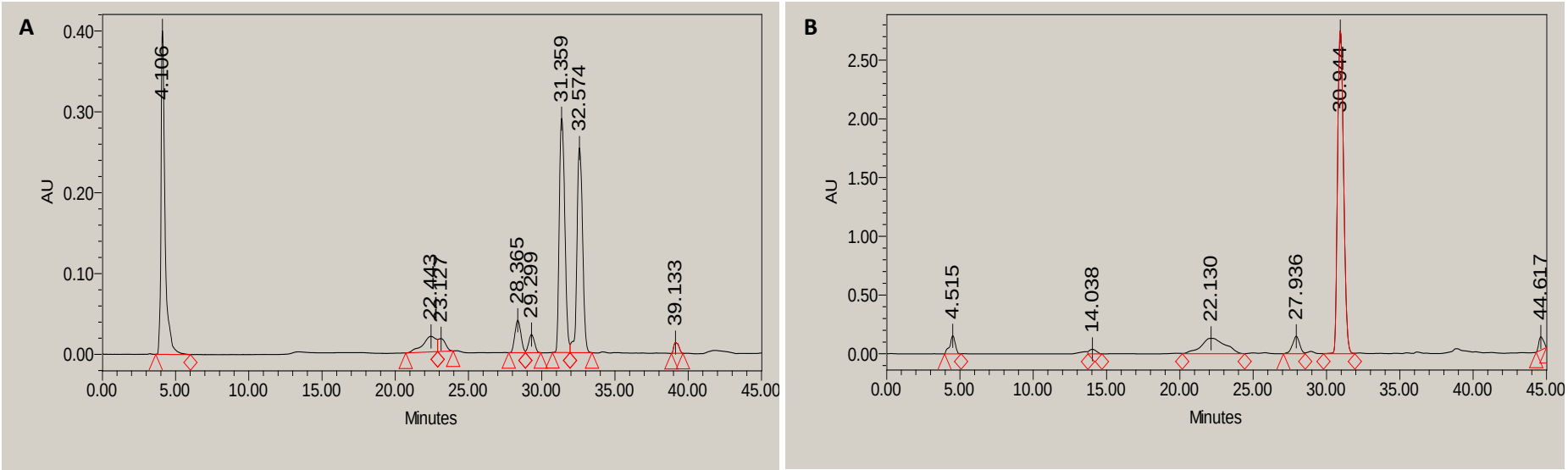


Figure 6-10 Typical HPLC chromatogram of *M. foetida* leaf A) crude extracts and B) saponified extracts. HPLC conditions are described in Section 2.10.

Table 6-2 Qualitative phenolic acid evaluation in *Momordica* extracts and SPE F3 identified by HPLC using standards and retention time (RT).

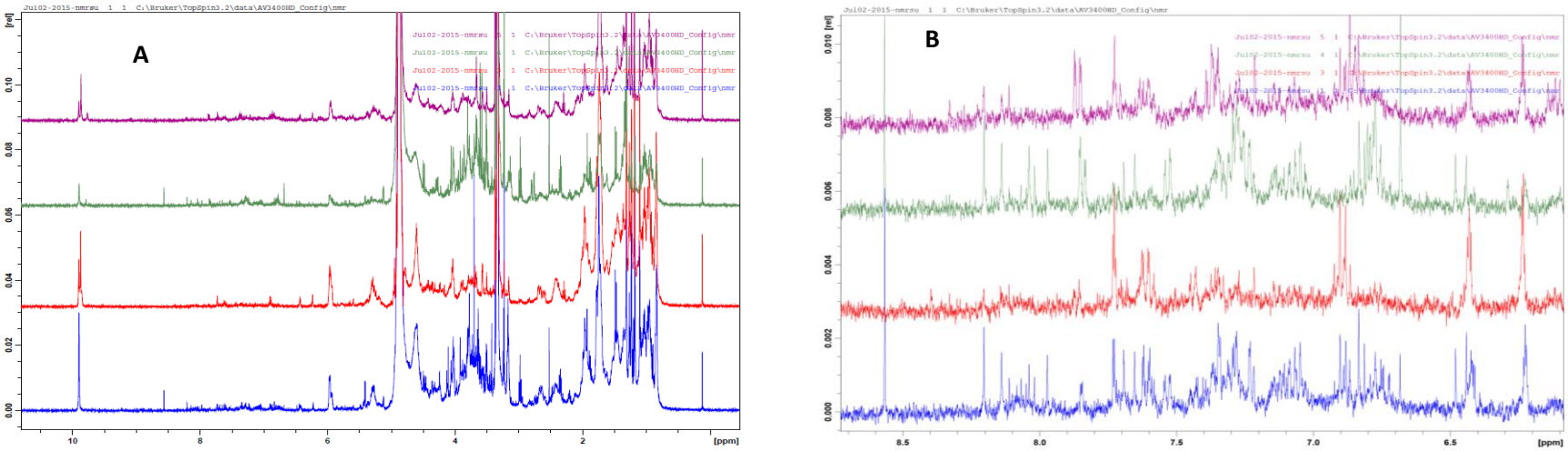
Phenolic acids	*M. balsamina* leaves		*M. balsamina* stem		*M. foetida* leaves		*M. foetida* stem	
	Crude	Elute	Crude	Elute	Crude	Elute	Crude	Elute
Gallic acid	+	+	+	+	+	+	+	+
P-coumaric acid	-	-	-	-	+	-	+	-
Ferulic acid	+	-	+	+	-	-	-	-
Epigallocatechin gallate	+	+	-	-	-	-	-	-
Sinapic acid	-	-	+	-	+	-	+	-
Myricetin	+	+	-	-	-	-	-	-
Kaempferol	-	-	-	-	-	+	-	+
Epicatechin	-	-	+	-	-	-	-	-

(+) = Present (-) = Absent

6.2.5 LCMS analysis

Preliminary LCMS analyses of *Momordica* crude extracts and SPE fractions were performed following the procedure as described in Section 2.12. Data presented are for the liquid chromatograms (LC) only. Analysis of the MS results was not possible due to the complexity of the fragmentations displayed. It is also worth noting that reference standards were not used for this part and thus chromatogram peaks could not be compared.

LCMS chromatograms of *Momordica* extracts showed clear differences. There were more peaks displayed in *M. balsamina* crude extracts than in *M. foetida* crude extracts (Figure 6-12). The SPE fraction 3 displayed fewer peaks in *M. balsamina* extracts compared to the crude extracts yet for *M. foetida* leaf extracts, fraction 3 displayed more peaks than the crude extract (Figure 6-13). The SPE fraction 3 of *M. foetida* stem did not display any peaks yet peaks were observed in fraction 1 (F1), and fraction 2 (F2) (Figure 6-14).



M. balsamina leaves crude

M. balsamina SPE fraction 3

M. balsamina stem crude

M. balsamina stem SPE fraction 3

Figure 6-11 (A) ^{1}H-NMR spectrum of crude and SPE fraction 3s (F3s) of *M. balsamina* leaf and stem. (B) Aromatic area in the range of 6.0 – 8.5 ppm.

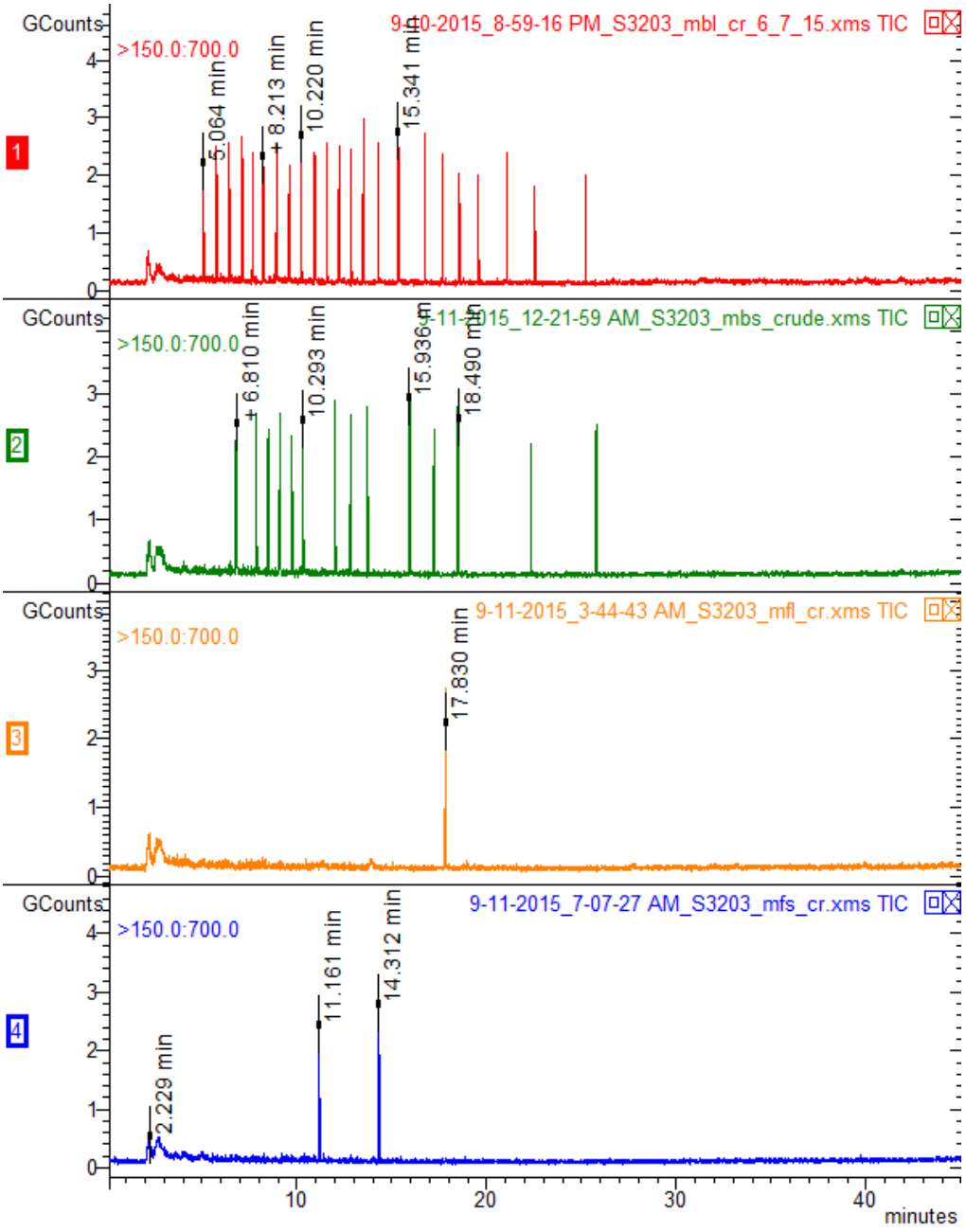


Figure 6-12 LCMS chromatograms of crude extracts of 1) *M. balsamina* leaf, 2) *M. balsamina* stem, 3) *M. foetida* leaf and 4) *M. foetida* stem.

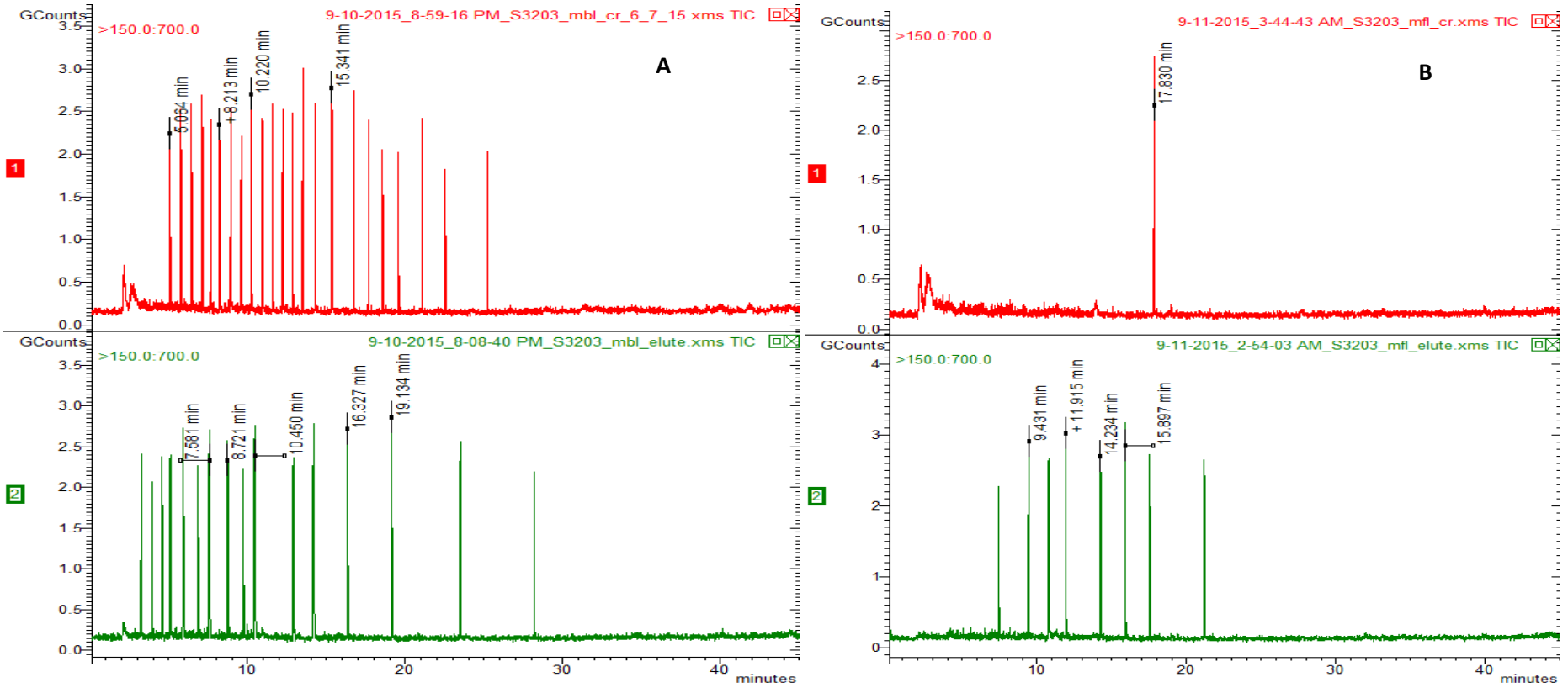


Figure 6-13 LCMS chromatograms comparing crude and SPE fraction 3 for A) *M. balsamina* leaf and B) *M. foetida* leaf; 1) crude and 2) F3.

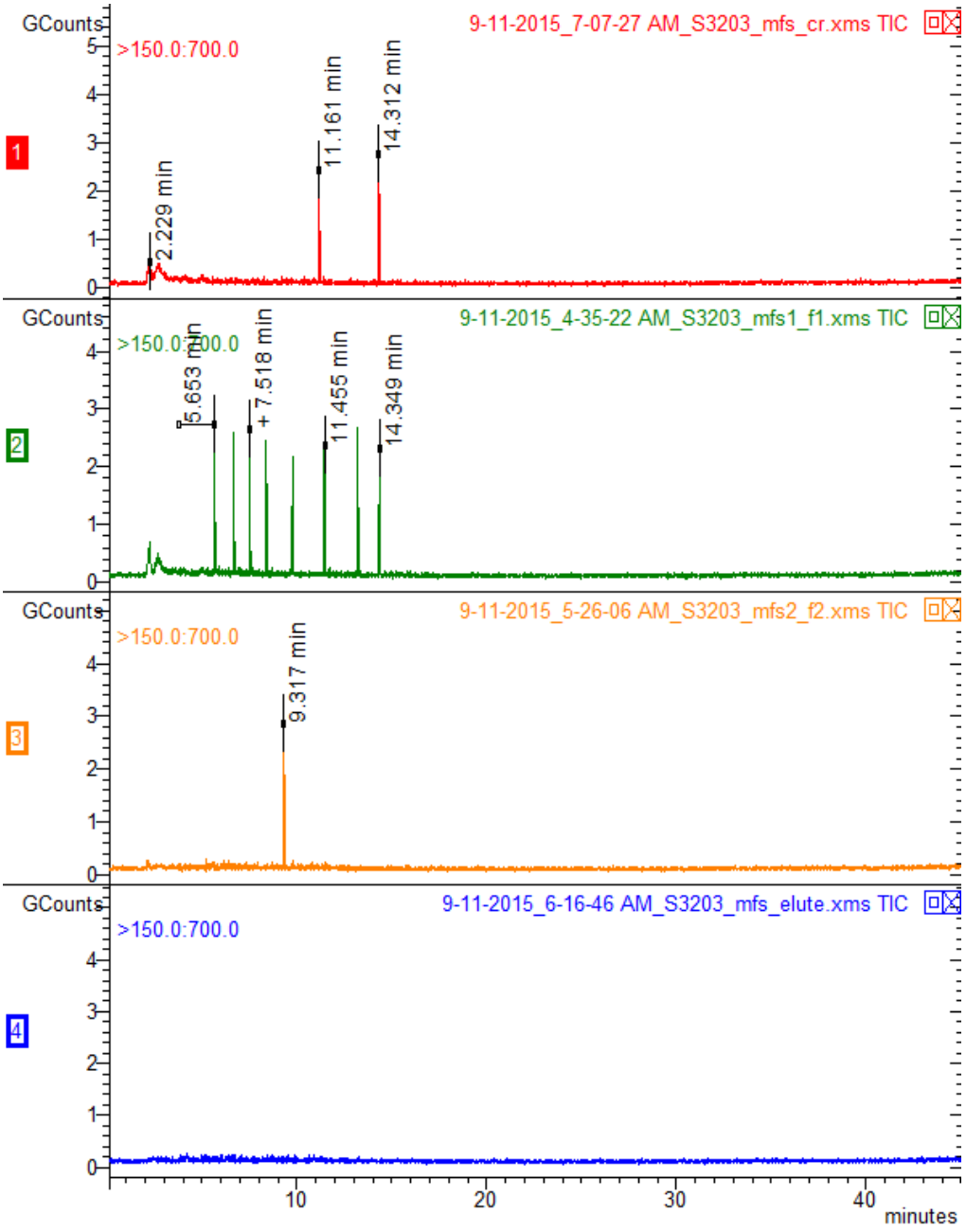


Figure 6-14 LCMS chromatograms of *M. foetida* stem crude and SPE fractions: 1 - crude extract; 2 – SPE fraction 1(F1) 3 – SPE fraction 2 (F2), and 4 –SPE fraction 3 (F3).

6.3 Discussion

The FTIR spectra observed were similar to those expected from any plant material and, as expected, from visual observation of the spectra, un-extracted tissues and extracts were shown to be very similar. Infrared spectroscopy works best on pure substances rather than complex mixtures. If a sample composition is complex, its spectrum will also be complex and it will be hard to know which infrared bands are due to which molecules (Smith 1998). The samples appeared to have strong bands associated with hydrogen bonded O-H stretching absorption around 3250 cm^{-1} and C-H stretching absorption around 2900 cm^{-1} (Coates 2000; Corredor *et al.* 2009). In the fingerprint region between 1800 and 900 cm^{-1}, there were many absorption bands observed which are probably associated with amides, carboxylic acids as well as alcohols and phenols (Coates 2000). These are all to be expected in any plant tissue. Perhaps more surprising was the strong similarity between the spectra from intact tissues and their crude extracts. This would suggest that the methanol-water extraction is extracting a wide range of compounds and that these are generally representative of all those present in the original tissue.

Despite the similarities in the spectra, PCA plots were able to cluster samples according to their growth conditions, tissue source and species thus suggesting that whilst the general composition of the tissues and extracts may be similar there are some more specific differences present. The PCA plots for un-extracted tissue suggested that there was a stronger environmental effect rather than genetic effect on these differences. The differences observed suggested that growth conditions had an important influence on the results. PCA plots for extracted tissue on the other hand, demonstrated differences based on plant

species, plant tissue extracted and to some extent level of antimicrobial activity suggesting combined action from different compounds. An interesting observation was displayed in *M. balsamina* extracts where leaf extracts sourced either from Swaziland or glasshouse were grouped together with *M. balsamina* stem sourced from Swaziland and not stem extracts sourced from the glasshouse. Clustering indicated that there may be a chemical basis for the groupings.

Total soluble phenolics in *Momordica* plants were present at varied levels. This study displayed the highest total phenolic content in leaf extracts with a range of 34.39 to 47.94 mg GAE/g, followed by stem extracts with a range of 21.02 to 26.19 mg GAE/g and least by fruit extracts with a range of 3.97 to 12.97 mg GAE/g. These results are in agreement with a study that reported the highest phenolic content in aqueous extracts of *M. charantia* leaf (474 mg GAE/g) followed by stem (259 mg GAE/g) and least in ripe fruit (224 mg GAE/g) (Kubola and Siriamornpun 2008). Also, worth noting is that the study reported much higher phenolic content than that displayed in this thesis and this was expected as the cited study used aqueous extracts which have been shown by many authors to generally contain higher phenolic content compared to other solvent extracts. Maisuthisakul *et al.* (2007) showed that ethanol extracts of *M. charantia* leaf and bud contained 50.9 mg GAE/g phenolics and that this was about average among the 26 medicinal plants that were studied. Molehin and Adefega (2014) on the other hand measured total phenolic content of aqueous and ethanolic extracts of *M. foetida* leaves of 3.93 and 5.55 mg GAE/g, respectively. Song *et al.* (2010) reported total phenolic content of methanol extracts of *Momordica giosvenori* Swingle as 12.22 mg GAE/g and

this was considerably lower than most of the 56 Chinese medicinal plants that were studied. The differences seen could be a result of the different varieties, extraction solvent as well as environmental factors as observed in the FTIR results.

