Phytocompound profiling and assessment of antioxidant, antibacterial, anti-inflammatory, and cytotoxic activities of *Momordica balsamina* leaf extracts

by

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Dissertation

Submitted in fulfilment of the requirements for

the degree of

Master of Science (MSc)

In

Microbiology

in the

Faculty of Sciences, Agriculture, and Engineering

at the

University of Venda

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2021

Declaration

I, Mabasa Xitsakiso declare that this dissertation for the fulfilment of a Master in Science (MSc) degree in Microbiology at the University of Venda is my work. It has not been previously submitted to acquire a degree in this or any other institution and all the references herein were duly acknowledged.

20 April 2021

(Signature of Candidate) **Date**

Dedication

-This dissertation is dedicated to my parents, Mr. HF and Mrs. CH Mabasa together with my late grandmother Katekani Kate Khosa.

Acknowledgements

Firstly, I would like to express my sincerest gratitude to the Department of Biochemistry and Microbiology, the Dean Prof N Potgieter, and the HOD Prof AN Traore for granting me an opportunity to pursue this degree and be a part of their department.

I humbly appreciate my Supervisor Dr. MT Sigidi for playing such a humongous role in this study, her patience, and the knowledge transmitted. Thank you for believing in me, trusting me with this project, and pushing me out of my comfort zone to work hard.

To the Co-Supervisors, Dr. E Musie and Mr. LM Mathomu, your contributions and willingness to assist me in this field of endeavour are highly appreciated. Thank you for your guidance, time, and effort in helping me achieve the goal of this project and also for playing a role in my intellectual growth. ***NDO LIVHUWA NGA MAANDA***.

I would like to humbly express my profound gratefulness to the NRF (National Research Foundation) for their financial assistance. This study wouldn't have been a success without their "helping hand" and I deeply appreciate it.

Appreciation to Prof M Van Der Venter and her team at the Nelson Mandela Metropolitan University, Port Elizabeth in the Eastern Cape of South Africa for assisting in conducting the cytotoxicity and anti-inflammatory assays.

Huge thanks to Dr. NE Madala from the Department of Biochemistry at the University of Venda for benevolently assisting with an assay (UHPLC-qTOF-MS) on profiling of *M. balsamina* extracts, I appreciate your contribution to this study.

Indeed "Iron sharpens iron", to the mentors Mr. M Magwalivha and Ms. MV Masiphephethu, the sacrifices, and inputs you made are highly appreciated.

I would also like to bid thanks to my parents for transportation (collection of samples), their profound support, and their contribution to the success of this study.

Above all, I would like to thank The Lord God Almighty for giving me a spirit to "never throw in the towel" when things do not go as expected, the courage to work hard, and the strength to achieve the goal behind this study.

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List of abbreviations

%	Percentage
°C	Degrees Celsius
cfu/ml	Colony forming units per millimetre
cm	Centimetre
cm^{-1}	Cubic centimetre
Da	Daltons
eV	Electron volt
g	Gram
kV	Kilovolt
m/z	Mass to charge ratio
mg	Milligram
mg/ml	Milligrams per millilitre
min	Minute
ml	Milligram
ml/min	Millimetre per minute
mM	Millimolar
mm	Millimetre
nm	Nanometre
pg/ml	Picograms Per Millilitre
s	Second
µg/mL	Microgram per millilitre
µl	Microlitre
µm	Micrometre
$[K_3Fe(CN)_6]$	Potassium hexacynoferrate
AG	Aminoguanidine
$\mathbf{A_{DPPH}}$	Absorbance of the DPPH

ANOVA	Analysis of variance
A_S	Absorbance of the samples and DPPH
ATCC	America Type Culture Collection
BEA	Benzene/Ethanol/Ammonium hydroxide
CE	Collision energy
CEF	Chloroform/Ethyl acetate/Formic acid
CGA	Chlorogenic acid
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DC	Dendritic cell
DMEM	Dubelco's modified Eagle media
DMSO	Dimethyl sulfoxide
DPPH	2, 2 diphenyl-1-picrylhydrazyl
E. coli	*Escherichia coli*
EC	Effective concentration
E. faecalis	*Enterococcus faecalis*
Esp	Enterococcal Surface Protein
EMW	Ethyl acetate/Methanol/Water
FBS	Foetal bovine serum
$FeCl_3$	Ferric Chloride
FTIR	Fourier transform infrared transmission
HCA	Hydroxyl-cinnamic acid
HPLC	High Performance Liquid Chromatography
IC_{50}	Sample concentration causing 50% inhibition
IgE	Immunoglobulin E
IL-17	Interleukin-17
INT	*p*-iodonitrotetrazolium violet

iNOS	Inhibitor of nitric oxide synthesis
IPNI	International plant name index
IR	Infrared
5-LOX	5-lipoxygenase
LPS	Lipopolysaccharide
M. balsamina	*Momordica balsamina*
MeOH	Methanol
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
MTT	(3-4, 5-dimethly-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NO	Nitric oxide
P. mirabilis	*Proteus mirabilis*
PDA	Photo-diode array
PI	Propidium iodide
rpm	Rotations per minute
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSA	Radical Scavenging Activity
R_f	Retention factor
R_t	Retention time
STAT-6	Signal transducer and activator of transcription 6
TCA	Trichloro-acetic acid
TLC	Thin Layer Chromatography
TPTZ	2, 3, 5-triphenyl-1, 3, 4- triaza-2-azoniacyclopenta-1, 4-diene chloride
UHPLC-qTOF-MS	Ultra High-Performance Liquid Chromatography- quadrupole time-of-flight and mass spectrometry
UV-vis	Ultraviolet and visible spectrophotometry

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Abstract

Title: Phytocompound profiling and assessment of antioxidant, antibacterial, anti-inflammatory, and cytotoxic activities of *Momordica balsamina* leaf extracts.