The percentage recovery of total phenolics in SPE extracts was noted to be higher than total phenolics in the original extracts (Table 6-1). The increase was however not surprising due to possible interference of other substances which are likely to influence the quantification of phenolic compounds (Dai and Mumper 2010). Generally, the Folin-Ciocalteu method used in the current study measures total phenolics as well as other oxidative substrates that may interfere with the total phenolic measurement either in an inhibitory, additive or enhancing manner (Singleton and Rossi 1965; Singleton *et al.* 1999, Dai and Mumper 2010).

Results from this study showed a fair correlation between the antimicrobial activity of *Momordica* extracts and total phenolic content. This suggests that phenolic content could be partly responsible for the antimicrobial activity of the extracts. It is worth noting however that antimicrobial activity of *M. balsamina* leaf extract was higher than that of *M. foetida* leaf extract (reported in Chapter 4 of this thesis) whereas *M. foetida* leaf extract showed the highest total soluble phenolic content which might explain why the correlation coefficient is slightly above average ($R^2 = 0.579$). Antimicrobial activity of extracts of plants has been attributed to the presence of many bioactive secondary metabolites (Stanković *et al.* 2012). Phenolic compounds as well as other compounds such as terpenoids and alkaloids are all important in antimicrobial activity of plant extracts (Cowan 1999; Stanković *et al.* 2012).

The findings of the current study were in agreement with another study that confirmed a relationship between the phenolic content of extracts of *Teucrium* species and their biological activities (Stanković *et al.* 2012). Sanhueza *et al*. (2014) also confirmed a direct relationship between total phenolic content of grape pomace extract and its antimicrobial activity.

HPLC analysis is a widely used technique for the analysis of phenolic acids (Suárez *at al.* 1996; Proestos *et al.* 2005). A UV detector was used since all phenolic constituents display strong absorption in the UV area of the spectrum (Proestos *et al.* 2005; Proestos and Komaitis 2013). HPLC was and still is the preferred separation technique for the analysis of natural products (Steinmann and Ganzera 2011). HPLC results suggested that phenolic components in extracts varied based on species, extraction, and parts of tissues. A possible reason for the differences observed in plant species could be due to the differences of each species in responding to environmental stresses which can encourage a rise in phenolic acid components as a natural response for the plants to survive (Horax *et al.* 2005). The extraction method is also likely to result in differences in compounds identified in the plant extracts. HPLC analysis of crude extracts might be affected by the presence of nonpolar compounds such as chlorophyll, waxes and oils, which can interfere with the chromatographic determination of compounds since organic compounds are purified and pre-concentrated (Głowniak *et al.* 1996). The differences in phenolic acid components among the different parts of tissues could be an outcome of biochemical mechanisms in their synthesis throughout the growing period (Horax *et al.* 2005). The current study also revealed that gallic acid was the only phenolic compound thought to be present in all the crude extracts and

SPE fraction 3 of *M. balsamina* and *M. foetida* leaf and stem. These results were supported by findings which showed that gallic acid was the most predominant phenolic compound in all parts of bitter gourd (*M. charantia*) (Kubola and Siriamornpun 2008). Apart from gallic acid, the reported study identified other phenolic acids that were present in boiled water extracts of *M. charantia* leaf, stem and fruit as tannic acid, (+)-catechin, *p*-coumaric and benzoic acids. Tannic acid, (+)-catechin and benzoic acids were however not identified in this current study because methanol-water extracts were used while the reported study used boiled water extracts and different *Momordica* species.

Since some chromatograms were not clearly showing individual components, indicating the possibility of complex phenolics, this necessitated saponification of extracts to release conjugated phenolics. Hydrolysing glycosides or esters was important in determining phenolic content by HPLC as a substantial fraction is in the bound form (Proestos and Komaitis 2013). Saponification of samples seemed to release some phenolics but may also have degraded some of the phenolics that were unstable since there was an appreciable loss of peak intensity in several cases. Saponification is the most common means of releasing acids; however, they may decompose under these conditions (Stalikas 2007).

Similar to the FTIR results, visual NMR analysis of spectra revealed that there were both some similarities and a few clear differences observed in *Momordica* extracts from different tissues of the same species. Crude extracts had higher carbohydrates compared to the SPE F3s. NMR results indicated that there were some chemical groups that disappeared in SPE F3. This could

be the reason why antimicrobial activity of *M. balsamina* leaf extracts (SPE F3) was slightly lower and *M. balsamina* stem extracts (SPE F3) had no activity at all compared to their crude extracts which showed antimicrobial activity (shown in Section 4.3.4 and Table 4-2). Another variation observed included differences in intensities of the same signals in leaf and stem crude and SPE extracts of *M. balsamina*. Similar to the current study, other authors reported differences in intensities as well as smaller signals in the aromatic region for plant extracts compared to the aliphatic and sugar region (Ward *et al.* 2003; Choi *et al* 2004). Crude plant extracts usually contain large amounts of carbohydrates and lipids, thus the concentration of phenolics in the crude extracts may be too low to detect when using techniques such as NMR (Dai and Mumper 2010). Sensitivity is the disadvantage of using proton nuclear magnetic resonance analysis (^{1}H NMR) in natural products, yet it is the only technique which produces signals directly correlating with the amounts of analytes in the sample (Lewis *et al.* 2007).

The LCMS analysis also confirmed differences between plant species and plant parts which could be due to the genotypic and environmental differences within species, plant parts, sampling time and determination method (Demiray *et al.* 2009). These results also confirmed that there were some chemical compounds that disappeared in the SPE F3 particularly *M. balsamina*, as had previously been shown in the HPLC and NMR results. Fractionation of the extracts also seemed to remove some carbohydrates and other compounds thus enabling some compounds that were not showing in crude extracts to be more pronounced in the SPE F3s. Removing unwanted substances such as waxes and chlorophyll from the crude extracts may aid in analysis of complex

phenolics (Robbins 2003; Naczk and Shahidi 2004). Purification and concentrating the extracts is one of the crucial steps for a successful LCMS analysis (Sasidharan *et al.* 2011). Although results from the MS were obtained, these could not be analysed further due to the complexity of the fragmentation profiles and the absence of a suitable spectral library. Non-targeted analysis is more complex thus mass spectral libraries are of dominant importance (Halket *et al.* 2005). Focus on the development and application of mass spectral libraries, chemical derivatization and chromatographic retention data, are all imperative parameters when carrying out both targeted and non-targeted analyses (Halket *at al.* 2005). Analysis of secondary metabolites in plants is a challenging task due to their chemical diversity, usually low abundance and variability even within the same species (Steinmann and Ganzera 2011). Optimizing conditions and using standard compounds could help obtain better results.

6.4 Conclusion

This preliminary biochemical screening of *Momordica* extracts has shown differences in their chemical composition. Although it has not been possible to attribute these differences specifically to any particular group of compounds, phenolics have been shown to be potential candidates contributing to the antimicrobial activity of the extracts.

The presence of medicinally important constituents in the studied plants has been revealed. The presence of the phytochemicals in the *Momordica* extracts validates the traditional medicinal uses of this plant and may also explain why

the extracts have shown, in the present study, to have high antimicrobial and anti-proliferative activities.

Special emphasis should be placed on effective crude extracts clean up method such as SPE and hydrolysis of samples as a preparatory step for biochemical analysis of complex samples such as crude plant extracts. Also effective separation of complex plant extracts is highly dependent on the separation techniques selected.

Further work should be carried out to isolate, purify and characterize the active constituents responsible for the activity of the plants by use of techniques such as HPLC, NMR and LCMS. Possible mechanism of action of the components of these plants should also be studied.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

The frequency of life threatening diseases such as cancer and those caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality particularly in immunocompromised patients in developing countries (Al-Bari *et al.* 2006; Rahman *et al.* 2009). Recent trends in the search for natural alternatives for treatments of diseases have stimulated interest in plants. Bioactive plants are still in use as part of traditional practices in many parts of the world and are the major resource to manage human health in many developing countries including Swaziland. The aims of this study were therefore to evaluate the bioactive potential and biochemical compounds present in *Momordica balsamina* and *Momordica foetida* plants from Swaziland. The bioactive potential was sought in relation to antimicrobial activity and anti-proliferative activity of the *Momordica* plants.

Two *Momordica* plants were selected for this research because they are among the easiest to grow and widely available tropical vegetables in Swaziland and these have been traditionally associated with medicinal use. These plants have the potential to play an important role in the country's economy for industrial and domestic uses. Also, the continued use of these plants as food and for their medicinal properties depend on the knowledge of the chemical constituents that are present in the plant (Baxter *et al.* 1998).

7.1 Antimicrobial activity of Momordica plant extracts and their SPE fractions

A broad range of organisms consisting of Gram-positive and Gram-negative bacteria were selected to test the ability of *Momordica* extracts to inhibit their growth. Initial antimicrobial studies used a range of solvents - hot water, chloroform-methanol and methanol-water - to obtain extracts and screen for antimicrobial activity. This study revealed the importance of choice of extraction solvent as it had an impact on the antimicrobial activity of the plant extracts. Even though water is the solvent used in cooking and making infusions, water extracts did not display any antimicrobial activity. Methanol-water proved to be the best solvent for extraction and these extracts showed a considerable amount of antimicrobial activity compared to chloroform-methanol extracts. Successful isolation of compounds from plant material is mostly dependent on the type of solvent used in the extraction procedure (Parekh and Chanda 2007). Methanol-water extracts and SPE methanol eluted fraction (fraction 3) of *Momordica* plants displayed antimicrobial activity but only against Gram-positive bacteria. Among the extracts, leaves showed the strongest antimicrobial activity particularly against *L. monocytogenes* compared to other parts of the plant. Many researchers have already shown that Gram-positive bacteria are more susceptible to plant extracts compared to Gram-negative bacteria (Lin *et al.* 1999; Parekh and Chanda 2006; Parekh and Chanda 2007). One possible reason for the observed differences may be because Gram-positive bacteria are single layered making them more susceptible to plant extract treatment, whilst the Gram-negative bacterial cell wall has multiple layers (Yao and Moellering 1995; Cowan 1999).

Some parts of the *Momordica* plants such as *M. balsamina* fruit, *M. foetida* stem and *M. foetida* fruit failed to display antimicrobial activity against any of the bacteria tested. It was also shown that the SPE fractions of some crude extracts, such as that from *M. balsamina* stem, showed reduced or a lack of activity. This suggested that there may be compounds that work additively, or even synergistically, together in the crude extracts but that these separate into different fractions. Further study to understand the potential combined action of these compounds is required.

It is important to note that negative results do not necessarily indicate the absence of bioactive components. It is possible that active constituents may be present at too low a concentration in the crude extracts to show antimicrobial activity with the dose levels employed. Lack of activity can thus only be proven by using large doses. On the other hand, if the active principle is present in high quantities, there could be other compounds exerting properties antagonistic to that of the bioactive compounds (Parekh and Chanda 2007; PaVadlapudi and Naidu 2010).

It was shown in this study that environmental factors also played an important role in determining antimicrobial activity of the *Momordica* extracts. Extracts of plants sourced in Swaziland displayed a higher antimicrobial activity compared to those from plants that were grown in the UK glasshouse. Plant extract composition is known to differ according to climatic and environmental conditions (Hammer *et al.* 1999). Results in the literature can also be confounded as it may be possible that extracts from plants with a common name may actually be derived from different plant species (Hammer *et al.* 1999). Certain points should be considered for the study of antimicrobial

activity of plant extracts; it is important to define certain parameters such as plant material, techniques employed, growth medium and microorganisms or strain tested (Rios and Recio 2005).

7.2 Anti-proliferative activity of *Momordica* extracts

This study investigated the potential anti-proliferative activity of methanol-water extracts of *M. balsamina* and *M. foetida* leaf sourced from Swaziland or UK glasshouse against human liver hepatocellular carcinoma (HepG2) cells. Anti-proliferative activity of these extracts varied depending on plant species as well as growth conditions as already displayed in antimicrobial activity. Trouillas *et al.* (2003) also suggested that agronomic practices and environmental conditions could influence the plant components which may impact on anti-proliferative activity. These plant components are however not known. The highest anti-proliferative activity was observed on those cells treated with extracts of *M. balsamina* leaf sourced from Swaziland at a concentration of 0.25 mg/mL after both 24 and 48 h incubation. These doses are however quite high compared to other researchers who studied other *Momordica* species. Extracts of *M. foetida* leaf on the other hand displayed poorer anti-proliferative activity even at much higher concentrations (1 mg/mL). For both *M. balsamina* and *M. foetida*, increasing the concentrations resulted in cytotoxic effects. Cell passage effects were also observed in HepG2 cells where higher passage was suspected to have contributed to poorer anti-proliferative activity. There was a weak connection established between HepG2 cell inhibition and antimicrobial activity. Previous studies have

suggested that compounds with a high phenolic content, among other things, inhibit tumour promotion and cell proliferation (Huang *et al* 1994; Simon *et al.* 1998). The mechanism underlying the anti-proliferative activity of phenolic compounds is not fully understood (Trouillas *et al.* 2003). It has been suggested that the bioactive compounds in the plants bring about restoration of deranged health in cancer patients (Bisen 2016). The present thesis did not study the mechanism because anti-proliferative effects observed could be due to many components which were not identified in the extracts. This necessitates the need for further research to investigate and understand the compounds responsible for anti-proliferative activity and their potential mechanisms. Effects of the *Momordica* extracts on other human cancer cell-lines as well as normal cell-lines should also be studied. More research on these plants could provide a viable alternative treatment for cancer particularly in many developing countries (Bisen 2016).

7.3 Biochemical evaluation of *Momordica* extracts

A variety of techniques were used for the preliminary biochemical screening of *M. balsamina* and *M. foetida* plant extracts. This study revealed the possible chemical basis for grouping of extracts through PCA analysis. Similar to antimicrobial and anti-proliferative activity discussed above, it was shown that there were chemical differences in extracts based on plant species, plant part, extraction and environmental conditions. Leaves proved to have the highest total phenolic content particularly extracts of M. *foetida* leaf. Fractionation of extracts using SPE showed that most of the total phenolic content was

recovered in the methanol-eluted fraction (fraction 3) with the best recovery observed for *M. balsamina* leaf and the lowest recovery observed for *M. balsamina* stem. Extracts of *Momordica* plants sourced from Swaziland had a higher total phenolic content compared to those sourced from UK glasshouse. Growing conditions are known to have an impact on phenolic acid content (Wang and Zheng 2001). The extracts were shown to possibly contain gallic acid, ferulic acid, sinapic acid, myricetin, kaemferol, epigallocatechin gallate, *p*-coumaric acid, and epicatechin. Gallic acid was the only phenolic compound which seemed to be present in all the different extracts. Other techniques such as NMR and LCMS indicated the presence of other components such as carbohydrates in larger quantities. The high concentrations of carbohydrates was to be expected but do serve to mask other compounds and make detailed analysis difficult. This highlighted the importance of purifying and concentrating extracts prior to analysis for successful isolation of active compounds.

It can be highlighted from this study that there are several important factors for a successful qualitative and quantitative analysis of biochemical components of plant extracts. First is the extraction method and how it will impact on the active compounds extracted; second is purifying and concentration of compounds prior to analysis to remove all unwanted substances; third is choosing the right techniques to enable compound identification and lastly is having standard compounds as reference points (Stalikas 2007). These factors have impacted on results obtained on the chemical analysis of *Momordica* extracts in this study. The current study used methanol-water extracts together with SPE and saponification all which were

the basis for determining the chemical components for further separation and characterization. As has been recognised from the literature, other extraction methods could have shown the presence of phenolics in very high concentrations compared to the methanol-water extracts that were used in this study however, when used with the species studied here, such methods (e.g. aqueous extracts) failed to display any antimicrobial activity hence these were not used for biochemical analysis. Stalikas (2007) also suggested that a mixture of alcohol (methanol, ethanol) and water is recommended for efficient extraction of bioactive compounds, supporting the current approach used. It was also observed that using a number of biochemical analysis techniques is important for efficient identification and characterization of bioactive compounds hence this study employed several techniques. The relative success of each technique varied. FTIR spectrum analysis presented a typical set of complex peaks expected in plants but did not display any differences between plant species either in extracts or in original tissues. PCA plots did however group extracts based on species, plant part and level of antimicrobial activity. Total soluble phenolics indicated presence of phenolics in extracts depending on plant part and growing conditions. Fractionation of extracts using SPE showed good recovery of phenolic content. As observed in HPLC chromatograms, saponification of samples showed release of some conjugated phenolics but also degraded those phenolics that were unstable. The use of analyses such as NMR and LCMS displayed the presence of other components such as carbohydrates in high concentrations presented limitations in efficient identification of bioactive compounds. These observed limitations displayed the importance of further purification of samples to enable efficient isolation

of bioactive compounds. Also, as displayed by the LCMS results, analysis of complex plant extracts can be very challenging especially when there are no reference standards.

7.4 Conclusion

This study has confirmed that methanol-water extracts of *M. balsamina* and *M. foetida* have potent antibacterial and anti-proliferative activities that may be beneficial for medicinal applications. There is limited information on the compounds found in *Momordica* extracts making it difficult to speculate on which compounds might contribute to either antimicrobial activity or anti-proliferative activity. While there is an increased need for further research on isolation and identification of the compounds responsible for producing bioactivity, evidence from this study has displayed that extracts of *M. balsamina* and *M. foetida* have a variety of compounds that may be applied in food preservation and human health. This research has confirmed the influence of factors such as environment, growth conditions, preparation and analysis techniques on the chemical composition of *Momordica* plants and demonstrated their significance in the production of bioactive compounds.

Further studies could include:

1) Determining the active compounds after isolation and purification of components of extracts.
2) Evaluating the safety and toxicity of the extracts is essential if extracts are to be used for food preservation or medicinal purposes. These would include *in vitro* studies using cell lines as well as *in vivo* (acute

and sub-acute oral toxicity) studies on animal models, studying the mechanisms of action in antimicrobial and antiproliferative activity

3) Determining the effects of extracts invivo using animal models.
4) Establishing the positive and negative interactions of extracts with common antibiotics.

REFERENCES

Acquaviva, R., Di Giacomo, C., Vanella, L., Santangelo, R., Sorrenti, V., Barbagallo, I., Genovese, C., Mastrojeni, S., Ragusa, S. and Iauk, L., 2013. Antioxidant activity of extracts of *Momordica foetida* Schumach. et Thonn. *Molecules*, *18*(3), pp.3241-3249.

Ahmad, M.B., Shameli, K., Wan Yunus, W.M.Z., Ibrahim, N.A., Hamid, A.A. and Zargar, M., 2009. Synthesis and antibacterial activity of silver/montmorillonite nanocomposites. *Research Journal of Biological Sciences*, *4*(9), pp.1032-1036.

Akbar, S. and Al-Yahya, M.A., 2011. Screening of Saudi plants for phytoconstituents, pharmacological and antimicrobial properties. *Australian Journal of Medical Herbalism*, *23*(2), p.76.

Akinmoladun, A.C., Ibukun, E.O., Afor, E., Obuotor, E.M. and Farombi, E.O., 2007. Phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum*. *Scientific Research and Essays*, *2*(5), pp.163-166.

Akinyemi, K.O., Mendie, U.E., Smith, S.T., Oyefolu, A.O. and Coker, A.O., 2005. Screening of some medicinal plants used in south-west Nigerian traditional medicine for anti-Salmonella typhi activity. *Journal of Herbal Pharmacotherapy*, *5*(1), pp.45-60.

Akter, A., Neela, F.A., Khan, M.S.I., Islam, M.S. and Alam, M.F., 2010. Screening of ethanol, petroleum ether and chloroform extracts of medicinal plants, *Lawsonia inermis* L. and *Mimosa pudica* L. for antibacterial activity. *Indian Journal of Pharmaceutical Sciences*, *72*(3), p.388.

Al-Bari, M.A.A., Sayeed, M.A., Rahman, M.S. and Mossadik, M.A., 2006. Characterization and antimicrobial activities of a phenolic acid derivative produced by *Streptomyces bangladeshiensis* a novel species collected in Bangladesh. *Research Journal of Medical Sciences, 1*(2), pp. 77-81.

Alshehri, M.A., 2016. Anticancer activity of methanolic extract of *Momordica charantia* against human colon, liver and breast cancer cell lines- In vitro. *Journal of Biology, Agriculture and Healthcare*, *6*(6), pp.106-111.

Alzoreky, N.S. and Nakahara, K., 2003. Antibacterial activity of extracts from some edible plants commonly consumed in Asia. *International Journal of Food Microbiology*, *80*(3), pp.223-230.

Amusan, O.O., Sukati, N.A. and Shongwe, M.S., 2005. Some phytomedicines from Shiselweni region of Swaziland. *Journal of Natural Remedies*, *5*(1), pp.19-25.

Amusan, O.O., Sukati, N.A., Dlamini, P.S. and Sibandze, F.G., 2007. Some Swazi phytomedicines and their constituents. *African Journal of Biotechnology*, *6*(3).

Amusan, O.O., 2009. Herbal medicine in Swaziland: an overview. In *ACS Symposium Series* (Vol. 1021, pp. 31-49). Oxford University Press.

Aphane, J., Chadha, M.L. and Oluoch, M.O, 2002. Increasing the consumption of micronutrient-rich foods through production and promotion of indigenous foods. *FAO-AVRD International Workshop Proceedings, Arucha, Tanzania, 5-8 March 2002.*

Arnold, T.H., Wells, M.J. and Wehmeyer, A.S., 1985. Khoisan food plants: taxa with potential for future economic exploitation. In *Plants for Arid Lands* (pp. 69-86). Springer Netherlands.

Ayua, E. and Omware, J., 2013. Assessment of processing methods and preservation of African leafy vegetables in Siaya County, Kenya. *Global Journal of Biology Agriculture and Health Sciences*, *2*(2), pp.46-48.

Baartjies, Z., 2014. Health benefits of the most common indigenous foods in Swaziland. *Swazi Observer (08/02/2014).* www.observer.org.sz/index.php?news=58545 18/02/2016.