Background: The use of medicinal herbs has raised considerable interest worldwide attributed to their health-promoting effects. *Momordica balsamina* (*M. balsamina*) is a medicinal herb that has long been used to treat various ailments. Therefore, this study aims to profile the phytocompounds and assess antioxidant, antibacterial, anti-inflammatory, and cytotoxic activities of *M. balsamina* leaf extracts.

Methodology: Methanol and water were used as extraction solvents. Profiling of phytochemical constituents of *M. balsamina* extracts was done using TLC, phytochemical screening tests, UV-Vis, FTIR, and UHPLC-qTOF-MS analysis. Biological activities were assessed using *in vitro* bioactivity screening (cytotoxicity and anti-inflammatory) assays, an antioxidant assay using free radical scavenging (DPPH) activity, and determination of minimum inhibitory concentration using the serial micro broth dilution technique.

Results: Phytochemical screening revealed the presence of cardiac glycosides, flavonoids, saponins, tannins, and terpenoids in both extracts. The UV-VIS profile revealed various absorption bands ranging from 200 – 750 nm indicating the presence of flavonoids, phenolic compounds, tannins, terpenoids, carotenoids, chlorophyll, and alkaloids. FTIR spectra confirmed the presence of alkaloids, flavonoids, terpenes, anthraquinones, and phenolic compounds. The UHPLC-qTOF-MS detected flavonoid aglyclones such as quercetin, isorhamnetin, and kaempferol as well as dicaffeoylquinic, feruloyl isocitric and pseudolaroside A acids in the methanolic extract. Based on our knowledge, this is the first report on the presence of pseudolaroside A and feruloyl isocitric acid in *M. balsamina* leaves. UHPLC-qTOF-MS could not identify the compounds in the water extract. Both extracts had antioxidant potential and exhibited no antibacterial activity on gut-associated bacteria. *In vitro* cytotoxicity results showed that extracts were non-toxic against human colorectal adenocarcinoma (HT29 and Caco2), Vero, and RAW 264.7 cells. Methanolic extract showed anti-inflammatory activity on RAW 264.7 cells and water extract exhibited no activity.

Conclusion: *M. balsamina* leaves contain plethora secondary metabolites with no cytotoxic potential and may be used as antioxidant and anti-inflammatory agents.

Keywords: *Momordica balsamina*, antioxidant, anti-inflammatory, antibacterial, cytotoxicity

Outline of dissertation

This dissertation is divided into six (6) chapters and the outline is as follows:

Chapter 1: General introduction

This chapter gives a background of the study and focuses on the significance, hypothesis, objectives, research questions as well as approaches used to achieve the objectives.

Chapter 2: Literature review

This chapter gives a brief review of the study.

Chapter 3: Profiling of phytocompounds in *M. balsamina* leaf extracts

This includes TLC, phytochemical screening tests, UV-VIS, FTIR, and UHPLC-qTOF-MS.

Chapter 4: Assessment of biological activities

This includes antioxidant, antibacterial, *in vitro* cytotoxicity, and *in vitro* anti-inflammatory activity screening.

Chapter 5: General conclusion and recommendations

This chapter gives a summary of the current study.

Chapter 6: References

CHAPTER 1

General Introduction

1.1 Background and rationale

For centuries, humans have depended on the “nature’s gift” or plants as sources of food and medicine (Vanjala and Kavitha, 2016). Plant-derived extracts have been proven to contain secondary metabolites with therapeutic effects against myriad infections and diseases (Uchegbu *et al*., 2015). *Momordica* species have been reported as such plants and this is associated with the availability of bioactive compounds in this plant (Nagarani *et al*., 2014).

Momordica balsamina is generally known as African cucumber or pumpkin, Balsam apple or pear (Hassan and Umar, 2006). In South African tribes, it is locally called “Nkaka” in Tsonga, “Tshibavhe” in Tshivenda, and “inkaka” in Swati and Zulu (Kutu and Magongwa, 2017). It is an annual perennial herb from the family *Cucurbitaceae* characterized by soft stems as well as tendrils that climb up shrubs and boundary fields (Hassan and Umar, 2006; Thakur *et al*., 2011). It is widely distributed in Botswana, Namibia, Swaziland, and all South African provinces except the Western Cape. It has also been reported as autochthonous to India, Australia, Asia, tropical Africa, and Arabia (Ramalhete *et al*., 2011; Thakur *et al*., 2011; Souda *et al*., 2018).

The leaves, fruits, and seeds of *M. balsamina* contain secondary metabolites of medicinal significance such as anti-HIV (Bot *et al*., 2007), anti-plasmodial (Benoit-Vical *et al.,* 2006), antidiabetic (Siboto *et al.,* 2018; Kgopa *et al.*, 2020), nephroprotective (Abdulfatai and Aduwamai, 2018), anti-diarrheal (Otimenyin *et al*., 2008, Thakur *et al*., 2009), antiviral (Otimenyin *et al*., 2008, Thakur *et al*., 2009), antibacterial (Otimenyin *et al*., 2008, Thakur *et al*., 2009; Shamsuddeen *et al.,* 2010; Adamu *et al*., 2015; Ajji *et al* 2016; Souda *et al*., 2018), anti-inflammatory (Abdulfatai and Aduwamai, 2018) and hepatoprotective (Shamsuddeen *et al.,* 2010).

The dependence on herbal concoctions prepared from plants such as *M. balsamina* has recently increased due to the severity and escalating burden of various

diseases in humans. The continual usage and over-harvesting of these ayurvedic herbs for medicinal purposes bring an urge to scientifically validate the biological effects these extracts might have. Approval and validation of the use of medicinal plants to treat different ailments caused by pathogenic microbes could be of great significance especially for rural dwellers who have a lack of health care infrastructure and inadequate access to crucial, life-saving expensive modern medicine (Nthulane *et al*., 2020).

Amongst the Zulu people, *M. balsamina* concoction is used to treat stomach aches and ulcers (Mshelia *et al*., 2017; Guarniz *et al*., 2019). Nagarani *et al* (2014) highlighted that in most villages *Momordica* paste is used to eliminate intestinal worms in children and prevent irritation in the anus, this is done by applying the paste externally on the anus. According to Shamsuddeen *et al* (2010), *M. balsamina* has the potential to treat gastroenteritis, strongly suggesting that these leaves may be used to treat gut-related infections. Therefore, plant profiling is crucial in order to link the phytocompounds with the bioactivities of the plants.

Recently, the use of sophisticated techniques and scientific methods to profile and validate phytochemical compounds in medicinal plants has become more reliable (Chandra, 2019). Gbashi *et al* (2017) highlighted that the essence is to depict a resemblance of ethnopharmacological exposure of traditional healers who do not have access to metabolite extraction methods that are usually utilized by scientists in the laboratory. A vast majority of techniques are used in determining and estimating the phytocompounds present in different plants (Saxena and Saxena, 2012).

Chromatography and spectroscopy have become more effective and reliable tools used for phytochemical analysis (Patle *et al*., 2020). Fourier Transform Infrared (FTIR) spectroscopy is used to characterize and identify functional groups (Saxena and Saxena, 2012). Ultraviolet-Visible spectrophotometry (UV-VIS) is related to photon spectroscopy in the UV-visible region (Saxena and Saxena, 2012; Johnson and Syed Ali Fathima, 2018). This technique uses light that is in the visible ranges of the electromagnetic spectrum (Saxena and Saxena, 2012; Johnson and Syed Ali Fathima, 2018). The colour of chemicals involved affects the absorption and molecules undergo electron transition in these ranges (Saxena and Saxena, 2012).

In a nutshell, the focus of this study was to profile phytocompounds and assess antioxidant, antibacterial, anti-inflammatory, and cytotoxic activities of *M. balsamina* leaf extracts.

1.2 Purpose of study

1.2.1 Hypothesis of the study

M. balsamina leaves possess several secondary metabolites that exhibit biological activities.

1.2.2 Research questions

1.2.2.1 Which phytocompounds are present in *M. balsamina* leaf extracts?

1.2.2.2 Do *M. balsamina* leaf extracts exhibit any anti-inflammatory, antioxidant, cytotoxic, and antibacterial activities?

1.2.3 Aim and objectives

1.2.3.1 Aim of the study

To profile phytocompounds and assess the antioxidant, antibacterial, anti-inflammatory, and cytotoxic activities of *M. balsamina* leaf extracts.

1.2.3.2 Objectives

Part 1: Phytocompound profiling of *M. balsamina* leaf extracts

To extract compounds from *M. balsamina* and characterize the phytochemical constituents using TLC, phytochemical screening tests, UV-VIS, FTIR, and UHPLC-qTOF-MS analysis.

Part 2: Assessment of biological activities of *M. balsamina* leaf extracts

a) To determine the antioxidant activity of extracts using free radical scavenging assay.
b) To evaluate the antibacterial activity of *M. balsamina* leaf extracts using the Serial micro broth dilution technique.
c) To evaluate the cytotoxicity of *M. balsamina* leaf extracts against human colorectal adenocarcinoma (HT29 and Caco2), Vero, and RAW 264.7 cells and assess cell viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide).
d) To evaluate and assess the anti-inflammatory activity of *M. balsamina* leaf extracts on RAW 264.7 cells using NO (Nitric oxide) production.

CHAPTER 2

Literature review

2.1 Medicinal plants

For centuries medicinal plants have been used to treat and manage several diseases in humans (Nagarani *et al*., 2014; Masoko and Makgapeetja, 2015); the rapid increase in their use can be due to their accessibility, affordability, and cultural beliefs (Sigidi *et al*., 2016; 2017). They serve as rich biological resources of food supplements, drugs for traditional medicinal systems, and pharmaceuticals (Das *et al*., 2010). Hence, medicinal plants have recently received attention from scientific and pharmaceutical communities and many publications are still issued to date to validate the "claims" of their biological activities (Das *et al*., 2010). In South Africa, informal traders are known to sell herbal products that are usually employed as, immune boosters, detoxifiers, blood cleansers, and energy boosters (Matotoka and Masoko, 2018).

Recently there has been a rapid increase in the use of herbal products. In support of this speculation, not only rural dwellers are using them due to lack of health care infrastructure, but also people from urban areas, and this may be attributed to their skepticism on whether western medication can rather treat the mental aspect of ill-health and not only the disease itself (Matotoka and Masoko, 2018). Therefore, this has encouraged manufacturers and traders to make herbal remedies available for those who prefer to use them (Matotoka and Masoko, 2018).

The plant parts that are typically utilized as ingredients to prepare herbal concoctions include stems, leaves, barks, roots, or seeds (Masoko and Makgapeetja, 2015; Masoko and Matotoka, 2018). The severity of the ailment determines the complexity of the formulations (Masoko and Matotoka, 2018). Simple home remedies are employed for minor ailments such as gastrointestinal disorders and coughs, whereas more intricate methods of preparations are required for lethal conditions (Matotoka and Masoko, 2018).

Medicinal plants are used as decoctions that can either be applied topically on wounds or taken orally (Oliveira *et al*., 2016) and some have been reported as "evil spirit cleansers" (Sigidi *et al*., 2017). Unfortunately, the people who use these plants do not have training in the safe use of natural plant products. Therefore, it is of great significance for natural plant products to be standardized and preliminary studies

conducted to evaluate the possible risks such as undesirable side effects, overdose, and toxicity (Ala *et al*., 2018).

Over the past years, the application of drugs derived from natural products has contributed enormously to human health, such as, penicillin in the treatment of bacterial infectious ailments, streptomycin in the long-term management of tuberculosis, cyclosporin which possesses immunomodulatory effects, ecteinascidin-743 which exhibits anti-tumor activity and paeoniflorin a plant-derived anti-inflammatory monoterpene glycoside (Huang *et al*., 2019).