Bailey, C.J. and Day, C., 1989. Traditional plant medicines as treatments for diabetes. *Diabetes Care*, *12*(8), pp.553-564.

Baxter, H., Harborne, J.B. and Moss, G.P. eds., 1998. *Phytochemical dictionary: a handbook of bioactive compounds from plants*. CRC press.

Behera, T.K., Behera, S., Bharathi, L.K., John, K.J., Simon, P.W. and Staub, J.E., 2010. 2 Bitter gourd: Botany, horticulture, breeding. *Horticultural Reviews*, *37*, p.101.

Behera, T.K., John, K.J., Bharathi, L.K. and Karuppaiyan, R., 2011. *Momordica*. In *Wild crop relatives: genomic and breeding resources* (pp. 217-246). Springer Berlin Heidelberg.

Benoit-Vical, F., Grellier, P., Abdoulaye, A., Moussa, I., Ousmane, A., Berry, A., Ikhiri, K. and Poupat, C., 2006. In vitro and in vivo antiplasmodial activity of *Momordica balsamina* alone or in a traditional mixture. *Chemotherapy*,*52*(6), pp.288-292.

Bhattacharya, A., Sood, P. and Citovsky, V., 2010. The roles of plant phenolics in defence and communication during *Agrobacterium* and *Rhizobium* infection. *Molecular plant pathology*, *11*(5), pp.705-719.

Bisen, P. S., 2016. Nutritional therapy as a potent alternative to chemotherapy against cancer. *Journal of Cancer Science*, 8, pp 168-169.

Boily, Y. and Van Puyvelde, L., 1986. Screening of medicinal plants of Rwanda (Central Africa) for antimicrobial activity. *Journal of Ethnopharmacology*, *16*(1), pp.1-13.

Booth, G.M., Malmstrom, R.D., Kipp, E. and Paul, A., 2012. Cytotoxicity of selected medicinal and nonmedicinal plant extracts to microbial and cervical cancer cells. *BioMed Research International*, *2012*.

Booth, I.R., 2002. Stress and the single cell: intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. *International Journal of Food Microbiology*, *78*(1), pp.19-30.

Bosch, C.H., 2004a. *Momordica balsamina* L. In Grubben G J H and Denton O A (Editors). *PROTA*, *2*, pp.384-392.

Bosch, C.H., 2004b. *Momordica foetida* Schumach. [Internet] Record from PROTA4U. Grubben, G.J.H. & Denton, O.A. (Editors). PROTA (Plant Resources of Tropical Africa), Wageningen, Netherlands. <http://www.prota4u.org/search.asp>. 11/03/2013.

Bot, Y.S., Mgbojikwe, L.O., Nwosu, C., Abimiku, A., Dadik, J. and Damshak, D., 2007. Screening of the fruit pulp extract of *Momordica balsamina* for anti HIV property. *African Journal of Biotechnology*, *6*(1), pp.047-052.

Bwembya, G. C., Thwala, J. M. and Otieno, D. A, 2008. Nutritional analysis of some major micronutrients in indigenous Swazi vegetables. UNZA Conference 2008.

Bwembya, G.C., Thwala, J.M. and Otieno, D.A., 2014. Vitamin A and mineral content of some common vegetables consumed in Swaziland. *UNISWA Journal of Agriculture, Science and Technology (UREJAST)*, *15*(1).

Cappuccino, J.G. and Sherman, N., 2005. Microbiology: A laboratory manual, 6th. *Ontario, Canada: Pearson Education*.

Casimiri, V. and Burstein, C., 1998. Biosensor for L-lactate determination as an index of *E. coli* number in crude culture medium. *Analytica Chimica acta*, *361*(1), pp.45-53.

Choi, H.K., Choi, Y.H., Verberne, M., Lefeber, A.W., Erkelens, C. and Verpoorte, R., 2004. Metabolic fingerprinting of wild type and transgenic tobacco plants by 1 H NMR and multivariate analysis technique. *Phytochemistry*, *65*(7), pp.857-864.

Coates, J., 2000. Interpretation of infrared spectra, a practical approach. *Encyclopedia of Analytical Chemistry*.

Cosentino, S., Tuberoso, C.I.G., Pisano, B., Satta, M.L., Mascia, V., Arzedi, E. and Palmas, F., 1999. In-vitro antimicrobial activity and chemical composition of *Sardinian thymus* essential oils. *Letters in Applied Microbiology*, *29*(2), pp.130-135.

Corredor, D.Y., Salazar, J.M., Hohn, K.L., Bean, S., Bean, B. and Wang, D., 2009. Evaluation and characterization of forage sorghum as feedstock for fermentable sugar production. *Applied Biochemistry and Biotechnology*, *158*(1), pp.164-179.

Cowan, M.M., 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews*, *12*(4), pp.564-582.

Cuvelier, M.E., Berset, C. and Richard, H., 1994. Antioxidant constituents in sage (Salvia officinalis). *Journal of Agricultural and Food Chemistry*, *42*(3), pp.665-669.

Dai, J. and Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, *15*(10), pp.7313-7352.

Das, K., Tiwari, R.K.S. and Shrivastava, D.K., 2010. Techniques for evaluation of medicinal plant products as antimicrobial agents: Current methods and future trends. *Journal of Medicinal Plants Research*, *4*(2), pp.104-111.

Demiray, S., Pintado, M.E. and Castro, P.M.L., 2009. Evaluation of phenolic profiles and antioxidant activities of Turkish medicinal plants: *Tilia argentea, Crataegi folium* leaves and *Polygonum bistorta* roots. *World Academy of Science, Engineering and Technology*, *54*, pp.312-317.

DeWaal, C.S. and Robert, N., 2005. *Global & local: Food safety around the world*. Center for Science in the Public Interest.

Dlamini, C.S. and Geldenhuys, C.J., 2011. Quantities and values of selected forest foods harvested by eight villages adjacent to natural woodlands in the four ecological zones of rural Swaziland. *African Journal of Food Science*, *5*(13), pp.717-727.

Driscoll, A.J., Bhat, N., Karron, R.A., O'Brien, K.L. and Murdoch, D.R., 2012. Disk diffusion bioassays for the detection of antibiotic activity in body fluids: applications for the pneumonia etiology research for child health project. *Clinical Infectious Diseases*, *54*(suppl 2), pp.S159-S164.

Dube, M.A. and Musi, P.J., 2002. Analysis of indigenous knowledge in Swaziland: Implications for sustainable agricultural development. *African Technology Policy Studies Working Paper Series*, (34).

Duraipandiyan, V., Ayyanar, M. and Ignacimuthu, S., 2006. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from

Tamil Nadu, India. *BMC Complementary and Alternative Medicine*, *6*(1), p.1.

Edeoga, H.O., Okwu, D.E. and Mbaebie, B.O., 2005. Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology*, *4*(7), pp.685-688.

Eloff, J.N., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica*, *64*(8), pp.711-713.

Emerstorfer, F., Kneifel, W. and Hein, W., 2009. The role of plant-based antimicrobials in food and feed production with special regard to silage fermentation. *Die Bodenkultur*, *55*(60), p.3.

Ennajar, M., Bouajila, J., Lebrihi, A., Mathieu, F., Savagnac, A., Abderraba, M., Raies, A. and Romdhane, M., 2010. The influence of organ, season and drying method on chemical composition and antioxidant and antimicrobial activities of *Juniperus phoenicea* L. essential oils. *Journal of the Science of Food and Agriculture*, *90*(3), pp.462-470.

Faujdar, S., Paliwal, S. K., Mehta, S., Kalia, A. N., 2013. Pharmacognostical and phytochemical evaluation of *Momordica balsamina* fruits. *International Journal of Pharmaceutical Sciences Review and Research*, *19*(2), pp.77-79.

Flyman, M.V. and Afolayan, A.J., 2008. Effect of plant maturity on the mineral content of the leaves of *Momordica balsamina* L and *Vigna unguiculata* subsp. Sesquipedalis (L.) VERDC. *Journal of Food Quality*, *31*(5), pp.661-671.

Friedman, M., Henika, P.R. and Mandrell, R.E., 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* enterica. *Journal of Food Protection*, *65*(10), pp.1545-1560.

Froelich, S., Onegi, B., Kakooko, A., Siems, K., Schubert, C. and Jenett-Siems, K., 2007. Plants traditionally used against malaria: phytochemical and pharmacological investigation of *Momordica foetida*. *Revista Brasileira de Farmacognosia*, *17*(1), pp.1-17.

García-Alonso, J., Ros, G. and Periago, M.J., 2006. Antiproliferative and cytoprotective activities of a phenolic-rich juice in HepG2 cells. *Food Research International*, *39*(9), pp.982-991.

Garvey, M.I., Rahman, M.M., Gibbons, S. and Piddock, L.J., 2011. Medicinal plant extracts with efflux inhibitory activity against Gram-negative bacteria. *International Journal of Antimicrobial Agents*, *37*(2), pp.145-151.

Geidam, M.A., Pakmam, I. and Laminu, H., 2004. Effects of aqueous stem bark extract of *Momordica balsamina* Linn on serum electrolytes and some hematological parameters in normal and alcohol fed rats. *Pakistan Journal of Biological Sciences*, *7*(8), pp.1430-1432.

Geidam, M.A., Dauda, E. and Hamza, H.G., 2007. Effects of aqueous stem-bark extract of *Momordica balsamina* Linn on some serum enzymes in normal and ethanol fed rats. *Journal of Biological Sciences*, *7*(2), pp.397-400.

Geyid, A., Abebe, D., Debella, A., Makonnen, Z., Aberra, F., Teka, F., Kebede, T., Urga, K., Yersaw, K., Biza, T. and Mariam, B.H., 2005. Screening of some medicinal plants of Ethiopia for their anti-microbial properties and chemical profiles. *Journal of Ethnopharmacology*, *97*(3), pp.421-427.

Głowniak, K., Zgórka, G. and Kozyra, M., 1996. Solid-phase extraction and reversed-phase high-performance liquid chromatography of free phenolic acids in some *Echinacea* species. *Journal of Chromatography A*, *730*(1), pp.25-29.

Grover, J.K. and Yadav, S.P., 2004. Pharmacological actions and potential uses of *Momordica charantia*: a review. *Journal of Ethnopharmacology*, *93*(1), pp.123-132.

Halket, J.M., Waterman, D., Przyborowska, A.M., Patel, R.K., Fraser, P.D. and Bramley, P.M., 2005. Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. *Journal of Experimental Botany*, *56*(410), pp.219-243.

Hamill, F.A., Apio, S., Mubiru, N.K., Bukenya-Ziraba, R., Mosango, M., Maganyi, O.W. and Soejarto, D.D., 2003. Traditional herbal drugs of Southern Uganda, II: literature analysis and antimicrobial assays. *Journal of Ethnopharmacology*, *84*(1), pp.57-78.

Hamill, F.A., Apio, S., Mubiru, N.K., Mosango, M., Bukenya-Ziraba, R., Maganyi, O.W. and Soejarto, D.D., 2000. Traditional herbal drugs of southern Uganda, I. *Journal of Ethnopharmacology*, *70*(3), pp.281-300.

Hammer, K.A., Carson, C.F. and Riley, T.V., 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology*, *86*(6), pp.985-990.

Hansen, T.K. and Jakobsen, M., 1997. Possible role of microbial interactions for growth and sporulation of *Penicillium roqueforti* in Danablu. *Le Lait*, *77*(4), pp.479-488.

Harborne, A.J., 1998. *Phytochemical methods a guide to modern techniques of plant analysis*. Springer Science & Business Media.

Hassan, L.G. and Umar, K.J., 2006. Nutritional value of Balsam Apple (*Momordica balsamina* L.) leaves. *Pakistan Journal of Nutrition*, *5*(6), pp.522-529.

Huang, M.T., Ho, C.T., Wang, Z.Y., Ferraro, T., Lou, Y.R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J.D. and Conney, A.H., 1994. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Research*, *54*(3), pp.701-708.

High, C. and Shackleton, C.M., 2000. The comparative value of wild and domestic plants in home gardens of a South African rural village. *Agroforestry Systems*, *48*(2), pp.141-156.

Hostettmann, K. and Wolfender, J.L., 1997. The search for biologically active secondary metabolites. *Pesticide Science*, *51*(4), pp.471-482.

Horax, R., Hettiarachchy, N. and Islam, S., 2005. Total phenolic contents and phenolic acid constituents in 4 varieties of bitter melons (*Momordica charantia*) and antioxidant activities of their extracts. *Journal of Food Science*, *70*(4), pp.C275-C280.

How, V. L. J. and Parasakthi, N., 1978. Urinary Viable Counts – An evaluation of three methods. *Malaysian Journal of Pathology*, 1, pp77-84.

Hsiao, P.C., Liaw, C.C., Hwang, S.Y., Cheng, H.L., Zhang, L.J., Shen, C.C., Hsu, F.L. and Kuo, Y.H., 2013. Antiproliferative and hypoglycemic cucurbitane-type glycosides from the fruits of *Momordica charantia*. *Journal of Agricultural and Food Chemistry*, *61*(12), pp.2979-2986.

Hsieh, P.C., Siegel, S.A., Rogers, B., Davis, D. and Lewis, K., 1998. Bacteria lacking a multidrug pump: a sensitive tool for drug discovery. *Proceedings of the National Academy of Sciences*, *95*(12), pp.6602-6606.

Huang, M.T., Lou, Y.R., Ma, W., Newmark, H.L., Reuhl, K.R. and Conney, A.H., 1994. Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. *Cancer Research*, *54*(22), pp.5841-5847.

Hutchings, A. ed., 1996. *Zulu medicinal plants: an inventory*. University of Kwazulu Natal Press.

Ilango, K., Yoganandam, G.P., Usha, K., Priyanga, K.S., Ilansezhiyan, M. and Kalaiarasi, V., 2010. Evaluation of wound healing activity of hexane and methanolic extract of fruit pulp of *Momordica balsamina* Linn in albino rats. *International Journal of Pharmacy and Pharmaceutical Sciences*, *2*(1), pp.88-92.

Iwalokun, B.A., Gbenle, G.O., Adewole, T.A. and Akinsinde, K.A., 2001. Shigellocidal properties of three Nigerian medicinal plants: *Ocimum*

gratissimum, *Terminalia avicennoides*, and *Momordica balsamina*. *Journal of Health, Population and Nutrition*, pp.331-335.

Javanmardi, J., Stushnoff, C., Locke, E. and Vivanco, J.M., 2003. Antioxidant activity and total phenolic content of Iranian Ocimum accessions. *Food Chemistry*, *83*(4), pp.547-550.

Jeffrey, C., 1980. A review of the *Cucurbitaceae*. *Botanical Journal of the Linnean Society*, *81*(3), pp.233-247.

Jigam, A.A., Akanya, H.O. and Adeyemi, D.J., 2004. Antimicrobial and antiplasmodial effects of *Momordica balsamina*. *Nigerian Journal of Natural Products and Medicine*, *8*(1), pp.11-12.

Jilka, C., Strifler, B., Fortner, G.W., Hays, E.F. and Takemoto, D.J., 1983. In vivo antitumor activity of the bitter melon (*Momordica charantia*). *Cancer Research*, *43*(11), pp.5151-5155.

Jones, L.J., Gray, M., Yue, S.T., Haugland, R.P. and Singer, V.L., 2001. Sensitive determination of cell number using the CyQUANT® cell proliferation assay. *Journal of Immunological Methods*, *254*(1), pp.85-98.

Joseph John, K., Antony, V. T. and Roy, Y. C., 2007. On the occurrence, distribution and taxanomy of *Momordica subangulata* Blume ssp. *Renigera* (G. Don) de Wilde in India. *Genetic Resources and Crop Evolution*, *54*:1327-1332.

Kaefer, C.M. and Milner, J.A., 2008. The role of herbs and spices in cancer prevention. *The Journal of Nutritional Biochemistry*, *19*(6), pp.347-361.

Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S. and Heinonen, M., 1999. Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*, *47*(10), pp.3954-3962.

Kalia, A., Bhardwaj, N., and Gauttam, V., 2010. Evaluation of antidiabetic activity of *Momordica balsamina* Linn seeds in experimentally-induced diabetes. *Journal of Chemical and Pharmaceutical Research*, *2*(5), pp.701-707.

Karaman, I., Şahin, F., Güllüce, M., Öğütçü, H., Şengül, M. and Adıgüzel, A., 2003. Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. *Journal of Ethnopharmacology*, *85*(2), pp.231-235.

Karaye, I.U., Aliero, A.A., Muhammad, S. and Bilbis, L.S., 2013. Evaluation of nutrient and anti-nutrient contents of selected Nigerian *Cucurbits* seeds. *Research Journal of Pharmaceutical Biological Chemical Sciences*, *4*(1), pp.136-137.

Karumi, Y., Onyeyili, P. and Ogugbuaja, V.O., 2003. Anti-inflammatory and antinociceptive (analgesic) properties of *Momordical balsamina* Linn. (Balsam apple) leaves in rats. *Pakistan Journal of Biological Sciences (Pakistan), 6*(17), pp1515-1518.

Karumi, Y., Onyeyili, P.A. and Ogugbuaja, V.O., 2004. Identification of active principles of *M. balsamina* (Balsam Apple) leaf extract. *International Journal of Medical Sciences, 4*, pp.179-182.

Karumi, Y., Onyeyili, P.A. and Ogugbuaja, V.O., 2006. Toxicity studies and effects of *Momordica balsamina* (Balsam apple) aqueous extract on serum electrolytes and plasma trace elements. *Sahel Journal of Veterinary Sciences*, *5*(1), pp.13-19.

Kim, H., Moon, J.Y., Kim, H., Lee, D.S., Cho, M., Choi, H.K., Kim, Y.S., Mosaddik, A. and Cho, S.K., 2010. Antioxidant and antiproliferative activities of mango (*Mangifera indica* L.) flesh and peel. *Food Chemistry*, *121*(2), pp.429-436.

Kim, H.K., Choi, Y.H. and Verpoorte, R., 2010. NMR-based metabolomic analysis of plants. *Nature Protocols*, *5*(3), pp.536-549.

Kiremire, B.T., Musinguzi, E., Kikafunda, J.K. and Lukwago, F.B., 2010. Effects of vegetable drying techniques on nutrient content: a case study of south-western Uganda. *African Journal of Food, Agriculture, Nutrition and Development*, *10*(5).

Kubola, J. and Siriamornpun, S., 2008. Phenolic contents and antioxidant activities of bitter gourd (*Momordica charantia* L.) leaf, stem and fruit fraction extracts in vitro. *Food Chemistry*, *110*(4), pp.881-890.

Levy, S.B. and Marshall, B., 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine*, *10*, pp. S122-S129.

Lewis, I.A., Schommer, S.C., Hodis, B., Robb, K.A., Tonelli, M., Westler, W.M., Sussman, M.R. and Markley, J.L., 2007. Method for determining molar concentrations of metabolites in complex solutions from two-dimensional 1H-13C NMR spectra. *Analytical Chemistry*, *79*(24), pp.9385-9390.

Lin, J., Opoku, A.R., Geheeb-Keller, M., Hutchings, A.D., Terblanche, S.E., Jäger, A.K. and Van Staden, J., 1999. Preliminary screening of some traditional zulu medicinal plants for anti-inflammatory and anti-microbial activities. *Journal of Ethnopharmacology*, *68*(1), pp.267-274.

Lin, H.K., Hu, Y.C., Yang, L., Altuwaijri, S., Chen, Y.T., Kang, H.Y. and Chang, C., 2003. Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. *Journal of Biological Chemistry*, *278*(51), pp.50902-50907.

Lin, R.Y., 1992. A perspective on penicillin allergy. *Archives of internal medicine*, *152*(5), pp.930-937.

Long, C., 2005. Swaziland's Flora—siSwati names and uses. Swaziland National Trust Commission. http://www.sntc.org.sz/flora/chrislong.asp 03/02/2013.

Lou, Z., Wang, H., Lv, W., Ma, C., Wang, Z. and Chen, S., 2010. Assessment of antibacterial activity of fractions from burdock leaf against food-related bacteria. *Food Control*, *21*(9), pp.1272-1278.

Lu, Y.L., Liu, Y.H., Liang, W.L., Chyuan, J.H., Cheng, K.T., Liang, H.J. and Hou, W.C., 2011. Antibacterial and cytotoxic activities of different wild bitter gourd cultivars (*Momordica charantia* L. var. abbreviata Seringe). *Botanical Studies*, *52*, pp.427-434.

Mackeen, M.M., Ali, A.M., Lajis, N.H., Kawazu, K., Hassan, Z., Amran, M., Habsah, M., Mooi, L.Y. and Mohamed, S.M., 2000. Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. ex T. Anders. *Journal of Ethnopharmacology*, *72*(3), pp.395-402.

Madureira, A.M., Ramalhete, C., Mulhovo, S., Duarte, A. and Ferreira, M.J.U., 2012. Antibacterial activity of some African medicinal plants used traditionally against infectious diseases. *Pharmaceutical Biology*, *50*(4), pp.481-489.

Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jiménez, L., 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), pp.727-747.

Manoharan, G., 2011. *Anti–Cancer effects of Momordica charantia in-vitro* (Doctoral dissertation, University of Central Lancashire).

Manosroi, J., Dhumtanom, P. and Manosroi, A., 2006. Anti-proliferative activity of essential oil extracted from Thai medicinal plants on KB and P388 cell lines. *Cancer Letters*, *235*(1), pp.114-120.

Maisuthisakul, P., Suttajit, M. and Pongsawatmanit, R., 2007. Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. *Food Chemistry*, *100*(4), pp.1409-1418.

Marquis, V.O., Adanlawo, T.A. and Olaniyi, A.A., 1977. The effect of foetidin from *Momordica foetida* on blood glucose level of albino rats. *Planta Medica*, *31*(04), pp.367-374.