Medicinal plants possess therapeutic properties due to some phytochemical compounds (Johnson and Syed Ali Fathima, 2018). Plants also serve as sources of a plethora of bioactive compounds which exhibit an array of bioactivities against viruses, inflammation, tumours, and bacteria, among others (Huang *et al*., 2019). These phytocompounds have minor toxicity as side effects or fewer side effects (Johnson and Syed Ali Fathima, 2018, Nthulane *et al*., 2020) and can treat diseases without causing harm to human beings hence they are considered “man-friendly medicines” which makes them advantageous as compared to pharmaceutical agents (Banu and Catherine, 2015; Renuka *et al*., 2016).

Phytocompounds are bioactive chemical compounds naturally occurring in plants that serve as the plant’s natural defence system and provide colour, aroma, and flavour. They play a pivotal role in managing human diseases (Johnson and Syed Ali Fathima, 2018) such as diabetes (Mulaudzi *et al*., 2019), cancer (Ramalhete *et al*., 2010), sexually transmitted diseases (Nthulane *et al*., 2020), and many more. Phytochemical compounds are categorized into two paramount groups, firstly primary metabolites which are vital for the growth and development of the plant, these include; lipids, carbohydrates, and proteins (Patle *et al*., 2020). Secondly, secondary metabolites which are crucial in the defence mechanisms against foreign threats to the plant such as insects and pollutants, are seen as a source of novel antibiotics due to their health-promoting advantages such as antioxidant, anti-inflammatory, and antimicrobial properties (Masoko and Makgapeetja, 2015). These secondary metabolites include phenolic compounds, flavonoids, and saponins among others (Patle *et al*., 2020).

2.2.1 Phenolic compounds

The basic structure of phenolic compounds **(Figure 2.1)** such as caffeic acid, ferulic acid, gallic acid, and coumaric acid contain a phenolic ring (C_6H_5OH), a carboxylic acid (-COOH), and hydroxyl groups (-OH) (Baião *et al*., 2017; Patle *et al*., 2020). Chandra (2019) defined phenolic compounds as aromatic benzene ring compounds with single or multiple hydroxyl groups produced by plants as a mechanism of protection from a pathogen attack. Among phenolic compounds, flavonoids are considered as a significant class of biomolecules due to their medicinal property for human beings (Patle *et al*., 2020).

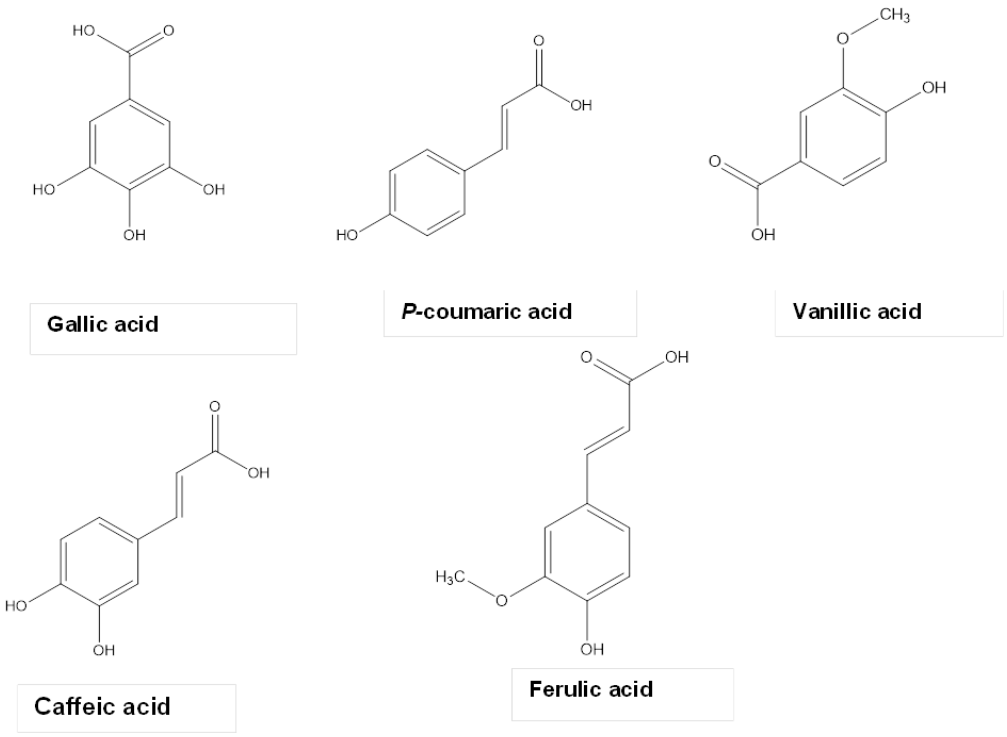


Figure 2.1: Chemical structures of phenolic compounds. Gallic acid and vanillin acid represent the benzoic acid subclass and possess a backbone that consists of 7 carbons (C6-C1). Ferulic acid, caffeic acid and p-coumaric acids belong to the cinammic acid subclass and are characterized by a backbone comprising 9 carbons (C6-C3) (adapted from Baião *et al*., 2017).

Phenolic compounds are secondary metabolites that play a role in maintaining the human body (Meenakshi *et al*., 2011) and have gained attention in traditional and modern medicine as possible sources of new therapeutics due to pharmaceutical properties such as anti-microbial and anti-inflammatory activities (Nthulane *et al*.,

2020). They are significant in herbals because of their ability to disrupt the cell wall of bacteria, interfere with the ATP pool, and alter its membrane potential thus leading to bacterial death (Oliveira *et al*., 2016). Furthermore, their presence indicates a possibility of antioxidant activity, and this activity has been reported to help in the prevention of many diseases through free-radical scavenging activity (Masoko and Eloff, 2007; Biradar *et al*., 2013; Karpagasundari and Kulothungan, 2014; Alara *et al*., 2018; Matotoka and Masoko, 2018). The antioxidant potential of phenolic compounds is in direct proportion to the hydroxyl (-OH) group present in a plant (Patle *et al*., 2020).