Masarirambi, M.T., Mavuso, V., Songwe, V.D. and Nkambule, T.P., 2010. Indigenous post-harvest handling and processing of traditional vegetables in Swaziland: a review. *African Journal of Agricultural Research*, *5*(24), pp.3333-3341.

Masoko, P. and Eloff, J.N., 2006. Bioautography indicates the multiplicity of antifungal compounds from twenty-four southern African Combretum species (*Combretaceae*). *African Journal of Biotechnology*, *5*(18).

Mayachiew, P., Devahastin, S., Mackey, B.M. and Niranjan, K., 2010. Effects of drying methods and conditions on antimicrobial activity of edible chitosan films enriched with galangal extract. *Food Research International*, *43*(1), pp.125-132.

Michaelidou, N. and Hassan, L.M., 2008. The role of health consciousness, food safety concern and ethical identity on attitudes and intentions towards organic food. *International Journal of Consumer Studies*, *32*(2), pp.163-170.

Mojab, F., Kamalinejad, M., Ghaderi, N. and Vahidipour, H.R., 2010. Phytochemical screening of some species of Iranian plants. *Iranian Journal of Pharmaceutical Research*, pp.77-82.

Molehin, O.R. and Adefegha, S.A., 2014. Comparative study of the aqueous and ethanolic extract of *Momordica foetida* on the phenolic content and antioxidant properties. *International Food Research Journal*, *21*(1), pp401-405.

Mothana, R.A., Lindequist, U., Gruenert, R. and Bednarski, P.J., 2009. Studies of the in vitro anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqotra. *BMC Complementary and Alternative Medicine*, *9*(1), p.1.

Mradu, G., Saumyakanti, S., Sohini, M. and Arup, M., 2012. HPLC profiles of standard phenolic compounds present in medicinal plants. *International Journal of Pharmacognosy and Phytochemical Research*, *4*(3), pp.162-167.

Naczk, M. and Shahidi, F., 2004. Extraction and analysis of phenolics in food. *Journal of Chromatography A*, *1054*(1), pp.95-111.

Nanasombat, S. and Teckchuen, N., 2009. Antimicrobial, antioxidant and anticancer activities of Thai local vegetables. *Journal of Medicinal Plants Research*, *3*(5), pp.443-449.

Ncube, N.S., Afolayan, A.J. and Okoh, A.I., 2008. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African Journal of Biotechnology*, *7*(12).

Ndam, L.M., Mih, A.M., Fongod, A.G.N., Tening, A.S., Tonjock, R.K., Enang, J.E. and Fujii, Y., 2014. Phytochemical screening of the bioactive compounds in twenty (20) Cameroonian medicinal plants. *International Journal of Current Microbiology and Applied Sciences*, *3*(12), pp.768-778.

Ndhlala, A.R., Finnie, J.F. and Van Staden, J., 2011. Plant composition, pharmacological properties and mutagenic evaluation of a commercial

Zulu herbal mixture: Imbiza ephuzwato. *Journal of Ethnopharmacology*, *133*(2), pp.663-674.

Nesamvuni, C., Steyn, N.P. and Potgieter, M.J., 2001. Nutritional value of wild, leafy plants consumed by the Vhavenda. *South African Journal of Science*, *97* pp51-54.

Nguta, J.M., Mbaria, J.M., Gakuya, D.W., Gathumbi, P.K., Kabasa, J.D. and Kiama, S.G., 2011. Biological screening of Kenyan medicinal plants using *Artemia salina* L.(*Artemiidae*). *Pharmacologyonline*, *2*.

Nkambule, T.P., 2008. *Antimicrobial properties of selected Asian herbs* (Master's Thesis, University of Florida).

O'Brien, T.F., 1987. Resistance of bacteria to antibacterial agents: report of Task Force 2. *Review of Infectious Diseases*, *9*(Supplement 3), pp.S244-S260.

O'Driscoll, L., Gammell, P., McKiernan, E., Ryan, E., Jeppesen, P.B., Rani, S. and Clynes, M., 2006. Phenotypic and global gene expression profile changes between low passage and high passage MIN-6 cells. *Journal of Endocrinology*, *191*(3), pp.665-676.

Ocheng, F., Bwanga, F., Joloba, M., Borg-Karlson, A.K., Gustafsson, A. and Obua, C., 2014. Antibacterial activities of extracts from Ugandan medicinal plants used for oral care. *Journal of Ethnopharmacology*, *155*(1), pp.852-855.

Odeleye, O.M. and Oyedeji, A.O., 2008. Antibacterial Activity of crude and fractions of *Momordica foetida* leaf extracts. *International Journal of Biomedical and Pharmaceutical Sciences*, *2*(2), pp.75-78.

Odeleye, O.M., Oyedeji, O.A. and Shode, F.O., 2009. Constituents of *Momordica foetida* and evaluation of their antimicrobial activity. *Planta Medica*, *75*(04), p.24.

Odeleye, O.M., 2010. *Investigation of chemical constituents, antimicrobial and antioxidant activities; and pharmacognostic characters of the leaves of momordica foetida and berkheya bergiana* (Doctoral dissertation, University of Zululand).

Odhav, B., Beekrum, S., Akula, U.S. and Baijnath, H., 2007. Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa. *Journal of Food Composition and Analysis*, *20*(5), pp.430-435.

Odusote, M.O. and Awaraka, C.N., 2004. *Momordica foetida* (cucurbitacea) A Potential Laxative Granule/Capsule. *Nigerian Quarterly Journal of Hospital Medicine*, *14*(2), pp.189-194.

Ogle, B.M. and Grivetti, L.E., 1985. Legacy of the chameleon: Edible wild plants in the kingdom of Swaziland, Southern Africa. A cultural,

ecological, nutritional study. Part IV-nutritional analysis and conclusions. *Ecology of Food and Nutrition*, *17*(1), pp.41-64.

Olaniyi, A.A., 1975. A neutral constituent of *Momordica foetida*. *Lloydia*, *38*(4), p.361.

Oloyede, O.I. and Aluko, O.M., 2012. Determination of antioxidant potential of Momordica Foetida leaf extract on tissue homogenate. *Science Journal of Medicine and Clinical Trial*, *2012*, pp1-4.

Otimenyin, O.S., Uguru, O.M. and Akanbi, B.E., 2008a. Antidiarrhea effect of aqueous extracts of *Momordica balsamina* and *Stachytarpheta indica* in Rats. *Journal of Natural Products*, *1*, pp.36-45.

Otimenyin, O., Sunday, U.M. and Ogbonna, A., 2008b. Antimicrobial and hypoglycemic effects of *Momordica balsamina*. Linn. *Journal of Natural Products*, *1*, pp.03-09.

Parekh, J., Jadeja, D. and Chanda, S., 2006. Efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. *Turkish Journal of Biology*, *29*(4), pp.203-210.

Parekh, J. and Chanda, S., 2007. Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. *African Journal of Biomedical Research*, *10*(2), pp175-181.

Parekh, J. and Chanda, S., 2008a. Antibacterial Activities of Aqueous and Alcoholic Extracts of 34 Indian Medicinal Plants against some Staphylococcus species. *Turkish Journal of Biology*, *32*(1), pp.63-71.

Parekh, J. and Chanda, S., 2008b. Phytochemical screening of some plants from western region of India. *Plant Archives*, *8*(2), pp.657-662.

Parish, M.E., and Davidson, P.M., 1993. *Methods for evaluation: In Antimicrobials in foods*, 2nd Ed. Marcel Dekker, Inc., New York, pp.597-615.

Peter, W. and Deogracious, O., 2006. The in vitro ascaricidal activity of selected indigenous medicinal plants used in ethnoveterinary practices in Uganda. *African Journal of Traditional, Complementary and Alternative Medicines*, *3*, pp.94-103.

Proestos, C., Chorianopoulos, N., Nychas, G.J. and Komaitis, M., 2005. RP-HPLC analysis of the phenolic compounds of plant extracts. Investigation of their antioxidant capacity and antimicrobial activity. *Journal of Agricultural and Food Chemistry*, *53*(4), pp.1190-1195.

Proestos, C. and Komaitis, M., 2013. Analysis of naturally occurring phenolic compounds in aromatic plants by RP-HPLC coupled to diode array detector (DAD) and GC-MS after silylation. *Foods*, *2*(1), pp.90-99.

Puri, M., 2010. "*Momordica balsamina*: A medicinal and neutraceutical plant for health care management". Comments: Biotechnological Potential of *M. balsamina* Revealed. *Current Pharmaceutical Biotechnology, 11*(2), pp.229-229.

Rage, R., Mitchen, J. and Wilding, G., 1990. DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water. *Analytical Biochemistry*, *191*(1), pp.31-34.

Rahman, F., Chowdhury, S., Rahman, M.M., Ahmed, D. and Hossain, A., 2009. Antimicrobial resistance pattern of gram-negative bacteria causing urinary tract infection. *Stamford Journal of Pharmaceutical Sciences*, *2*(1), pp.44-50.

Ramalhete, C., Lopes, D., Mulhovo, S., Rosário, V.E. and Ferreira, M.J.U., 2008, October. Antimalarial activity of some plants traditionally used in Mozambique. In *Workshop Plantas Medicinais e Fitoterapêuticas nos Trópicos. IICT/CCCM, 29, 30 e 31 de Outubro de*.

Ramalhete, C., Spengler, G., Martins, A., Martins, M., Viveiros, M., Mulhovo, S., Ferreira, M.J.U. and Amaral, L., 2011. Inhibition of efflux pumps in meticillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* resistant strains by triterpenoids from *Momordica balsamina*. *International Journal of Antimicrobial Agents*, *37*(1), pp.70-74.

Ramos, S., Alía, M., Bravo, L. and Goya, L., 2005. Comparative effects of food-derived polyphenols on the viability and apoptosis of a human hepatoma cell line (HepG2). *Journal of Agricultural and Food Chemistry*, *53*(4), pp.1271-1280.

Rauha, J.P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H. and Vuorela, P., 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology*, *56*(1), pp.3-12.

Rios, J.L. and Recio, M.C., 2005. Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology*, *100*(1), pp.80-84.

Rios, J.L., Recio, M.C. and Villar, A., 1988. Screening methods for natural products with antimicrobial activity: a review of the literature. *Journal of Ethnopharmacology*, *23*(2), pp.127-149.

Robbins, R.J., 2003. Phenolic acids in foods: an overview of analytical methodology. *Journal of Agricultural and Food Chemistry*, *51*(10), pp.2866-2887.

Robinson, R.W. and Decker-Walters, D.S., 1997. *Cucurbits*. Cab international, pp 97-101.

Rohloff, J., 2015. Analysis of phenolic and cyclic compounds in plants using derivatization techniques in combination with GC-MS-based metabolite profiling. *Molecules*, *20*(2), pp.3431-3462.

Rojas, J.J., Ochoa, V.J., Ocampo, S.A. and Muñoz, J.F., 2006. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: A possible alternative in the treatment of non-nosocomial infections. *BMC Complementary and Alternative Medicine*, *6*(1), p.2.

Roller, S., 1991. The biotechnological development of new food preservatives. *Biotechnology and Genetic Engineering Reviews*, *9*(1), pp.183-206.

Russell, A.D., 1991. Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology*, *71*(3), pp.191-201.

Russell, S.L., Gold, M.J., Hartmann, M., Willing, B.P., Thorson, L., Wlodarska, M., Gill, N., Blanchet, M.R., Mohn, W.W., McNagny, K.M. and Finlay, B.B., 2012. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO reports*, *13*(5), pp.440-447.

Saengsai, J., Kongtunjanphuk, S., Yoswatthana, N., Kummalue, T. and Jiratchariyakul, W., 2015. Antibacterial and antiproliferative activities of Plumericin, an Iridoid isolated from *Momordica charantia* vine. *Evidence-Based Complementary and Alternative Medicine*, *2015*.

Sanhueza, L., Tello, M., Vivanco, M., Mendoza, L. and Wilkens, M., 2014. Relation between antibacterial activity against food transmitted pathogens and total phenolic compounds in grape pomace extracts from Cabernet Sauvignon and Syrah varieties. *Advances in Microbiology*, *4*(5), pp225-232.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y., 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, *8*(1), pp.1-10.

Savage, A.K., Duynhoven, J.P., Tucker, G. and Daykin, C.A., 2011. Enhanced NMR-based profiling of polyphenols in commercially available grape juices using solid-phase extraction. *Magnetic Resonance in Chemistry*, *49*(S1), pp. S27-S36.

Schafer, H., 2005. The biogeography of *Momordica*. *Cucurbit Network News*, *12*(1):5

Sene, C.F., McCann, M.C., Wilson, R.H. and Grinter, R., 1994. Fourier-transform Raman and Fourier-transform infrared spectroscopy (an investigation of five higher plant cell walls and their components). *Plant Physiology*, *106*(4), pp.1623-1631.

Serra, A.T., Matias, A.A., Nunes, A.V., Leitão, M.C., Brito, D., Bronze, R., Silva, S., Pires, A., Crespo, M.T., San Romão, M.V. and Duarte, C.M., 2008. In vitro evaluation of olive-and grape-based natural extracts as potential preservatives for food. *Innovative Food Science & Emerging Technologies*,*9*(3), pp.311-319.

Shelef, L.A., Naglik, O.A. and Bogen, D.W., 1980. Sensitivity of some common food-borne bacteria to the spices sage, rosemary, and allspice. *Journal of Food Science*, *45*(4), pp.1042-1044.

Shettima, Y., Karumi, Y. and Addy, E.O., 2001. Effect of *Momordica balsamina* Linn. fruit on liver enzymes and histopathology in rats. *Nigerian Journal of Experimental and Applied Biology*, *2*, pp.195-198.

Shetty, K. and Labbe, R.G., 1998. Food-borne pathogens, health and role of dietary phytochemicals. *Asia Pacific Journal of Clinical Nutrition*, *7*, pp.270-276.

Shitanda, D. and Wanjala, N.V., 2006. Effect of different drying methods on the quality of jute (*Corchorus olitorius* L.). *Drying Technology*, *24*(1), pp.95-98.

Shunying, Z., Yang, Y., Huaidong, Y., Yue, Y. and Guolin, Z., 2005. Chemical composition and antimicrobial activity of the essential oils of *Chrysanthemum indicum*. *Journal of Ethnopharmacology*, *96*(1), pp.151-158.

Simon, A., Allais, D.P., Duroux, J.L., Basly, J.P., Durand-Fontanier, S. and Delage, C., 1998. Inhibitory effect of curcuminoids on MCF-7 cell proliferation and structure–activity relationships. *Cancer Letters*, *129*(1), pp.111-116.

Singh, U.P., Maurya, S., Singh, A. and Singh, M., 2011. Phenolic acids in some Indian cultivars of *Momordica charantia* and their therapeutic properties. *Journal of Medicinal Plants Research*, *5*, pp.3558-3560.

Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M., 1999. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*, *299*, pp.152-178.

Singleton, V.L. and Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*(3), pp.144-158.

Smith, B.C., 1998. *Infrared spectral interpretation: a systematic approach*. CRC press.

Smith, J.W., Johnson III, J.E. and Cluff, L.E., 1966. Studies on the epidemiology of adverse drug reactions: an evaluation of penicillin allergy. *New England Journal of Medicine*, *274*(18), pp.998-1002.

Song, F.L., Gan, R.Y., Zhang, Y., Xiao, Q., Kuang, L. and Li, H.B., 2010. Total phenolic contents and antioxidant capacities of selected Chinese medicinal plants. *International Journal of Molecular Sciences*, *11*(6), pp.2362-2372.

Stalikas, C.D., 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. *Journal of Separation Science*, *30*(18), pp.3268-3295.

Stanković, M.S., Stefanović, O., Čomić, L., Topuzović, M., Radojević, I. and Solujić, S., 2012. Antimicrobial activity, total phenolic content and flavonoid concentrations of *Teucrium* species. *Central European Journal of Biology*,*7*(4), pp.664-671.

Steinmann, D. and Ganzera, M., 2011. Recent advances on HPLC/MS in medicinal plant analysis. *Journal of Pharmaceutical and Biomedical Analysis*, *55*(4), pp.744-757.

Suárez, B., Picinelli, A. and Mangas, J.J., 1996. Solid-phase extraction and high-performance liquid chromatographic determination of polyphenols in apple musts and ciders. *Journal of Chromatography A*, *727*(2), pp.203-209.

Sun, J., Chu, Y.F., Wu, X. and Liu, R.H., 2002. Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry*, *50*(25), pp.7449-7454.

Tabuti, J.R., 2008. Herbal medicines used in the treatment of malaria in Budiope county, Uganda. *Journal of ethnopharmacology*, *116*(1), pp.33-42.

Tabuti, J.R., Kukunda, C.B. and Waako, P.J., 2010. Medicinal plants used by traditional medicine practitioners in the treatment of tuberculosis and related ailments in Uganda. *Journal of Ethnopharmacology*, *127*(1), pp.130-136.

Talib, W.H. and Mahasneh, A.M., 2010. Antiproliferative activity of plant extracts used against cancer in traditional medicine. *Scientia Pharmaceutica*, *78*(1), p.33.

Tepe, B., Daferera, D., Sokmen, A., Sokmen, M. and Polissiou, M., 2005. Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (*Lamiaceae*). *Food Chemistry*, *90*(3), pp.333-340.

Thakur, G.S., Bag, M., Sanodiya, B.S., Bhadauriya, P., Debnath, M., Prasad, G.B.K.S. and Bisen, P.S., 2009. *Momordica balsamina*: a medicinal and neutraceutical plant for health care management. *Current Pharmaceutical Biotechnology*, *10*(7), pp.667-682.

Trouillas, P., Calliste, C.A., Allais, D.P., Simon, A., Marfak, A., Delage, C. and Duroux, J.L., 2003. Antioxidant, anti-inflammatory and antiproliferative properties of sixteen water plant extracts used in the Limousin countryside as herbal teas. *Food Chemistry*, *80*(3), pp.399-407.

Vadlapudi, V. and Naidu, K.C., 2010. In-vitro bioactivity of Indian medicinal plants *Lantana camara* and *Mimosa pudica* against important pathogens. *Annals of Biological Research*, *1*(1), pp.98-101.

Vaghasiya, Y., Dave, R. and Chanda, S., 2011. Phytochemical analysis of some medicinal plants from western region of India. *Research Journal of Medicinal Plants*, *5*(5), pp.567-576.

Valgas, C., Souza, S.M.D., Smânia, E.F. and Smânia Jr, A., 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, *38*(2), pp.369-380.

van de Venter, M., Roux, S., Bungu, L.C., Louw, J., Crouch, N.R., Grace, O.M., Maharaj, V., Pillay, P., Sewnarian, P., Bhagwandin, N. and Folb, P., 2008. Antidiabetic screening and scoring of 11 plants traditionally used in South Africa. *Journal of Ethnopharmacology*, *119*(1), pp.81-86.

Waako, P.J., Gumede, B., Smith, P. and Folb, P.I., 2005. The in vitro and in vivo antimalarial activity of *Cardiospermum halicacabum* L. and *Momordica foetida* Schumch. Et Thonn. *Journal of Ethnopharmacology*, *99*(1), pp.137-143.

Wang, P., Henning, S.M. and Heber, D., 2010. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *PloS One*, *5*(4), p.e10202.

Wang, S.Y. and Zheng, W., 2001. Effect of plant growth temperature on antioxidant capacity in strawberry. *Journal of Agricultural and Food Chemistry*, *49*(10), pp.4977-4982.

Ward, J.L., Harris, C., Lewis, J. and Beale, M.H., 2003. Assessment of 1 H NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of Arabidopsis thaliana. *Phytochemistry*, *62*(6), pp.949-957.

Welman, M., 2004. *Momordica balsamina* L. [Internet] PlantZAfrica. SA Nationa Biodiversity Institute, Pretoria, South Africa, 2004. http://www.plantzafrica.com/plantklm/momordicabalsam.htm 14/02/2013

Wilson, K. and Walker, J. eds., 2010. *Principles and techniques of biochemistry and molecular biology*. Cambridge University Press.

Yadav, R.N.S. and Agarwala, M., 2011. Phytochemical analysis of some medicinal plants. *Journal of Phytology*, *3*(12).

Yao, J. and Moellering, R. 1995. Antibacterial agents. In: Murray, P., Baron, E., Pfaller, M., Tenover, F. and Yolken, R. (Eds.), *Manual of Clinical Microbiology*. ASM Press, Washington, DC, pp. 1281–1290.

Zaika, L.L., 1988. Spices and herbs: their antimicrobial activity and its determination1. *Journal of Food Safety*, *9*(2), pp.97-118.

Zheng, W. and Wang, S.Y., 2001. Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, *49*(11), pp.5165-5170.

Złotek, U., Mikulska, S., Nagajek, M. and Świeca, M., 2015. The effect of different solvents and number of extraction steps on the polyphenol content and antioxidant capacity of basil leaves (*Ocimum basilicum* L.) extracts. *Saudi Journal of Biological Sciences*, *23*(5), pp.628-633.

APPENDICES

CHAPTER 2

Appendix 2-1. Composition of media used in the study

1. Baird-Parker agar: tryptone, 10 g; 'Lab-Lemco' powder, 5 g; yeast extract, 1 g; sodium pyruvate, 10 g; glycine, 12 g; lithium chloride, 5 g; agar, 20 g; egg yolk emulsion, 50 ml; potassium tellurite, 0.1 g; water, 1 l; pH 6.8±0.2.

2. Brain heart infusion (BHI): calf brain infusion solids, 12.5 g; beef heart infusion solids, 5 g; proteose peptone, 100 g; glucose, 2 g; sodium chloride, 5 g; disodium phosphate, 2.5 g; water, 1 l; pH 7.4±0.2.