2.2.2 Flavonoids

Flavonoids (**Figure 2.2**) are secondary plant metabolites that vary from one another and exist as either aglyclones (Madala *et al*., 2016), unsaturated, or as glycosides which are connected to a sugar molecule and sugars can be linked through a C- or O- glycosylation as either monosaccharides, disaccharides or oligosaccharides (Ferreyra *et al*., 2012; Makita *et al.,* 2016). Their structure is comprised of a pair of benzene rings (A and B) that are attached to a heterocyclic ring (C) that contains an oxygen molecule (Maleki *et al*., 2019).

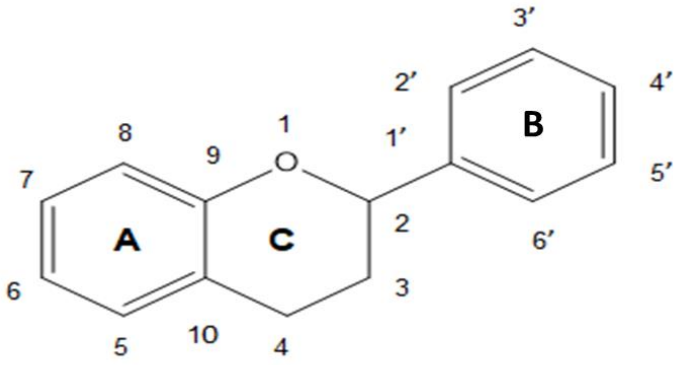
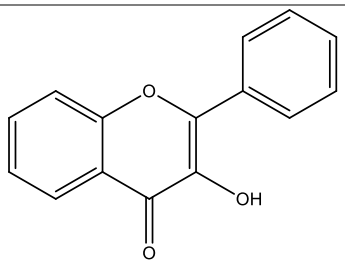
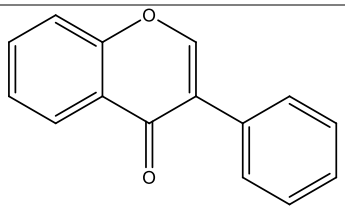
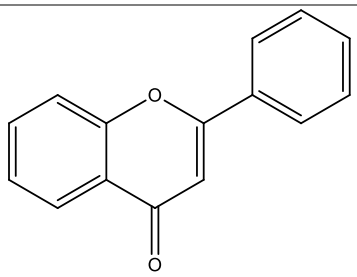
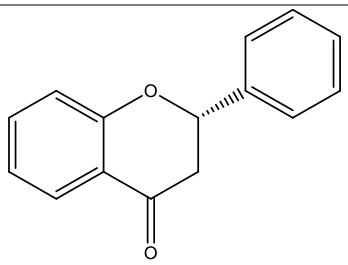
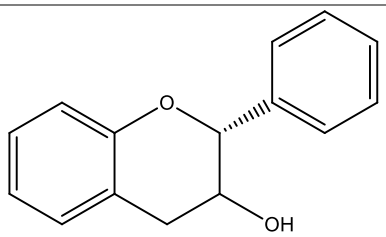
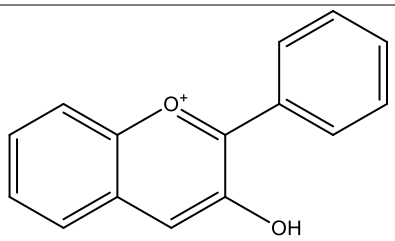


Figure 2.2: Basic structure of flavonoids is comprised of a pair of benzene rings (**A**) and (**B)** that are attached to a heterocyclic ring (**C**) that contains an oxygen molecule (adapted from Maleki *et al*., 2019).

These compounds may be divided into subclasses based on four factors, namely, the attachment between the rings B and C, the structure of the B ring, hydroxylation, and glycosylation patterns of the three rings (Maleki *et al*., 2019). They may be divided into 6 major classes including anthocyanidins, flavanols, flavanones, isoflavanones, flavones, and flavonols (**Table 2.1)** (Kumar and Pandey, 2013).

Table 2.1: Flavonoid subclasses, structures, and types

Subclass	Structure	Type
Flavonol		Galadin Kaempferol Myricetin Quercetin
Flavone		Apigenin Chysin Luteolin

Isoflavone		Daidzein Genistein Glycitein
Flavanone		Eriodicytol Hesperetin Naringenin
Flavanol		Catechin Epicatechin Gallocatechin
Anthocyanodin		Cyanidin

***Adapted from Maleki *et al.* (2019).**

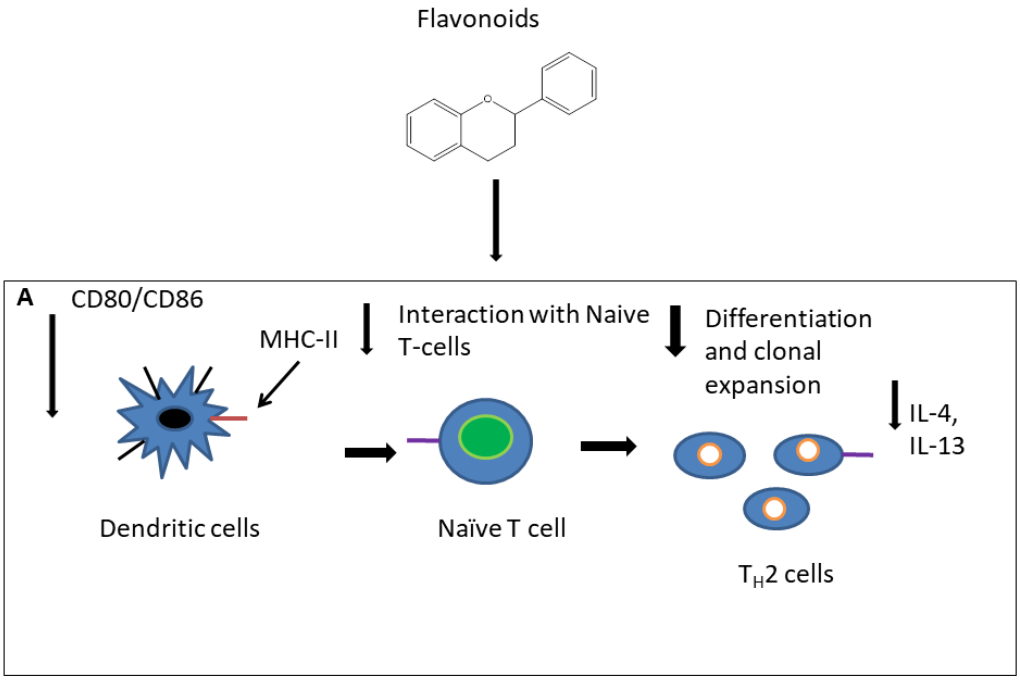
Flavonoids play a crucial role in cell maturation, activation, signalling transduction, and cytokine production in immune cells (**Figure 2.3**). They have been reported as inhibitors of dendritic cell (DC) maturation by suppressing markers such as CD 80 and CD 86 which are crucial in the activation of CD 4+ T cells and are upregulated during the maturation of dendritic cells. This results in the inhibition of cytokine secretion and proliferative response (Maleki *et al*., 2019).