3. MacConkey agar no.3: peptone, 20 g; lactose, 10 g; bile salts No. 3, 1.5 g; sodium chloride, 5 g; neutral red, 0.03 g; crystal violet, 0.001 g; agar, 15 g; water, 1 l; pH 7.1±0.2.

4. Maximum recovery diluent: peptone, 1 g; sodium chloride, 8.5 g; water, 1 l; pH 7.0±0.2.

5. Phosphate buffered saline: sodium chloride, 8 g; KCl, 0.2 g; Na2HPO4, 1.44 g; KH2PO4, 0.24 g; Tween 20, 2 ml; water, 1 l; pH 7.2±0.2.

6. Tryptone soy agar: casein peptone (pancreatic), 15 g; sodium chloride, 5 g; soya peptone (papainic), 5 g, water, 1 l; pH 7.3±0.2.

7. Tryptone soya broth: casein peptone (pancreatic), 17 g; dipotassium hydrogen phosphate, 2.5 g; glucose, 2.5 g; sodium chloride, 5 g; soya peptone (papain digest), 3 g, water, 1 l; pH 7.3±0.2.

8. Mueller hinton agar: beef, dehydrated infusion, 300 g; casein hydrolysate, 17.5 g; starch, 1.5 g, water, 1 l; pH 7.3±0.2.

9. Mueller hinton broth: beef, dehydrated infusion, 300 g; casein hydrolysate, 17.5 g; starch, 1.5 g, water, 1 l; pH 7.3±0.2.

10. Sorbitol MacConkey: peptone, 20 g; sorbitol, 10 g; bile salst no. 3, 1.5 g; sodium chloride, 5 g; neutral red, 0.03g; crystal violet, 0.001 g; agar, 15 g; water, 1 l; pH 7.1±0.2.

11. Listeria modified oxford agar: Columbia blood agar base, 39 g; aesculin, 1 g; ferric ammonium citrate, 0.5 g; lithium chloride, 15 g; water, 1 l; pH 7.0±0.2.

12. XLD agar: yeast extract, 3 g; L-lysineHCl, 5 g; xylose, 3.75 g; lactose, 7.5 g; sucrose, 7.5 g; sodium desoxycholate, 1 g; sodium chloride, 5 g; sodium thiosulphate, 6.8 g; ferric ammonium citrate, 0.8 g; phenol red, 0.08 g; agar, 12.5 g; water, 1 l; pH 7.4±0.2.

13. Dulbecco's modified eagle's medium: calcium chloride, 0.2 g; ferric nitrate $9H_2O$, 0.0001 g; magnesium sulfate (anhydrous), 0.09767 g; potassium chloride, 0.4 g; sodium bicarbonate, 3.7 g; sodium chloride, 6.4 g; sodium phosphate monobasic (anhydrous), 0.109 g; L-Arginine HCl, 0.084 g; L-Cystine 2HCl, 0,0626 g; glycine, 0.03 g; L-Histidine HCl H_2O, 0.042 g; L-Isoleucine, 0.105 g; L-lysine HCl, 0.146g; L-Methionine, 0.03 g; L-Phenylalanine, 0.066; L-Serine, 0.042 g; L-Theonine, 0.095 g; L-Trptophan, 0.016 g; L-Tyrosne 2NA $2H_2O$, 0.10379 g; L-Valine, 0.094 g; choline chloride, 0.004 g; folic acid, 0.004 g; *myo*-Inositol, 0.0072 g; niacinamide, 0.004 g; D-Pantothenic acid (henicalcium), 0.004 g; pyridoxine HCl, 0.004 g; riboflavin, 0.0004 g; thiamine HCl, 0.004 g; D-Glucose, 4.5 g; phenol red Na, 0.0159 g; pyruvic acid Na, 0.11 g; water, 1 l.

CHAPTER 3

Appendix 3-1. Paired t-test comparing drying methods (oven-dried and air-dried) for water extracts.

Paired t test	
P value	0.4964
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.7720 df=3
Number of pairs	4
How big is the difference?	
Mean of differences	-0.001668
SD of differences	0.004322
SEM of differences	0.002161
95% confidence interval	-0.008546 to 0.005209
R squared	0.1657
How effective was the pairing?	
Correlation coefficient (r)	0.9969
P value (one tailed)	0.0016
P value summary	**
Was the pairing significantly effective?	Yes

Appendix 3-2. Paired t-test comparing drying methods (oven-dried and air-dried) for chloroform-methanol extracts.

Paired t test	
P value	0.0700
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=2.762 df=3
Number of pairs	4
How big is the difference?	
Mean of differences	0.002758
SD of differences	0.001997
SEM of differences	0.0009983
95% confidence interval	-0.0004195 to 0.005935
R squared	0.7178
How effective was the pairing?	
Correlation coefficient (r)	0.9958
P value (one tailed)	0.0021
P value summary	**
Was the pairing significantly effective?	Yes

Appendix 3-3. ANOVA (Tukey's multiple comparison) comparing yield between extraction solvents for oven dried extracts.

Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**
M. balsamina leaf				
Water vs. Chloroform-methanol (5:3 v/v)	0.1566	0.1549 to 0.1584	Yes	****
Water vs. Methanol-water (7:3 v/v)	0.08287	0.08113 to 0.08461	Yes	****
Chloroform-methanol (5:3 v/v) vs. Methanol-water (7:3 v/v)	-0.07377	-0.07551 to -0.07203	Yes	****
M. balsamina stem				
Water vs. Chloroform-methanol (5:3 v/v)	0.07204	0.07030 to 0.07378	Yes	****
Water vs. Methanol-water (7:3 v/v)	-0.00361	-0.005350 to -0.001870	Yes	****
Chloroform-methanol (5:3 v/v) vs. Methanol-water (7:3 v/v)	-0.07565	-0.07739 to -0.07391	Yes	****

Appendix 3-3. ANOVA (Tukey's multiple comparison) comparing yield between extraction solvents for oven dried extracts (continued).

M. foetida leaf				
Water vs. Chloroform-methanol (5:3 v/v)	0.1062	0.1045 to 0.1079	Yes	****
Water vs. Methanol-water (7:3 v/v)	0.06021	0.05847 to 0.06195	Yes	****
Chloroform-methanol (5:3 v/v) vs. Methanol-water (7:3 v/v)	-0.0460	-0.04774 to -0.04426	Yes	****
M. foetida stem				
Water vs. Chloroform-methanol (5:3 v/v)	0.07554	0.07380 to 0.07728	Yes	****
Water vs. Methanol-water (7:3 v/v)	-0.01096	-0.01270 to -0.009217	Yes	****
Chloroform-methanol (5:3 v/v) vs. Methanol-water (7:3 v/v)	-0.08650	-0.08824 to -0.08476	Yes	****

Extraction solvents (water, chloroform-methanol and methanol-water) were significantly different ($P < 0.05$).

CHAPTER 4

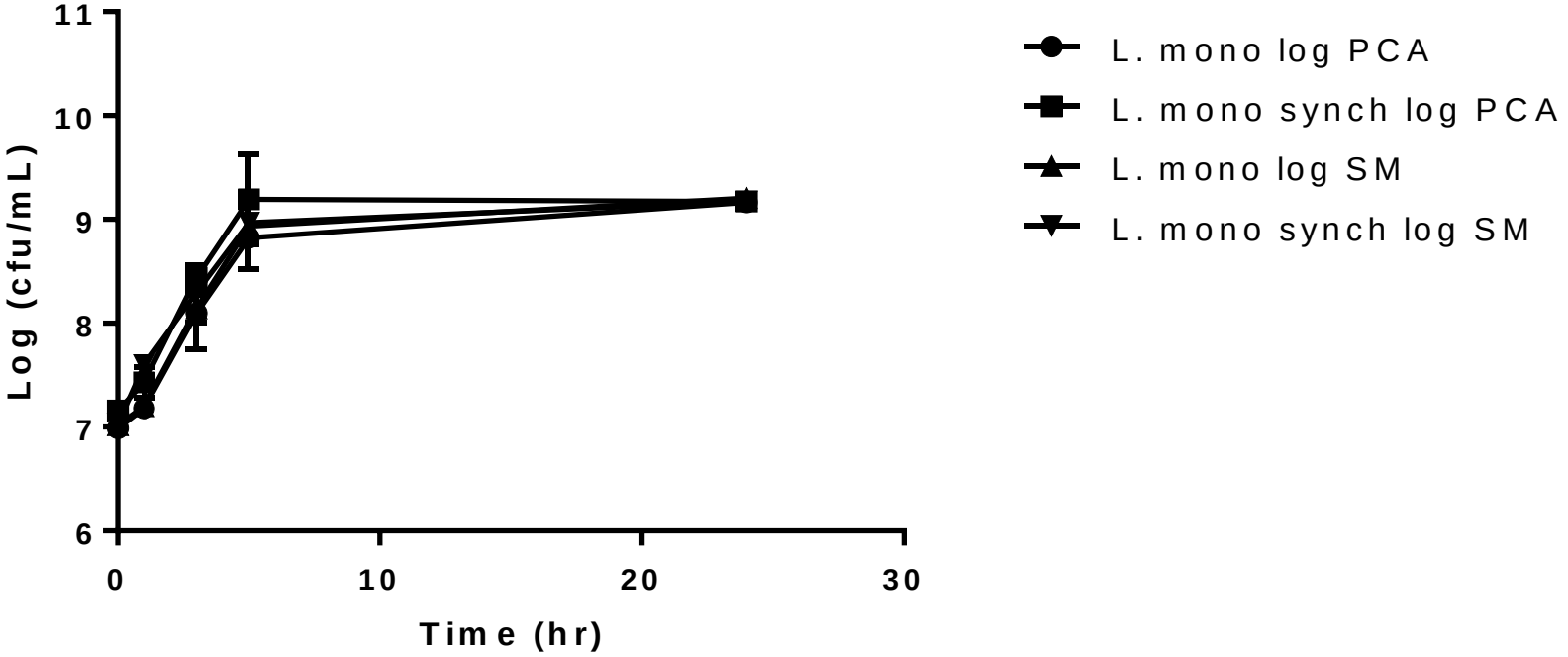
Appendix 4-1. Two-way analysis of variance (ANOVA) comparing inhibition zone of Swaziland (SD) and UK glasshouse (GH) extracts of *Momordica* plants.

Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Interaction	6.189	< 0.0001	****	Yes	
Row Factor	90.82	< 0.0001	****	Yes	
Column Factor	2.804	< 0.0001	****	Yes	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	178.3	20	8.913	F (20, 66) = 107.0	P < 0.0001
Row Factor	2615	10	261.5	F (10, 66) = 3139	P < 0.0001
Column Factor	80.75	2	40.37	F (2, 66) = 484.5	P < 0.0001
Residual	5.500	66	0.08333		

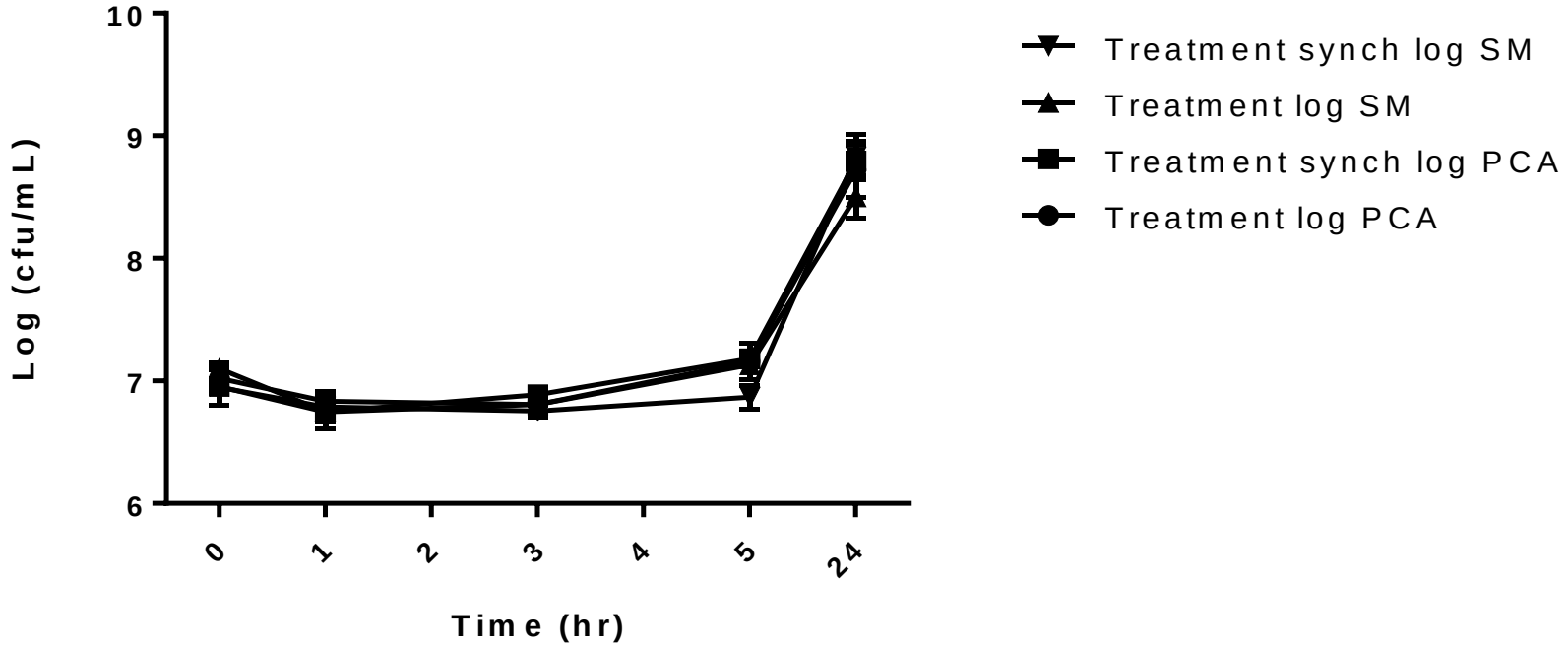
Appendix 4-2 Two-way ANOVA comparing sensitivity of bacteria to *Momordica* extracts.

Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Interaction	49.01	< 0.0001	****	Yes	
Row Factor	22.56	< 0.0001	****	Yes	
Column Factor	26.71	< 0.0001	****	Yes	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	236.8	44	5.381	F (44, 120) = 78.14	P < 0.0001
Row Factor	109.0	4	27.25	F (4, 120) = 395.7	P < 0.0001
Column Factor	129.0	11	11.73	F (11, 120) = 170.3	P < 0.0001
Residual	8.263	120	0.06886		

Appendix 4-3. Effect of culture synchronization and media of growth of *L. monocytogenes* (control).



Appendix 4-4. Effect of culture synchronization and media of growth of *L. monocytogenes* treated with 2 mg/mL *M. balsamina* leaf extract.



Appendix 4-5. Two-way ANOVA comparing the effect of normal and synchronised culture treated with *M. balsamina* leaf extracts on the log count (cfu/mL) when PCA and selective media were used.

Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Time	96.87	< 0.0001	****	Yes	
Treatment	0.2003	0.1059	ns	No	
Interaction: Time x Treatment	1.276	0.0024	**	Yes	
Interaction: Time x Subjects	0.7312				
Interaction: Treatment x Subjects	0.1260				
Subjects	0.1402				
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Time	31.54	4	7.886	F (4, 8) = 265.0	P < 0.0001
Treatment	0.06523	3	0.02174	F (3, 6) = 3.181	P = 0.1059
Interaction: Time x Treatment	0.4154	12	0.03462	F (12, 24) = 3.863	P = 0.0024
Interaction: Time x Subjects	0.2381	8	0.02976		
Interaction: Treatment x Subjects	0.04102	6	0.006836		
Subjects	0.04566	2	0.02283		
Residual	0.2151	24	0.008962		

Appendix 4-6. Tukey's multiple comparison test determining differences in crude and SPE extracts method 1 and method 2.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Crude Method 1 vs. Crude Method 2	-0.4583	-1.059 to 0.1427	No	ns
Crude Method 1 vs. SPE Phenolic Method 1	1.750	1.149 to 2.351	Yes	****
Crude Method 1 vs. SPE Phenolic Method 2	1.417	0.8156 to 2.018	Yes	****
Crude Method 2 vs. SPE Phenolic Method 1	2.208	1.607 to 2.809	Yes	****
Crude Method 2 vs. SPE Phenolic Method 2	1.875	1.274 to 2.476	Yes	****
SPE Phenolic Method 1 vs. SPE Phenolic Method 2	-0.3333	-0.9344 to 0.2677	No	ns

.

CHAPTER 5

Appendix 5-1. ANOVA comparing (Tukey's multiple comparison) effect of *M. balsamina* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells.

Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**
SD 0				
0 mg/mL vs. 0.06 mg/mL	1.052	-1.460 to 3.564	No	ns
0 mg/mL vs. 0.125 mg/mL	0.9720	-1.540 to 3.484	No	ns
0 mg/mL vs. 0.25 mg/mL	-0.9452	-3.457 to 1.567	No	ns
0 mg/mL vs. 0.5 mg/mL	0.1463	-2.366 to 2.658	No	ns
0 mg/mL vs. 0.75 mg/mL	0.7580	-1.754 to 3.270	No	ns
0 mg/mL vs. 1 mg/mL	0.07259	-2.439 to 2.584	No	ns
0.06 mg/mL vs. 0.125 mg/mL	-0.08023	-2.592 to 2.432	No	ns
0.06 mg/mL vs. 0.25 mg/mL	-1.997	-4.509 to 0.5145	No	ns
0.06 mg/mL vs. 0.5 mg/mL	-0.9059	-3.418 to 1.606	No	ns
0.06 mg/mL vs. 0.75 mg/mL	-0.2942	-2.806 to 2.218	No	ns
0.06 mg/mL vs. 1 mg/mL	-0.9796	-3.491 to 1.532	No	ns
0.125 mg/mL vs. 0.25 mg/mL	-1.917	-4.429 to 0.5947	No	ns
0.125 mg/mL vs. 0.5 mg/mL	-0.8256	-3.338 to 1.686	No	ns
0.125 mg/mL vs. 0.75 mg/mL	-0.2140	-2.726 to 2.298	No	ns

Appendix 5-1. ANOVA comparing (Tukey's multiple comparison) effect of *M. balsamina* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (*cont.d*).

0.125 mg/mL vs. 1 mg/mL	-0.8994	-3.411 to 1.613	No	ns
0.25 mg/mL vs. 0.5 mg/mL	1.092	-1.420 to 3.603	No	ns
0.25 mg/mL vs. 0.75 mg/mL	1.703	-0.8087 to 4.215	No	ns
0.25 mg/mL vs. 1 mg/mL	1.018	-1.494 to 3.530	No	ns
0.5 mg/mL vs. 0.75 mg/mL	0.6117	-1.900 to 3.124	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.07374	-2.586 to 2.438	No	ns
0.75 mg/mL vs. 1 mg/mL	-0.6854	-3.197 to 1.826	No	ns
GH 0				
0 mg/mL vs. 0.06 mg/mL	0.3210	-2.191 to 2.833	No	ns
0 mg/mL vs. 0.125 mg/mL	0.3185	-2.193 to 2.830	No	ns
0 mg/mL vs. 0.25 mg/mL	-0.7569	-3.269 to 1.755	No	ns
0 mg/mL vs. 0.5 mg/mL	-0.6622	-3.174 to 1.850	No	ns
0 mg/mL vs. 0.75 mg/mL	-0.2210	-2.733 to 2.291	No	ns
0 mg/mL vs. 1 mg/mL	-0.4338	-2.946 to 2.078	No	ns
0.06 mg/mL vs. 0.125 mg/mL	-0.002475	-2.514 to 2.509	No	ns
0.06 mg/mL vs. 0.25 mg/mL	-1.078	-3.590 to 1.434	No	ns
0.06 mg/mL vs. 0.5 mg/mL	-0.9832	-3.495 to 1.529	No	ns
0.06 mg/mL vs. 0.75 mg/mL	-0.5420	-3.054 to 1.970	No	ns
0.06 mg/mL vs. 1 mg/mL	-0.7548	-3.267 to 1.757	No	ns
0.125 mg/mL vs. 0.25 mg/mL	-1.075	-3.587 to 1.436	No	ns
0.125 mg/mL vs. 0.5 mg/mL	-0.9807	-3.493 to 1.531	No	ns

Appendix 5-1. ANOVA comparing (Tukey's multiple comparison) effect of *M. balsamina* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (*cont.d*).

0.125 mg/mL vs. 0.75 mg/mL	-0.5395	-3.051 to 1.972	No	ns
0.125 mg/mL vs. 1 mg/mL	-0.7523	-3.264 to 1.760	No	ns
0.25 mg/mL vs. 0.5 mg/mL	0.09475	-2.417 to 2.607	No	ns
0.25 mg/mL vs. 0.75 mg/mL	0.5360	-1.976 to 3.048	No	ns
0.25 mg/mL vs. 1 mg/mL	0.3231	-2.189 to 2.835	No	ns
0.5 mg/mL vs. 0.75 mg/mL	0.4412	-2.071 to 2.953	No	ns
0.5 mg/mL vs. 1 mg/mL	0.2284	-2.284 to 2.740	No	ns
0.75 mg/mL vs. 1 mg/mL	-0.2128	-2.725 to 2.299	No	ns
SD 24				
0 mg/mL vs. 0.06 mg/mL	0.1076	-2.404 to 2.619	No	ns
0 mg/mL vs. 0.125 mg/mL	-0.5235	-3.035 to 1.988	No	ns
0 mg/mL vs. 0.25 mg/mL	1.023	-1.489 to 3.535	No	ns
0 mg/mL vs. 0.5 mg/mL	4.994	2.482 to 7.506	Yes	****
0 mg/mL vs. 0.75 mg/mL	5.726	3.214 to 8.238	Yes	****
0 mg/mL vs. 1 mg/mL	4.425	1.913 to 6.937	Yes	****
0.06 mg/mL vs. 0.125 mg/mL	-0.6311	-3.143 to 1.881	No	ns
0.06 mg/mL vs. 0.25 mg/mL	0.9155	-1.596 to 3.427	No	ns
0.06 mg/mL vs. 0.5 mg/mL	4.886	2.374 to 7.398	Yes	****
0.06 mg/mL vs. 0.75 mg/mL	5.619	3.107 to 8.131	Yes	****
0.06 mg/mL vs. 1 mg/mL	4.317	1.805 to 6.829	Yes	****

Appendix 5-1. ANOVA comparing (Tukey's multiple comparison) effect of *M. balsamina* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (*cont.d*).