Research has shown that these secondary metabolites can reduce the release of prostaglandin or histamine from mast cells or hinder pro-inflammatory cytokine production in neutrophils and other immune cells (Maleki *et al*., 2019). They cohere to cytokine receptors such as IL-17RA which is a subunit of the IL-17 receptor, resulting in attenuated signalling (Zhu *et al*., 2017). Flavonoids may also hinder downstream

signalling from receptors such as IgE which has high affinity and other receptors at the site of inflammation (Zhu *et al*., 2017).

Flavonoids also modulate protein kinases by inhibiting transcription factors such as NF-κB which regulate adhesion molecules, cytokines, and chemokines involved in inflammatory processes (Maleki *et al*., 2019). These compounds have been reported to regulate the activity of IκB and NF-κB, with a direct impact on cell activation (Chen *et al*., 2018; Maleki *et al*., 2019). During inflammation, NF-κB is inhibited by an inhibitory molecule IκB which is then phosphorylated and degraded.

As a result, translocation of NF-κB from the cytoplasm to the nucleus, where the expression of dissimilar pro-inflammatory genes is induced (Maleki *et al*., 2019). These bioactive compounds can also regulate master regulatory transcription factors for signal transducer and activator of transcription 6 (STAT-6) and CD+T helper 2 (Th2) cytokines such as GATA-3 (Maleki *et al*., 2019).



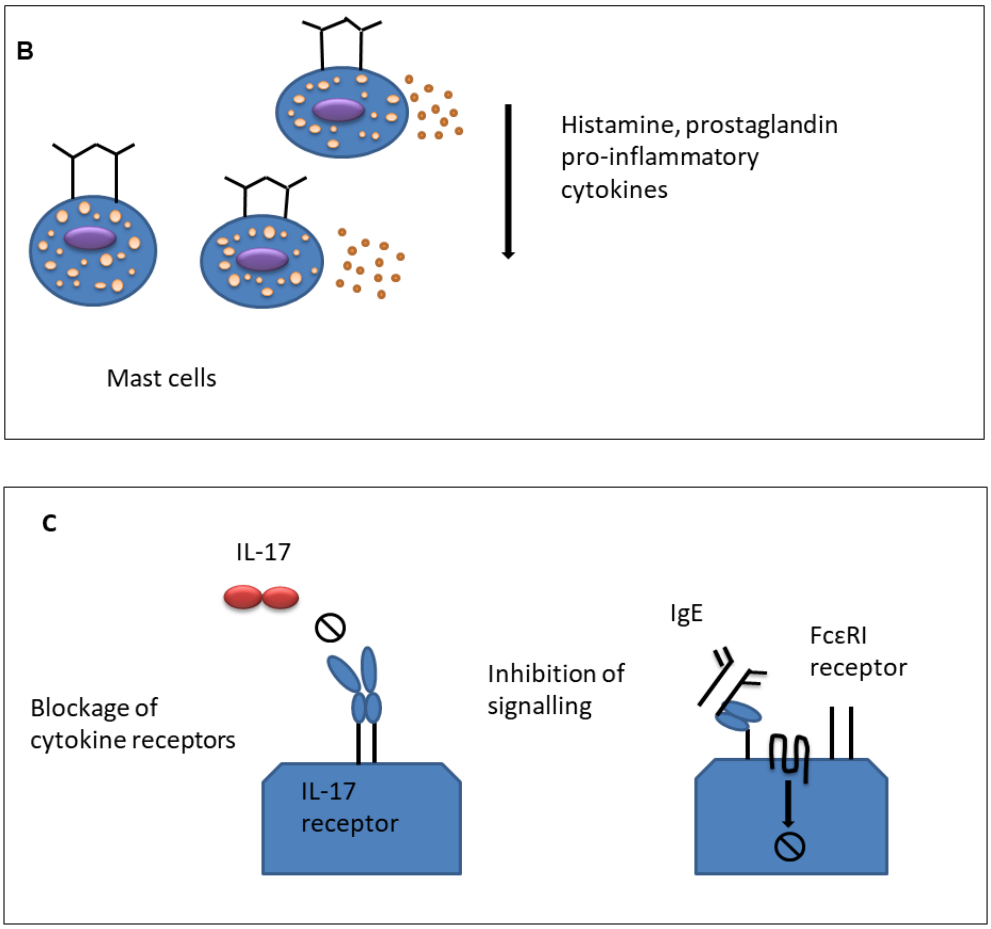


Figure 2.3: Impact of flavonoids in immune cells. Flavonoids can hinder the maturation of dendritic cells (DCs) by suppression of the expression of markers responsible for maturation such as CD80/CD86, thus decreasing the proliferative response of CD4+ T cells (**A**). Flavonoids can reduce the release of histamine, prostaglandin, and cytokines from mast cells (**B**) as well as decrease signalling (**C**) by binding to cytokine receptors or FcεRI (adapted from Maleki *et al* (2019).