0.125 mg/mL vs. 0.25 mg/mL	1.547	-0.9653 to 4.058	No	ns
0.125 mg/mL vs. 0.5 mg/mL	5.517	3.005 to 8.029	Yes	****
0.125 mg/mL vs. 0.75 mg/mL	6.250	3.738 to 8.762	Yes	****
0.125 mg/mL vs. 1 mg/mL	4.948	2.436 to 7.460	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	3.971	1.459 to 6.483	Yes	****
0.25 mg/mL vs. 0.75 mg/mL	4.703	2.191 to 7.215	Yes	****
0.25 mg/mL vs. 1 mg/mL	3.402	0.8899 to 5.914	Yes	**
0.5 mg/mL vs. 0.75 mg/mL	0.7327	-1.779 to 3.245	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.5688	-3.081 to 1.943	No	ns
0.75 mg/mL vs. 1 mg/mL	-1.302	-3.813 to 1.210	No	ns
GH 24				
0 mg/mL vs. 0.06 mg/mL	1.208	-1.304 to 3.720	No	ns
0 mg/mL vs. 0.125 mg/mL	1.054	-1.458 to 3.566	No	ns
0 mg/mL vs. 0.25 mg/mL	0.6980	-1.814 to 3.210	No	ns
0 mg/mL vs. 0.5 mg/mL	2.824	0.3121 to 5.336	Yes	*
0 mg/mL vs. 0.75 mg/mL	7.477	4.965 to 9.989	Yes	****
0 mg/mL vs. 1 mg/mL	7.446	4.934 to 9.958	Yes	****
0.06 mg/mL vs. 0.125 mg/mL	-0.1537	-2.666 to 2.358	No	ns
0.06 mg/mL vs. 0.25 mg/mL	-0.5099	-3.022 to 2.002	No	ns
0.06 mg/mL vs. 0.5 mg/mL	1.616	-0.8958 to 4.128	No	ns
0.06 mg/mL vs. 0.75 mg/mL	6.270	3.758 to 8.781	Yes	****

Appendix 5-1. ANOVA comparing (Tukey's multiple comparison) effect of *M. balsamina* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (*cont.d*).

0.06 mg/mL vs. 1 mg/mL	6.238	3.726 to 8.750	Yes	****
0.125 mg/mL vs. 0.25 mg/mL	-0.3562	-2.868 to 2.156	No	ns
0.125 mg/mL vs. 0.5 mg/mL	1.770	-0.7421 to 4.282	No	ns
0.125 mg/mL vs. 0.75 mg/mL	6.423	3.911 to 8.935	Yes	****
0.125 mg/mL vs. 1 mg/mL	6.392	3.880 to 8.904	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	2.126	-0.3859 to 4.638	No	ns
0.25 mg/mL vs. 0.75 mg/mL	6.779	4.267 to 9.291	Yes	****
0.25 mg/mL vs. 1 mg/mL	6.748	4.236 to 9.260	Yes	****
0.5 mg/mL vs. 0.75 mg/mL	4.653	2.142 to 7.165	Yes	****
0.5 mg/mL vs. 1 mg/mL	4.622	2.110 to 7.134	Yes	****
0.75 mg/mL vs. 1 mg/mL	-0.03113	-2.543 to 2.481	No	ns
SD 48				
0 mg/mL vs. 0.06 mg/mL	0.1581	-2.354 to 2.670	No	ns
0 mg/mL vs. 0.125 mg/mL	0.6162	-1.896 to 3.128	No	ns
0 mg/mL vs. 0.25 mg/mL	5.490	2.978 to 8.001	Yes	****
0 mg/mL vs. 0.5 mg/mL	11.27	8.762 to 13.79	Yes	****
0 mg/mL vs. 0.75 mg/mL	9.926	7.414 to 12.44	Yes	****
0 mg/mL vs. 1 mg/mL	8.726	6.214 to 11.24	Yes	****
0.06 mg/mL vs. 0.125 mg/mL	0.4581	-2.054 to 2.970	No	ns
0.06 mg/mL vs. 0.25 mg/mL	5.331	2.820 to 7.843	Yes	****
0.06 mg/mL vs. 0.5 mg/mL	11.12	8.604 to 13.63	Yes	****

Appendix 5-1. ANOVA comparing (Tukey's multiple comparison) effect of *M. balsamina* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (*cont.d*).

0.06 mg/mL vs. 0.75 mg/mL	9.768	7.256 to 12.28	Yes	****
0.06 mg/mL vs. 1 mg/mL	8.568	6.056 to 11.08	Yes	****
0.125 mg/mL vs. 0.25 mg/mL	4.873	2.361 to 7.385	Yes	****
0.125 mg/mL vs. 0.5 mg/mL	10.66	8.146 to 13.17	Yes	****
0.125 mg/mL vs. 0.75 mg/mL	9.310	6.798 to 11.82	Yes	****
0.125 mg/mL vs. 1 mg/mL	8.110	5.598 to 10.62	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	5.785	3.273 to 8.297	Yes	****
0.25 mg/mL vs. 0.75 mg/mL	4.437	1.925 to 6.949	Yes	****
0.25 mg/mL vs. 1 mg/mL	3.236	0.7243 to 5.748	Yes	**
0.5 mg/mL vs. 0.75 mg/mL	-1.348	-3.860 to 1.164	No	ns
0.5 mg/mL vs. 1 mg/mL	-2.548	-5.060 to -0.03659	Yes	*
0.75 mg/mL vs. 1 mg/mL	-1.201	-3.713 to 1.311	No	ns
GH 48				
0 mg/mL vs. 0.06 mg/mL	0.2644	-2.248 to 2.776	No	ns
0 mg/mL vs. 0.125 mg/mL	-0.4559	-2.968 to 2.056	No	ns
0 mg/mL vs. 0.25 mg/mL	0.9644	-1.548 to 3.476	No	ns
0 mg/mL vs. 0.5 mg/mL	8.992	6.480 to 11.50	Yes	****
0 mg/mL vs. 0.75 mg/mL	9.320	6.808 to 11.83	Yes	****
0 mg/mL vs. 1 mg/mL	9.720	7.208 to 12.23	Yes	****
0.06 mg/mL vs. 0.125 mg/mL	-0.7202	-3.232 to 1.792	No	ns

Appendix 5-1. ANOVA comparing (Tukey's multiple comparison) effect of *M. balsamina* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (*cont.d*).

0.06 mg/mL vs. 0.25 mg/mL	0.7000	-1.812 to 3.212	No	ns
0.06 mg/mL vs. 0.5 mg/mL	8.728	6.216 to 11.24	Yes	****
0.06 mg/mL vs. 0.75 mg/mL	9.055	6.544 to 11.57	Yes	****
0.06 mg/mL vs. 1 mg/mL	9.455	6.944 to 11.97	Yes	****
0.125 mg/mL vs. 0.25 mg/mL	1.420	-1.092 to 3.932	No	ns
0.125 mg/mL vs. 0.5 mg/mL	9.448	6.936 to 11.96	Yes	****
0.125 mg/mL vs. 0.75 mg/mL	9.776	7.264 to 12.29	Yes	****
0.125 mg/mL vs. 1 mg/mL	10.18	7.664 to 12.69	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	8.028	5.516 to 10.54	Yes	****
0.25 mg/mL vs. 0.75 mg/mL	8.355	5.844 to 10.87	Yes	****
0.25 mg/mL vs. 1 mg/mL	8.755	6.244 to 11.27	Yes	****
0.5 mg/mL vs. 0.75 mg/mL	0.3279	-2.184 to 2.840	No	ns
0.5 mg/mL vs. 1 mg/mL	0.7279	-1.784 to 3.240	No	ns
0.75 mg/mL vs. 1 mg/mL	0.4001	-2.112 to 2.912	No	ns

Appendix 5-2. ANOVA comparing effect of *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (HepG2 cell Passage 33).

Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**
SD 0				
0 mg/mL vs. 0.25 mg/mL	0.7800	-1.249 to 2.809	No	ns
0 mg/mL vs. 0.5 mg/mL	-0.1200	-2.149 to 1.909	No	ns
0 mg/mL vs. 1 mg/mL	-0.1200	-2.149 to 1.909	No	ns
0 mg/mL vs. 2 mg/mL	-0.3700	-2.399 to 1.659	No	ns
0 mg/mL vs. 4 mg/mL	-0.2800	-2.309 to 1.749	No	ns
0.25 mg/mL vs. 0.5 mg/mL	-0.9000	-2.929 to 1.129	No	ns
0.25 mg/mL vs. 1 mg/mL	-0.9000	-2.929 to 1.129	No	ns
0.25 mg/mL vs. 2 mg/mL	-1.150	-3.179 to 0.8789	No	ns
0.25 mg/mL vs. 4 mg/mL	-1.060	-3.089 to 0.9689	No	ns
0.5 mg/mL vs. 1 mg/mL	0.0	-2.029 to 2.029	No	ns
0.5 mg/mL vs. 2 mg/mL	-0.2500	-2.279 to 1.779	No	ns
0.5 mg/mL vs. 4 mg/mL	-0.1600	-2.189 to 1.869	No	ns
1 mg/mL vs. 2 mg/mL	-0.2500	-2.279 to 1.779	No	ns
1 mg/mL vs. 4 mg/mL	-0.1600	-2.189 to 1.869	No	ns
2 mg/mL vs. 4 mg/mL	0.09000	-1.939 to 2.119	No	ns
GH 0				
0 mg/mL vs. 0.25 mg/mL	0.1000	-1.929 to 2.129	No	ns
0 mg/mL vs. 0.5 mg/mL	-0.01000	-2.039 to 2.019	No	ns
0 mg/mL vs. 1 mg/mL	-0.7000	-2.729 to 1.329	No	ns
0 mg/mL vs. 2 mg/mL	-0.4500	-2.479 to 1.579	No	ns
0 mg/mL vs. 4 mg/mL	-1.690	-3.719 to 0.3389	No	ns
0.25 mg/mL vs. 0.5 mg/mL	-0.1100	-2.139 to 1.919	No	ns
0.25 mg/mL vs. 1 mg/mL	-0.8000	-2.829 to 1.229	No	ns

Appendix 5-2. ANOVA comparing effect of *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (HepG2 cell Passage 33) (*cont.d*).

0.25 mg/mL vs. 2 mg/mL	-0.5500	-2.579 to 1.479	No	ns
0.25 mg/mL vs. 4 mg/mL	-1.790	-3.819 to 0.2389	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.6900	-2.719 to 1.339	No	ns
0.5 mg/mL vs. 2 mg/mL	-0.4400	-2.469 to 1.589	No	ns
0.5 mg/mL vs. 4 mg/mL	-1.680	-3.709 to 0.3489	No	ns
1 mg/mL vs. 2 mg/mL	0.2500	-1.779 to 2.279	No	ns
1 mg/mL vs. 4 mg/mL	-0.9900	-3.019 to 1.039	No	ns
2 mg/mL vs. 4 mg/mL	-1.240	-3.269 to 0.7889	No	ns
SD 24				
0 mg/mL vs. 0.25 mg/mL	-0.2608	-2.618 to 2.097	No	ns
0 mg/mL vs. 0.5 mg/mL	-0.4107	-2.896 to 2.074	No	ns
0 mg/mL vs. 1 mg/mL	-0.1249	-2.482 to 2.232	No	ns
0 mg/mL vs. 2 mg/mL	6.115	3.758 to 8.472	Yes	****
0 mg/mL vs. 4 mg/mL	6.337	3.980 to 8.695	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	-0.1499	-2.507 to 2.208	No	ns
0.25 mg/mL vs. 1 mg/mL	0.1359	-2.087 to 2.358	No	ns
0.25 mg/mL vs. 2 mg/mL	6.376	4.153 to 8.598	Yes	****
0.25 mg/mL vs. 4 mg/mL	6.598	4.375 to 8.821	Yes	****
0.5 mg/mL vs. 1 mg/mL	0.2858	-2.072 to 2.643	No	ns
0.5 mg/mL vs. 2 mg/mL	6.526	4.168 to 8.883	Yes	****
0.5 mg/mL vs. 4 mg/mL	6.748	4.390 to 9.105	Yes	****
1 mg/mL vs. 2 mg/mL	6.240	4.017 to 8.463	Yes	****
1 mg/mL vs. 4 mg/mL	6.462	4.239 to 8.685	Yes	****
2 mg/mL vs. 4 mg/mL	0.2221	-2.001 to 2.445	No	ns

Appendix 5-2. ANOVA comparing effect of *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (HepG2 cell Passage 33) (*cont.d*).

GH 24				
0 mg/mL vs. 0.25 mg/mL	-0.009998	-2.039 to 2.019	No	ns
0 mg/mL vs. 0.5 mg/mL	-0.9600	-2.989 to 1.069	No	ns
0 mg/mL vs. 1 mg/mL	0.0	-2.029 to 2.029	No	ns
0 mg/mL vs. 2 mg/mL	6.720	4.691 to 8.749	Yes	****
0 mg/mL vs. 4 mg/mL	7.940	5.911 to 9.969	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	-0.9500	-2.979 to 1.079	No	ns
0.25 mg/mL vs. 1 mg/mL	0.009998	-2.019 to 2.039	No	ns
0.25 mg/mL vs. 2 mg/mL	6.730	4.701 to 8.759	Yes	****
0.25 mg/mL vs. 4 mg/mL	7.950	5.921 to 9.979	Yes	****
0.5 mg/mL vs. 1 mg/mL	0.9600	-1.069 to 2.989	No	ns
0.5 mg/mL vs. 2 mg/mL	7.680	5.651 to 9.709	Yes	****
0.5 mg/mL vs. 4 mg/mL	8.900	6.871 to 10.93	Yes	****
1 mg/mL vs. 2 mg/mL	6.720	4.691 to 8.749	Yes	****
1 mg/mL vs. 4 mg/mL	7.940	5.911 to 9.969	Yes	****
2 mg/mL vs. 4 mg/mL	1.220	-0.8089 to 3.249	No	ns
SD 48				
0 mg/mL vs. 0.25 mg/mL	-0.4662	-2.951 to 2.019	No	ns
0 mg/mL vs. 0.5 mg/mL	-1.409	-3.894 to 1.076	No	ns
0 mg/mL vs. 1 mg/mL	1.157	-1.200 to 3.515	No	ns
0 mg/mL vs. 2 mg/mL	9.040	6.772 to 11.31	Yes	****
0 mg/mL vs. 4 mg/mL	9.214	6.945 to 11.48	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	-0.9424	-3.427 to 1.543	No	ns
0.25 mg/mL vs. 1 mg/mL	1.623	-0.7339 to 3.981	No	ns
0.25 mg/mL vs. 2 mg/mL	9.507	7.238 to 11.78	Yes	****

Appendix 5-2. ANOVA comparing effect of *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts on proliferation of HepG2 cells (HepG2 cell Passage 33) (*cont.d*).

0.25 mg/mL vs. 4 mg/mL	9.680	7.412 to 11.95	Yes	****
0.5 mg/mL vs. 1 mg/mL	2.566	0.2085 to 4.923	Yes	*
0.5 mg/mL vs. 2 mg/mL	10.45	8.181 to 12.72	Yes	****
0.5 mg/mL vs. 4 mg/mL	10.62	8.354 to 12.89	Yes	****
1 mg/mL vs. 2 mg/mL	7.883	5.755 to 10.01	Yes	****
1 mg/mL vs. 4 mg/mL	8.057	5.929 to 10.18	Yes	****
2 mg/mL vs. 4 mg/mL	0.1733	-1.856 to 2.202	No	ns
GH 48				
0 mg/mL vs. 0.25 mg/mL	0.5500	-1.479 to 2.579	No	ns
0 mg/mL vs. 0.5 mg/mL	1.450	-0.5789 to 3.479	No	ns
0 mg/mL vs. 1 mg/mL	5.700	3.671 to 7.729	Yes	****
0 mg/mL vs. 2 mg/mL	13.10	11.07 to 15.13	Yes	****
0 mg/mL vs. 4 mg/mL	12.26	10.23 to 14.29	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	0.9000	-1.129 to 2.929	No	ns
0.25 mg/mL vs. 1 mg/mL	5.150	3.121 to 7.179	Yes	****
0.25 mg/mL vs. 2 mg/mL	12.55	10.52 to 14.58	Yes	****
0.25 mg/mL vs. 4 mg/mL	11.71	9.681 to 13.74	Yes	****
0.5 mg/mL vs. 1 mg/mL	4.250	2.221 to 6.279	Yes	****
0.5 mg/mL vs. 2 mg/mL	11.65	9.621 to 13.68	Yes	****
0.5 mg/mL vs. 4 mg/mL	10.81	8.781 to 12.84	Yes	****
1 mg/mL vs. 2 mg/mL	7.400	5.371 to 9.429	Yes	****
1 mg/mL vs. 4 mg/mL	6.560	4.531 to 8.589	Yes	****
2 mg/mL vs. 4 mg/mL	-0.8400	-2.869 to 1.189	No	ns

Appendix 5-3. ANOVA comparing *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts (Passage 47).

Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**
0 SD				
0 mg/mL vs. 0.25 mg/mL	0.2917	-1.572 to 2.155	No	ns
0 mg/mL vs. 0.5 mg/mL	-0.3978	-2.261 to 1.465	No	ns
0 mg/mL vs. 0.75 mg/mL	-0.8801	-2.743 to 0.9832	No	ns
0 mg/mL vs. 1 mg/mL	-0.8578	-2.721 to 1.005	No	ns
0 mg/mL vs. 1.5 mg/mL	-0.003014	-1.979 to 1.973	No	ns
0 mg/mL vs. 2 mg/mL	0.2798	-1.696 to 2.256	No	ns
0.25 mg/mL vs. 0.5 mg/mL	-0.6895	-2.553 to 1.174	No	ns
0.25 mg/mL vs. 0.75 mg/mL	-1.172	-3.035 to 0.6915	No	ns
0.25 mg/mL vs. 1 mg/mL	-1.149	-3.013 to 0.7138	No	ns
0.25 mg/mL vs. 1.5 mg/mL	-0.2947	-2.271 to 1.682	No	ns
0.25 mg/mL vs. 2 mg/mL	-0.01182	-1.988 to 1.964	No	ns
0.5 mg/mL vs. 0.75 mg/mL	-0.4822	-2.346 to 1.381	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.4600	-2.323 to 1.403	No	ns
0.5 mg/mL vs. 1.5 mg/mL	0.3948	-1.581 to 2.371	No	ns
0.5 mg/mL vs. 2 mg/mL	0.6777	-1.299 to 2.654	No	ns
0.75 mg/mL vs. 1 mg/mL	0.02226	-1.841 to 1.886	No	ns
0.75 mg/mL vs. 1.5 mg/mL	0.8771	-1.099 to 2.853	No	ns
0.75 mg/mL vs. 2 mg/mL	1.160	-0.8164 to 3.136	No	ns
1 mg/mL vs. 1.5 mg/mL	0.8548	-1.121 to 2.831	No	ns
1 mg/mL vs. 2 mg/mL	1.138	-0.8386 to 3.114	No	ns
1.5 mg/mL vs. 2 mg/mL	0.2829	-1.800 to 2.366	No	ns

Appendix 5-3. ANOVA comparing *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts (Passage 47) (*cont.d*).

24 SD				
0 mg/mL vs. 0.25 mg/mL	0.6105	-1.253 to 2.474	No	ns
0 mg/mL vs. 0.5 mg/mL	0.7605	-1.103 to 2.624	No	ns
0 mg/mL vs. 0.75 mg/mL	-0.02551	-1.889 to 1.838	No	ns
0 mg/mL vs. 1 mg/mL	0.5977	-1.266 to 2.461	No	ns
0 mg/mL vs. 1.5 mg/mL	3.626	1.763 to 5.490	Yes	****
0 mg/mL vs. 2 mg/mL	3.792	1.929 to 5.656	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	0.1500	-1.713 to 2.013	No	ns
0.25 mg/mL vs. 0.75 mg/mL	-0.6360	-2.499 to 1.227	No	ns
0.25 mg/mL vs. 1 mg/mL	-0.01276	-1.876 to 1.851	No	ns
0.25 mg/mL vs. 1.5 mg/mL	3.016	1.153 to 4.879	Yes	****
0.25 mg/mL vs. 2 mg/mL	3.182	1.319 to 5.045	Yes	****
0.5 mg/mL vs. 0.75 mg/mL	-0.7860	-2.649 to 1.077	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.1627	-2.026 to 1.701	No	ns
0.5 mg/mL vs. 1.5 mg/mL	2.866	1.003 to 4.729	Yes	***
0.5 mg/mL vs. 2 mg/mL	3.032	1.169 to 4.895	Yes	****
0.75 mg/mL vs. 1 mg/mL	0.6233	-1.240 to 2.487	No	ns
0.75 mg/mL vs. 1.5 mg/mL	3.652	1.789 to 5.515	Yes	****
0.75 mg/mL vs. 2 mg/mL	3.818	1.955 to 5.681	Yes	****
1 mg/mL vs. 1.5 mg/mL	3.029	1.165 to 4.892	Yes	****
1 mg/mL vs. 2 mg/mL	3.195	1.331 to 5.058	Yes	****
1.5 mg/mL vs. 2 mg/mL	0.1658	-1.697 to 2.029	No	ns
48 SD				
0 mg/mL vs. 0.25 mg/mL	0.1627	-1.920 to 2.246	No	ns
0 mg/mL vs. 0.5 mg/mL	-1.024	-3.000 to 0.9523	No	ns

Appendix 5-3. ANOVA comparing *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts (Passage 47) (*cont.d*).

0 mg/mL vs. 0.75 mg/mL	-2.113	-4.089 to -0.1367	Yes	*
0 mg/mL vs. 1 mg/mL	-1.741	-3.718 to 0.2349	No	ns
0 mg/mL vs. 1.5 mg/mL	3.190	1.214 to 5.167	Yes	****
0 mg/mL vs. 2 mg/mL	5.759	3.783 to 7.735	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	-1.187	-3.163 to 0.7896	No	ns
0.25 mg/mL vs. 0.75 mg/mL	-2.276	-4.252 to -0.2995	Yes	*
0.25 mg/mL vs. 1 mg/mL	-1.904	-3.880 to 0.07215	No	ns
0.25 mg/mL vs. 1.5 mg/mL	3.028	1.051 to 5.004	Yes	***
0.25 mg/mL vs. 2 mg/mL	5.596	3.620 to 7.573	Yes	****
0.5 mg/mL vs. 0.75 mg/mL	-1.089	-2.952 to 0.7742	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.7175	-2.581 to 1.146	No	ns
0.5 mg/mL vs. 1.5 mg/mL	4.214	2.351 to 6.078	Yes	****
0.5 mg/mL vs. 2 mg/mL	6.783	4.920 to 8.646	Yes	****
0.75 mg/mL vs. 1 mg/mL	0.3716	-1.492 to 2.235	No	ns
0.75 mg/mL vs. 1.5 mg/mL	5.303	3.440 to 7.167	Yes	****
0.75 mg/mL vs. 2 mg/mL	7.872	6.009 to 9.735	Yes	****
1 mg/mL vs. 1.5 mg/mL	4.932	3.068 to 6.795	Yes	****
1 mg/mL vs. 2 mg/mL	7.500	5.637 to 9.364	Yes	****
1.5 mg/mL vs. 2 mg/mL	2.569	0.7054 to 4.432	Yes	**
0 GH				
0 mg/mL vs. 0.25 mg/mL	-0.1363	-1.837 to 1.565	No	ns
0 mg/mL vs. 0.5 mg/mL	-0.02902	-1.730 to 1.672	No	ns
0 mg/mL vs. 0.75 mg/mL	-0.4453	-2.146 to 1.256	No	ns
0 mg/mL vs. 1 mg/mL	-0.4497	-2.151 to 1.251	No	ns
0 mg/mL vs. 1.5 mg/mL	-0.1943	-1.895 to 1.507	No	ns
0 mg/mL vs. 2 mg/mL	0.5287	-1.172 to 2.230	No	ns

Appendix 5-3. ANOVA comparing *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts (Passage 47) (*cont.d*).

0.25 mg/mL vs. 0.5 mg/mL	0.1073	-1.594 to 1.808	No	ns
0.25 mg/mL vs. 0.75 mg/mL	-0.3090	-2.010 to 1.392	No	ns
0.25 mg/mL vs. 1 mg/mL	-0.3134	-2.014 to 1.388	No	ns
0.25 mg/mL vs. 1.5 mg/mL	-0.05805	-1.759 to 1.643	No	ns
0.25 mg/mL vs. 2 mg/mL	0.6650	-1.036 to 2.366	No	ns
0.5 mg/mL vs. 0.75 mg/mL	-0.4163	-2.117 to 1.285	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.4207	-2.122 to 1.280	No	ns
0.5 mg/mL vs. 1.5 mg/mL	-0.1653	-1.866 to 1.536	No	ns
0.5 mg/mL vs. 2 mg/mL	0.5577	-1.143 to 2.259	No	ns
0.75 mg/mL vs. 1 mg/mL	-0.004408	-1.705 to 1.697	No	ns
0.75 mg/mL vs. 1.5 mg/mL	0.2509	-1.450 to 1.952	No	ns
0.75 mg/mL vs. 2 mg/mL	0.9739	-0.7270 to 2.675	No	ns
1 mg/mL vs. 1.5 mg/mL	0.2553	-1.446 to 1.956	No	ns
1 mg/mL vs. 2 mg/mL	0.9784	-0.7226 to 2.679	No	ns
1.5 mg/mL vs. 2 mg/mL	0.7230	-0.9779 to 2.424	No	ns
24 GH				
0 mg/mL vs. 0.25 mg/mL	0.7491	-0.9518 to 2.450	No	ns
0 mg/mL vs. 0.5 mg/mL	1.096	-0.8053 to 2.998	No	ns
0 mg/mL vs. 0.75 mg/mL	0.7820	-0.9190 to 2.483	No	ns
0 mg/mL vs. 1 mg/mL	0.9966	-0.7044 to 2.697	No	ns
0 mg/mL vs. 1.5 mg/mL	3.528	1.828 to 5.229	Yes	****
0 mg/mL vs. 2 mg/mL	3.571	1.870 to 5.272	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	0.3473	-1.554 to 2.249	No	ns
0.25 mg/mL vs. 0.75 mg/mL	0.03288	-1.668 to 1.734	No	ns
0.25 mg/mL vs. 1 mg/mL	0.2475	-1.453 to 1.948	No	ns

Appendix 5-3. ANOVA comparing *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts (Passage 47) (*cont.d*).

0.25 mg/mL vs. 1.5 mg/mL	2.779	1.078 to 4.480	Yes	****
0.25 mg/mL vs. 2 mg/mL	2.822	1.121 to 4.523	Yes	****
0.5 mg/mL vs. 0.75 mg/mL	-0.3145	-2.216 to 1.587	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.09987	-2.002 to 1.802	No	ns
0.5 mg/mL vs. 1.5 mg/mL	2.432	0.5303 to 4.334	Yes	**
0.5 mg/mL vs. 2 mg/mL	2.475	0.5731 to 4.376	Yes	**
0.75 mg/mL vs. 1 mg/mL	0.2146	-1.486 to 1.916	No	ns
0.75 mg/mL vs. 1.5 mg/mL	2.746	1.046 to 4.447	Yes	****
0.75 mg/mL vs. 2 mg/mL	2.789	1.088 to 4.490	Yes	****
1 mg/mL vs. 1.5 mg/mL	2.532	0.8309 to 4.233	Yes	***
1 mg/mL vs. 2 mg/mL	2.575	0.8737 to 4.276	Yes	***
1.5 mg/mL vs. 2 mg/mL	0.04278	-1.658 to 1.744	No	ns
48 GH				
0 mg/mL vs. 0.25 mg/mL	1.446	-0.4174 to 3.309	No	ns
0 mg/mL vs. 0.5 mg/mL	1.444	-0.3400 to 3.228	No	ns
0 mg/mL vs. 0.75 mg/mL	0.6607	-1.491 to 2.812	No	ns
0 mg/mL vs. 1 mg/mL	0.9657	-1.011 to 2.942	No	ns
0 mg/mL vs. 1.5 mg/mL	7.369	5.585 to 9.153	Yes	****
0 mg/mL vs. 2 mg/mL	7.028	5.244 to 8.812	Yes	****
0.25 mg/mL vs. 0.5 mg/mL	-0.001990	-1.786 to 1.782	No	ns
0.25 mg/mL vs. 0.75 mg/mL	-0.7852	-2.937 to 1.366	No	ns
0.25 mg/mL vs. 1 mg/mL	-0.4802	-2.457 to 1.496	No	ns
0.25 mg/mL vs. 1.5 mg/mL	5.923	4.139 to 7.707	Yes	****
0.25 mg/mL vs. 2 mg/mL	5.582	3.798 to 7.366	Yes	****

Appendix 5-3. ANOVA comparing *M. foetida* leaf Swaziland sourced and UK glasshouse grown extracts (Passage 47) (*cont.d*).

0.5 mg/mL vs. 0.75 mg/mL	-0.7832	-2.866 to 1.300	No	ns
0.5 mg/mL vs. 1 mg/mL	-0.4782	-2.380 to 1.424	No	ns
0.5 mg/mL vs. 1.5 mg/mL	5.925	4.224 to 7.626	Yes	****
0.5 mg/mL vs. 2 mg/mL	5.584	3.883 to 7.285	Yes	****
0.75 mg/mL vs. 1 mg/mL	0.3050	-1.945 to 2.555	No	ns
0.75 mg/mL vs. 1.5 mg/mL	6.709	4.625 to 8.792	Yes	****
0.75 mg/mL vs. 2 mg/mL	6.367	4.284 to 8.451	Yes	****
1 mg/mL vs. 1.5 mg/mL	6.403	4.502 to 8.305	Yes	****
1 mg/mL vs. 2 mg/mL	6.062	4.161 to 7.964	Yes	****
1.5 mg/mL vs. 2 mg/mL	-0.3412	-2.042 to 1.360	No	ns

CHAPTER 6

Appendix 6-1. Tukey's multiple comparisons for total soluble phenolic content of crude extracts only (GH, SD, leaves, stem and fruit).

Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**
Leaves				
M. balsamina SD vs. *M. balsamina* GH	0.07500	-9.147 to 9.297	No	ns
M. balsamina SD vs. *M. foetida* SD	-13.48	-22.70 to -4.253	Yes	**
M. balsamina SD vs. *M. foetida* GH	-3.875	-13.10 to 5.347	No	ns
M. balsamina GH vs. *M. foetida* SD	-13.55	-22.77 to -4.328	Yes	**
M. balsamina GH vs. *M. foetida* GH	-3.950	-13.17 to 5.272	No	ns
M. foetida SD vs. *M. foetida* GH	9.600	0.3777 to 18.82	Yes	*
Stem				
M. balsamina SD vs. *M. balsamina* GH	5.175	-4.047 to 14.40	No	ns
M. balsamina SD vs. *M. foetida* SD	1.575	-7.647 to 10.80	No	ns
M. balsamina SD vs. *M. foetida* GH	4.300	-4.922 to 13.52	No	ns

Appendix 6-1. Tukey's multiple comparisons for total soluble phenolic content of crude extracts only (GH, SD, leaves, stem and fruit) (*cont.d*).

M. balsamina GH vs. *M. foetida* SD	-3.600	-12.82 to 5.622	No	ns
M. balsamina GH vs. *M. foetida* GH	-0.8750	-10.10 to 8.347	No	ns
M. foetida SD vs. *M. foetida* GH	2.725	-6.497 to 11.95	No	ns
Fruit				
M. balsamina SD vs. *M. balsamina* GH	0.1300	-9.092 to 9.352	No	ns
M. balsamina SD vs. *M. foetida* SD	-6.920	-16.14 to 2.302	No	ns
M. balsamina SD vs. *M. foetida* GH	-8.145	-17.37 to 1.077	No	ns
M. balsamina GH vs. *M. foetida* SD	-7.050	-16.27 to 2.172	No	ns
M. balsamina GH vs. *M. foetida* GH	-8.275	-17.50 to 0.9473	No	ns
M. foetida SD vs. *M. foetida* GH	-1.225	-10.45 to 7.997	No	ns

Appendix 6-2. Tukey's multiple comparisons for crude extracts and SPE fractions of *Momordica* extracts sourced from Swaziland.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Extract				
M. balsamina leaves vs. *M. balsamina* stem	11.00	4.038 to 17.96	Yes	**
M. balsamina leaves vs. *M. foetida* leaves	-32.83	-39.80 to -25.87	Yes	****
M. balsamina leaves vs. *M. foetida* stem	21.67	14.70 to 28.63	Yes	****
M. balsamina stem vs. *M. foetida* leaves	-43.83	-50.80 to -36.87	Yes	****
M. balsamina stem vs. *M. foetida* stem	10.67	3.705 to 17.63	Yes	**
M. foetida leaves vs. *M. foetida* stem	54.50	47.54 to 61.46	Yes	****
F1				
M. balsamina leaves vs. *M. balsamina* stem	-3.042	-10.00 to 3.920	No	ns
M. balsamina leaves vs. *M. foetida* leaves	-7.875	-14.84 to -0.9132	Yes	*
M. balsamina leaves vs. *M. foetida* stem	-0.5417	-7.503 to 6.420	No	ns
M. balsamina stem vs. *M. foetida* leaves	-4.833	-11.80 to 2.128	No	ns
M. balsamina stem vs. *M. foetida* stem	2.500	-4.462 to 9.462	No	ns
M. foetida leaves vs. *M. foetida* stem	7.333	0.3716 to 14.30	Yes	*

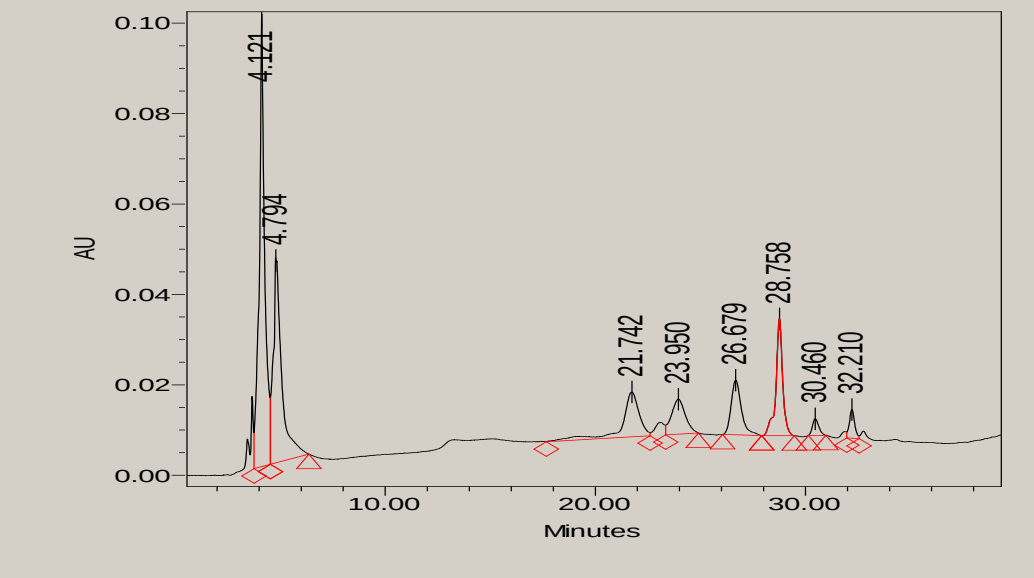
Appendix 6-2. Tukey's multiple comparisons for crude extracts and SPE fractions of *Momordica* extracts sourced from Swaziland (*cont.d*).

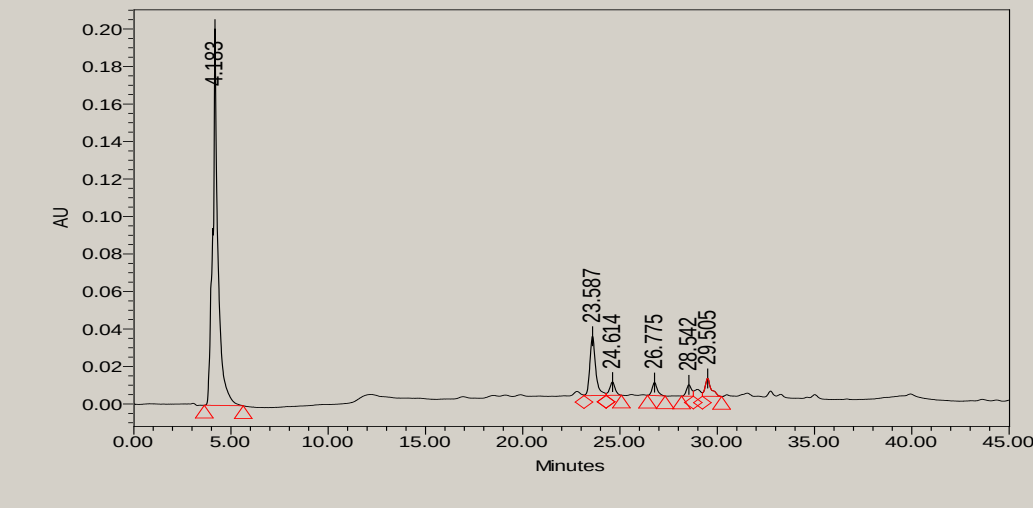
F2				
M. balsamina leaves vs. *M. balsamina* stem	1.375	-5.587 to 8.337	No	ns
M. balsamina leaves vs. *M. foetida* leaves	1.667	-5.295 to 8.628	No	ns
M. balsamina leaves vs. *M. foetida* stem	6.458	-0.5034 to 13.42	No	ns
M. balsamina stem vs. *M. foetida* leaves	0.2917	-6.670 to 7.253	No	ns
M. balsamina stem vs. *M. foetida* stem	5.083	-1.878 to 12.05	No	ns
M. foetida leaves vs. *M. foetida* stem	4.792	-2.170 to 11.75	No	ns
F3				
M. balsamina leaves vs. *M. balsamina* stem	27.38	20.41 to 34.34	Yes	****
M. balsamina leaves vs. *M. foetida* leaves	-14.83	-21.80 to -7.872	Yes	****
M. balsamina leaves vs. *M. foetida* stem	31.33	24.37 to 38.30	Yes	****
M. balsamina stem vs. *M. foetida* leaves	-42.21	-49.17 to -35.25	Yes	****
M. balsamina stem vs. *M. foetida* stem	3.958	-3.003 to 10.92	No	ns
M. foetida leaves vs. *M. foetida* stem	46.17	39.20 to 53.13	Yes	****

Appendix 6-3. Sidak's multiple comparisons test for phenolic content crude and SPE fraction 3.

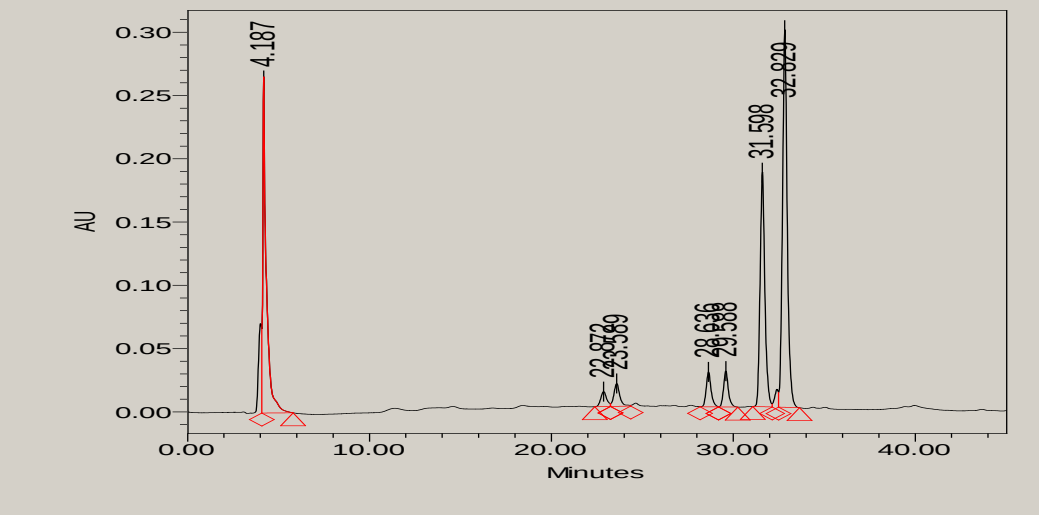
Sidak's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**
Extract – Fraction 3				
M. balsamina leaves	7.125	-1.335 to 15.59	No	ns
M. balsamina stem	23.50	15.04 to 31.96	Yes	****
M. foetida leaves	25.13	16.66 to 33.59	Yes	****
M. foetida stem	16.79	8.331 to 25.25	Yes	***

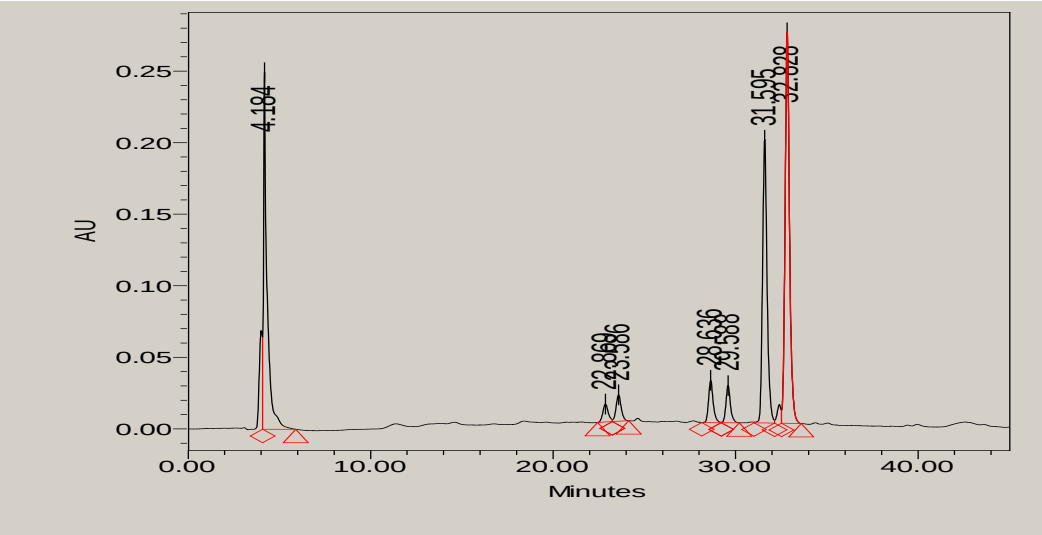
Appendix 6-4. HPLC chromatograms of *M. balsamina* leaf and *M. balsamina* stem extracts.