2.2.3 Saponins

Saponins are secondary metabolites that are classified as protective molecules found in plants namely, 'phytoprotectants', meaning they exhibit antimicrobial and anti-insect activity or 'phytoanticins', meaning they are activated by plant's enzymes in response to a pathogen attack or tissue damage (Desai *et al*., 2009). There are eleven (11) classes of saponins: dammaranes, lupanes, cucurbitanes, cycloartanes, hopanes, lanostanes, oleananes, tirucallanes, taraxasteranes, steroids, and ursanes, with oleanane as the most common skeleton that exists in the plant kingdom (Kregiel *et al*., 2017).

They are characterized by a sugar molecule typically containing rhamnose, glucose, xylose, glucuronic acid, methyl pentose, or galactose, these are glycosidically attached to a sapogenin which is known as a hydrophobic aglyclone such as a steroid

(**Figure 2.4**) or a triterpenoid (**Figure 2.4**) (Desai *et al*., 2009; Kregiel *et al*., 2017). The ability of saponins to foam is attributed to a non-polar sapogenin and water-soluble side-chain combined (Desai *et al*., 2009).

Saponins with oligosaccharide or carbohydrate groups linked to the C-3 position are monodesmosidic, whereas bidesmosidic saponins are those containing carbohydrates connected to both C-3 and C-26 or C-28 positions (Kregiel *et al.,* 2017). Numerous types of saponins are an outcome of a variety of attachment positions, carbohydrates, and aglyclones. The carbohydrate chains of saponins include D-galactose, D-glucose, D-fructose, D-glucuronic acid, D, xylose, D-apiose, L-rhamnose, and L-arabinose (Kregiel *et al*., 2017).

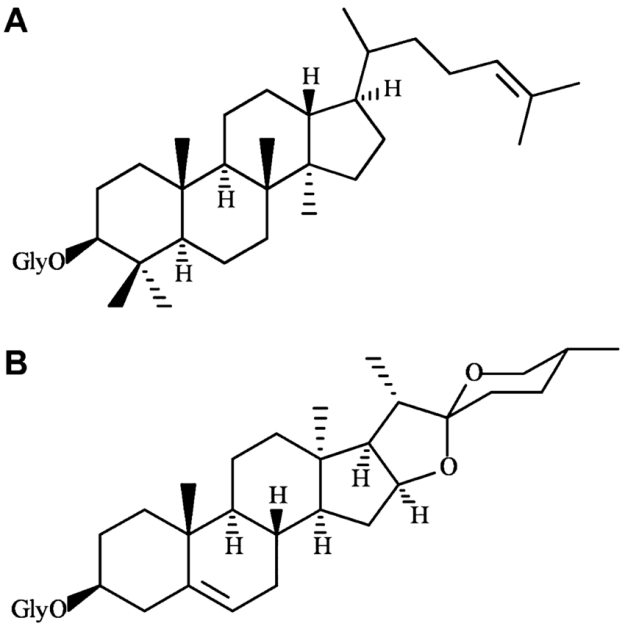


Figure 2.4: Saponins are subdivided into triterpernoid (**A**) and steroidal (**B**) glycosides. Steroidal saponins are comprised of 27 carbon atoms that form the basic structures and usually show spirostanol (16, β22:22α,26-diepoxy-cholestan) or furostanol (16β,22-epoxycholestan) form (Kregiel *et al.,* 2017).

Saponins have been reported to have anti-tumour activity (**Figure 2.5**). The first report regarding the anti-tumour activity of saponins was done by Ebbensen *et al* (1976). Sharma *et al* (2021) highlighted that the first *in vivo* study for examining the anti-tumour activity of triterpenoid saponins was done on a mouse model.

Furthermore, Sharma *et al* (2021) reported that saponins from *Bolbostemma paniculatum* extracts were capable of obstructing the growth of melanoma in mice, Saikosaponin A was reported as a potential inhibitor of ovarian cancer cell proliferation and these results were proven by Yu *et al* (1995).