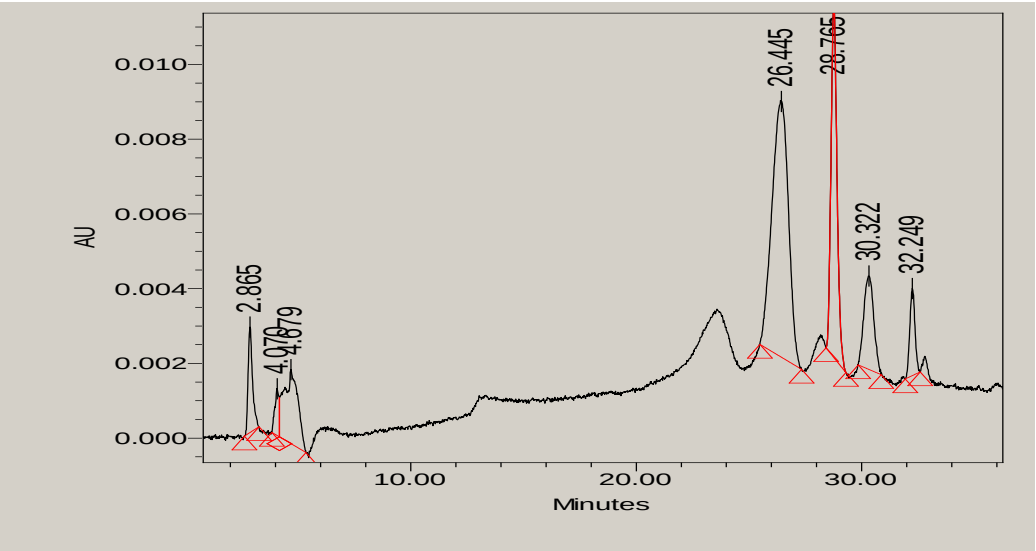


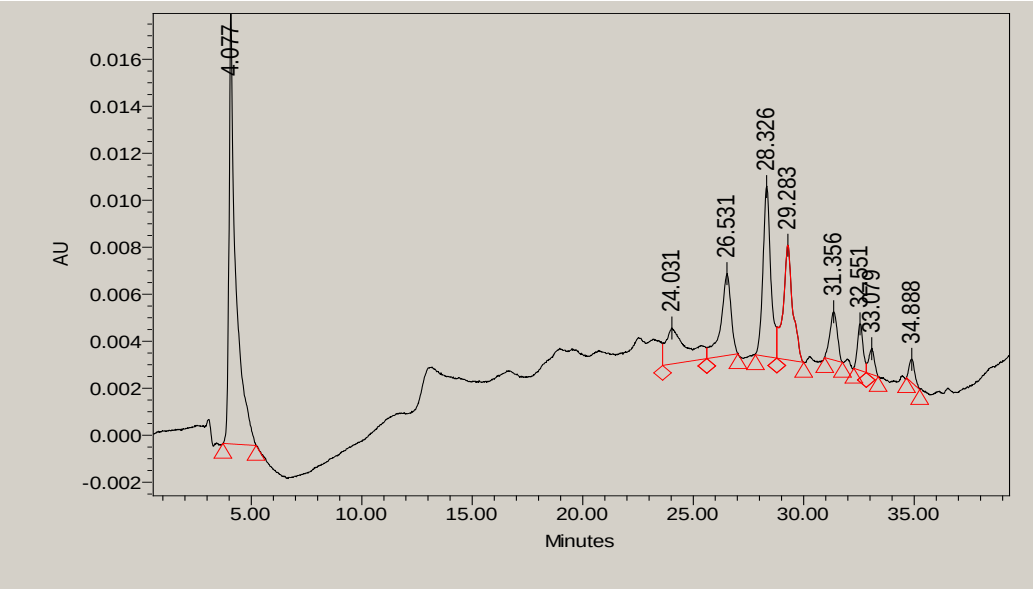
Appendix 6-5. HPLC chromatograms of *M. foetida* leaf and *M. foetida* stem extracts.



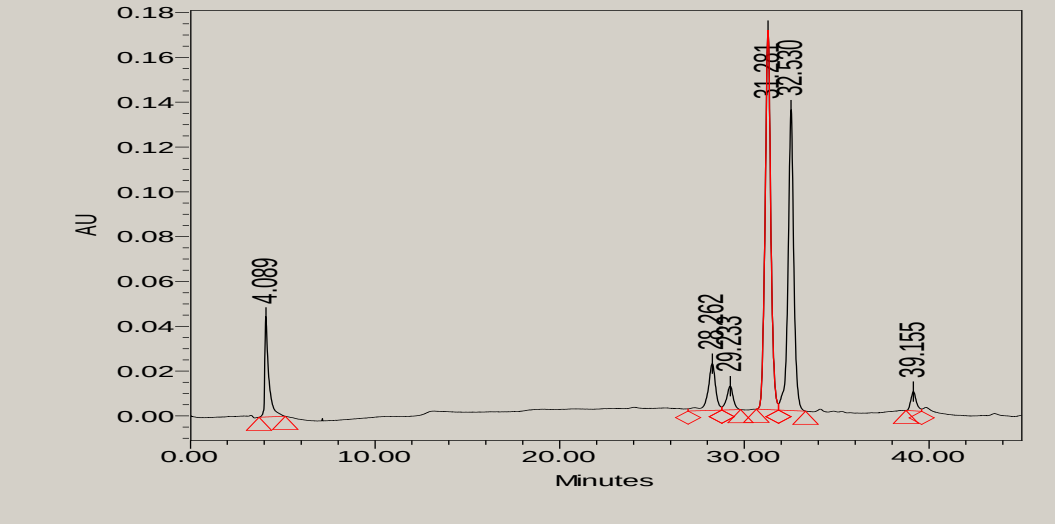


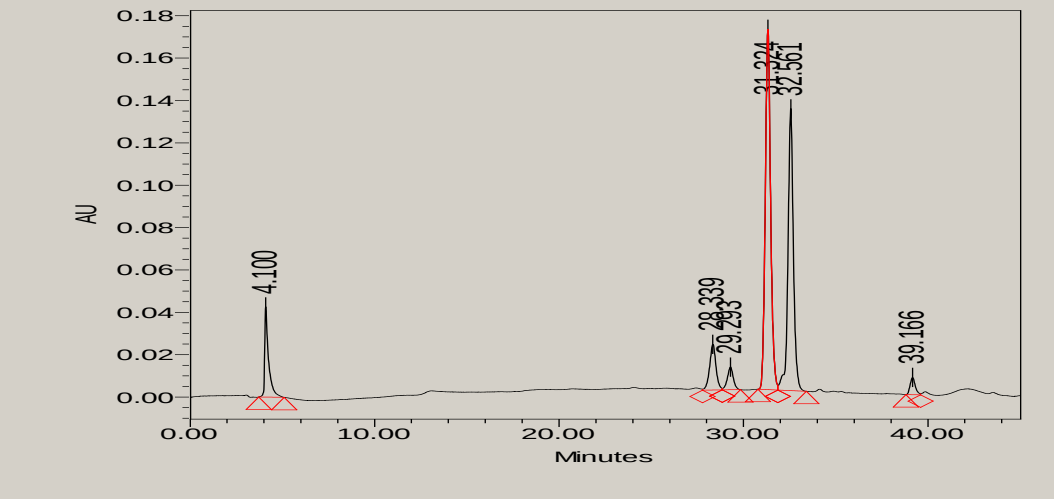
Appendix 6-6. HPLC chromatograms of SPE fraction 3s of *M. balsamina* leaf and *M. balsamina* stem extracts.



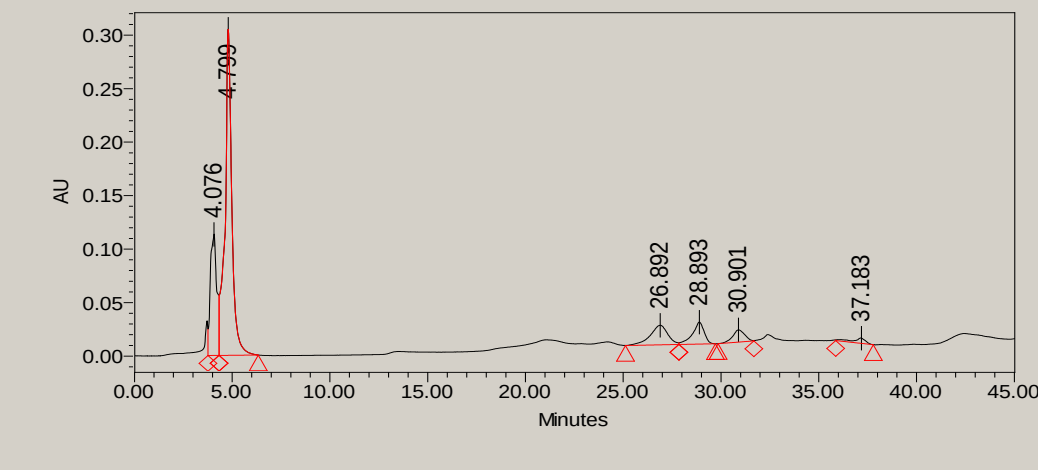


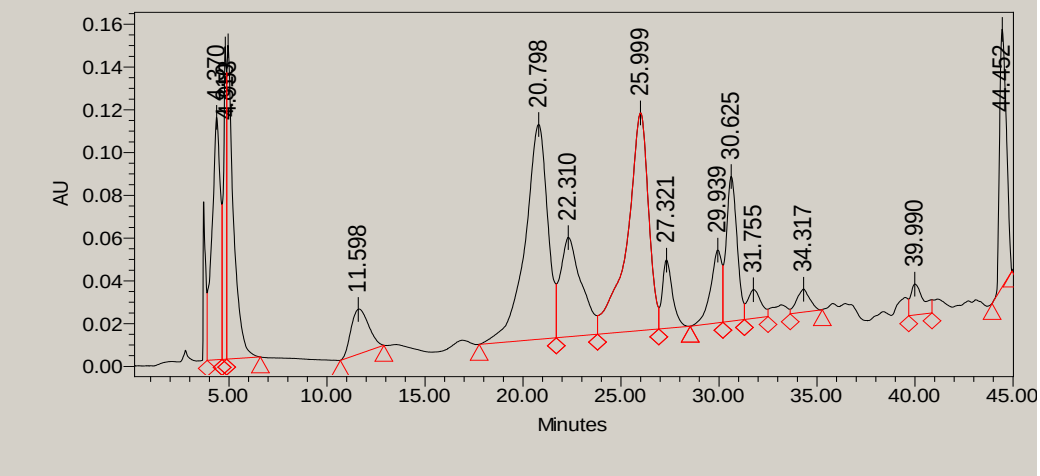
Appendix 6-7. HPLC chromatograms of SPE fraction 3s of *M. foetida* leaf and *M. foetida* stem extracts.



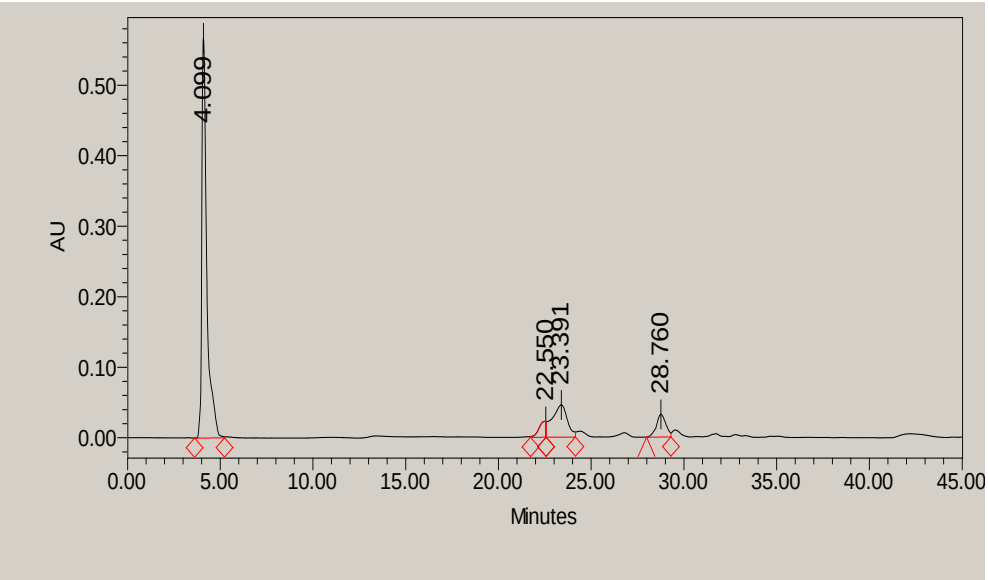


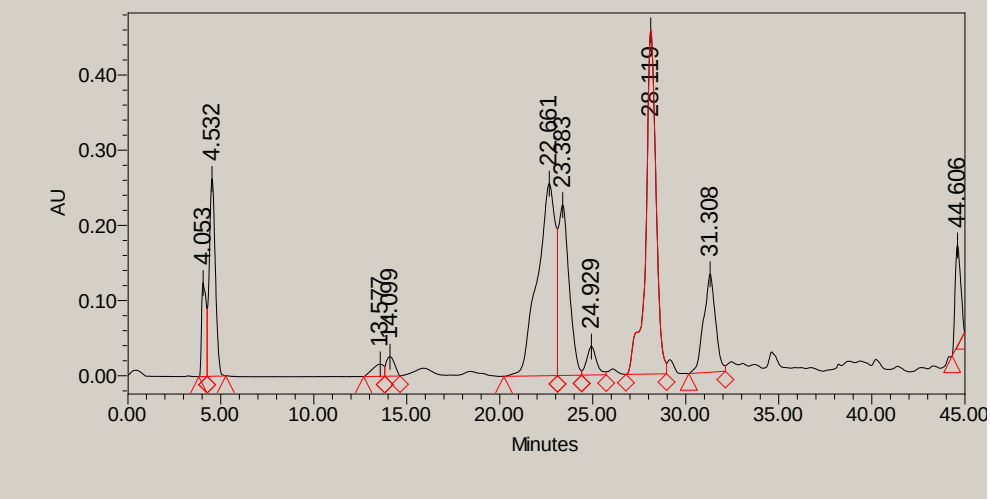
Appendix 6-8. HPLC chromatograms of *M. balsamina* leaf crude and hydrolysed extract.



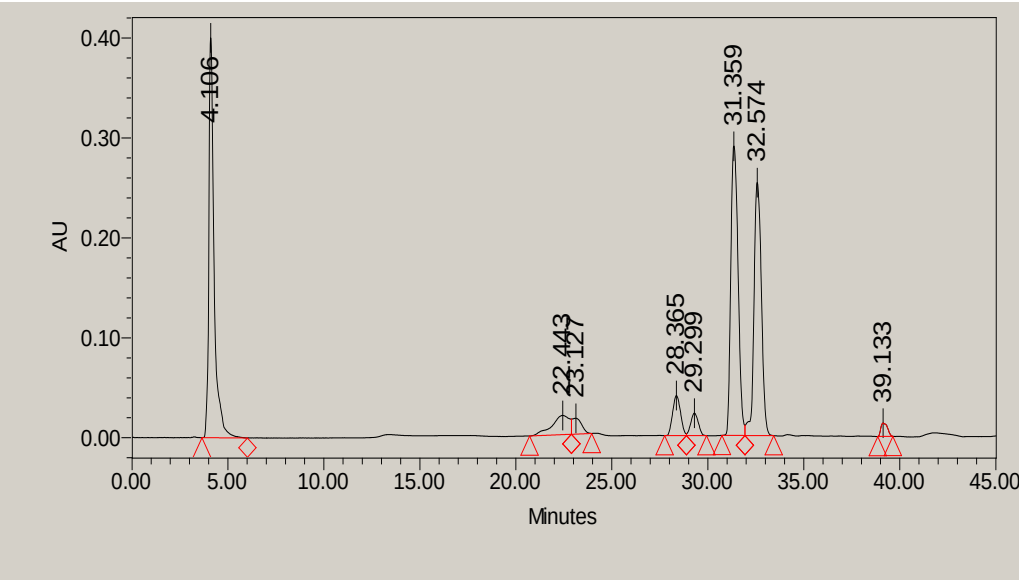


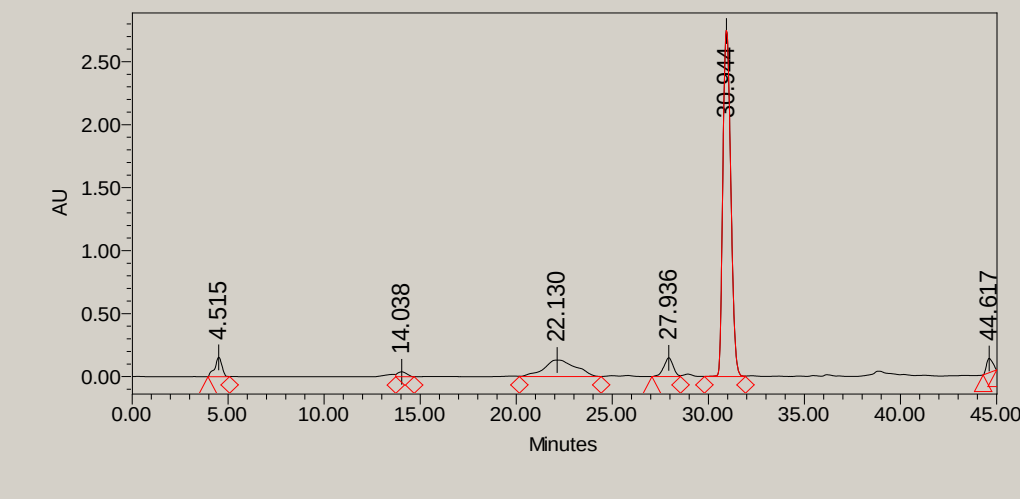
Appendix 6-9. HPLC chromatograms of *M. balsamina* stem crude and hydrolysed extract.



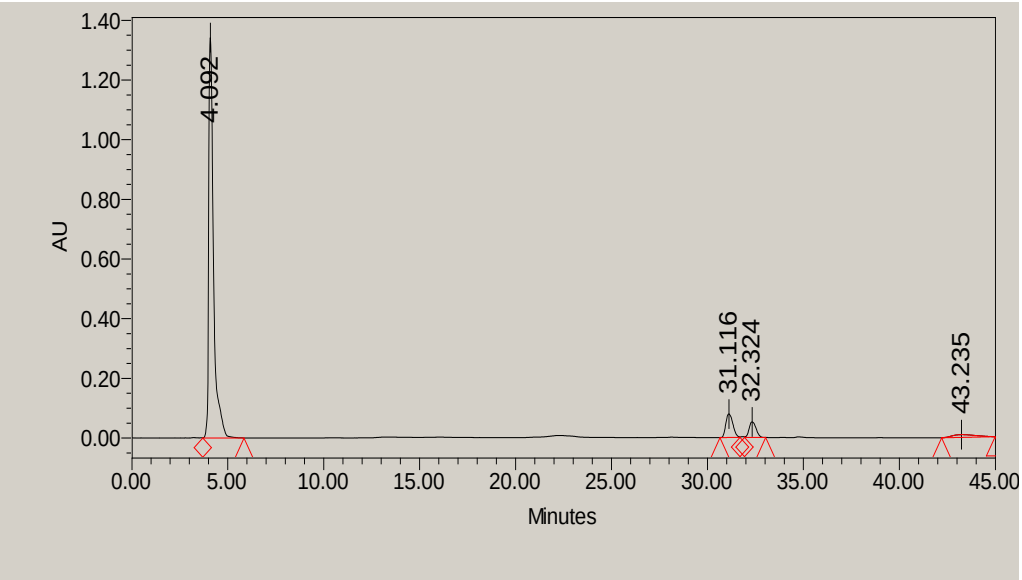


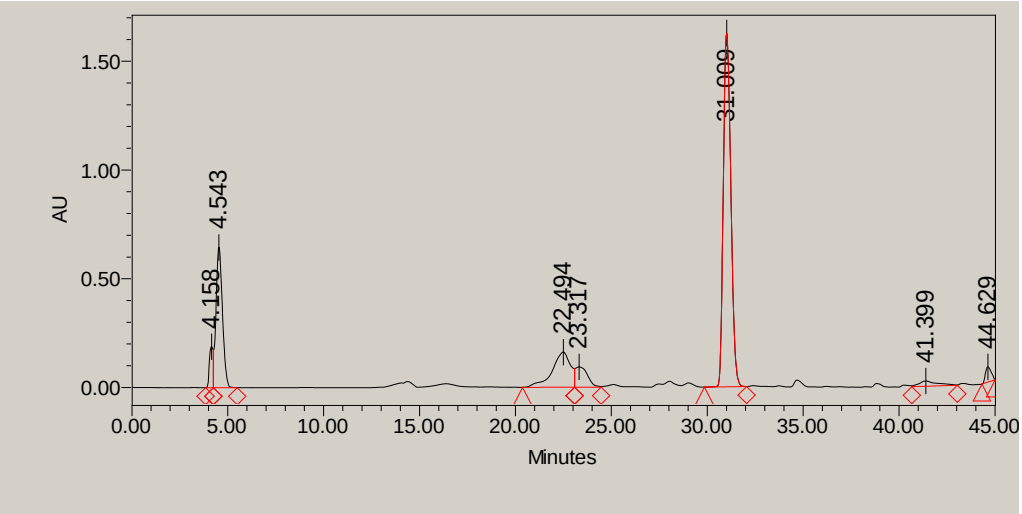
Appendix 6-10. HPLC chromatograms of *M. foetida* leaf crude and hydrolysed extract.





Appendix 6-11. HPLC chromatograms of *M. foetida* stem crude and hydrolysed extract.





Appendix 6-12. Chemical structure of common phenolic and aromatic compounds (Rohloff 2015)