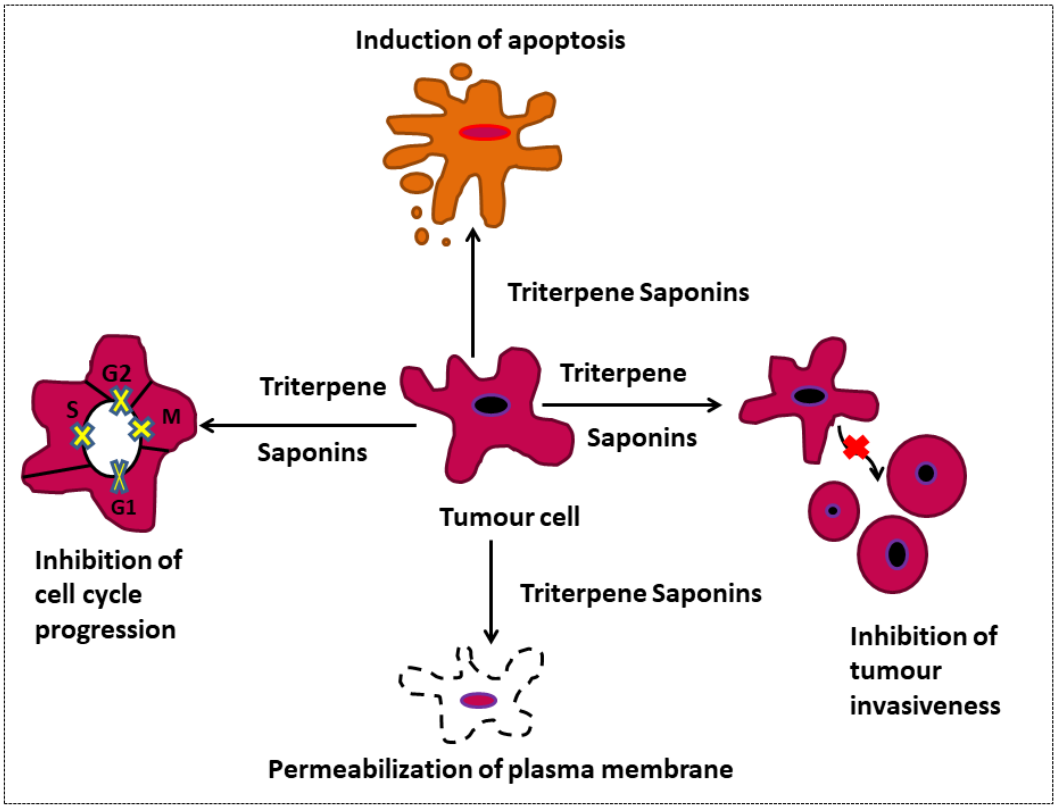


Figure 2.5: Effects of Saponins on tumour cells. These are classified into four categories: apoptotic induction, inhibition of cell cycle progression, inhibition of tumour invasiveness and Permeabilization of the plasma membrane (Figure adapted from Sharma *et al* (2021).

2.3 Biological activities

The biological activity of phytocompounds is assigned to their significance in the ability of the plant to survive. Some compounds are synthesized to protect plants against microbial attacks (Matotoka and Masoko, 2018). Phytochemical compounds possess therapeutic properties and physiological effects such as antimicrobial, anti-inflammatory, and cardioprotective effects (Alara *et al*., 2018).

2.3.1 Antioxidant activity

In this day and age, free radicals are generated by the entry of toxic substances into the body through the consumption of food and water thus resulting in emergence of diseases in the human body (Patle *et al*., 2020). Masoko and Eloff (2007) defined free radicals as natural by-products of human metabolism. These molecules are charged and capable of attacking cells by destroying cellular membranes and interacting with proteins, enzymes, and nucleic acids present in cells (Masoko and Eloff, 2007, Hiransai *et al*., 2016). Research has shown that flavonoids and phenolic compounds can prevent the damage caused by free radicals (Patle *et al*., 2020) ascribed to their capability to quench free radicals, strongly suggesting that these compounds have antioxidant activity (Meenakshim *et al*., 2011; Nagarani *et al*., 2014; Anokwuru *et al*., 2017).

An antioxidant is a molecule that obstructs the oxidation of other molecules. During oxidation, an electron or hydrogen from substances is transferred to an oxidizing agent (Moharram and Youssef, 2014). Oxidation reactions can produce free radicals capable of initiating a chain reaction in a cell thus causing cell damage or death (Moharram and Youssef, 2014). These molecules terminate chain reactions by eradicating free radical intermediates and preventing other oxidative reactions (Moharram and Youssef, 2014). Antioxidants are often reducing agents such as polyphenols and are responsible for defence mechanisms of the organism against an attack of free radical associated pathologies (Moharram and Youssef, 2014).

2.3.2 Anti-inflammatory activity

Inflammation is a crucial and complex host's defensive mechanism that is intended to eliminate the initial cause of cell injury induced by microbial infections (Soonthornsit *et al*., 2017; Maleki *et al*., 2019). Initially, immune cells migrate from blood vessels and mediators such as adhesion molecules, cytokines, and chemokines released at the site of damage (Kumar and Pandey, 2013). Inflammatory cells are then recruited and reactive oxygen species (ROS), reactive nitrogen species (RNS), and pro-inflammatory cytokines are released to eradicate foreign pathogens and thus repair injured tissues (Kumar and Pandey, 2013; Maleki *et al*., 2019). The chronicity

of inflammation is dependent on the production of different proteases, ROS and RNS that lead to tissue damage, cell proliferation, and fibrosis during an inflammatory response (Maleki *et al*., 2019).

2.3.3 Cytotoxicity

In vitro, toxicological studies use broad analyses in determining cell viability and cytotoxicity resulting from exposure to chemical substances. As a result, the establishments from these *in vitro* cytotoxicity assays may be employed in predicting the possible human toxicities (Matotoka and Masoko, 2018). Steenkamp and Gouws (2006) highlighted that various cell lines exhibit dissimilar sensitivities towards plant extracts. For instance, some plants have been reported to have cytotoxic effects on cancer cells, whereas others activate certain parameters on the immune system as a way to destroy cancer (Steenkamp and Gouws, 2006).

2.3.4 Antibacterial activity

The development of novel drugs to combat diseases has not stopped microbes to develop ways to survive (Masoko and Makgapeetja, 2015). However, plants develop new natural antimicrobials than man-made remedies and they remain promising for the discovery of new biologically active compounds (Masoko and Makgapeetja, 2015). The medicinal plant must contain the lowest MIC value in each microbe tested for it to be considered as a good candidate plant with antimicrobial activity (Nthulane *et al*., 2020).

The antibacterial activity of plant substances is associated with the phytochemical compounds that are present in the plant such as steroids, saponins, alkaloids, and many more which play a crucial role in protecting the plant against pathogenic microbes (Bukar *et al*., 2009; Shamsuddeen *et al*., 2010).

2.4 The gut microbiota

The gut microbiota has been reported as an “essential component” that is mainly composed of bacterial species, such as *Lactobacillus acidophilus* and *Escherichia coli* (Duda-Chodak, 2012). The gut flora is involved in essential human

biological processes such as regulating the development of epithelial intestinal mucosa (Wang *et al*., 2017). If not regulated, this may lead to a series of severe illnesses also known as dysbiosis, which is an imbalance in the structure and function of the gut-associated with emergence of diseases such as colorectal cancer, inflammatory bowel disease, and colitis (Jahani-Sherafat *et al*., 2018).

The gut microbiota is responsible for influencing innate immunity by providing a physical protective barrier against foreign pathogens through the release of antimicrobial substances (Wang *et al*., 2017). It is also responsible for metabolizing compounds that are present in the diet such as polyphenols, these influence human health due to free-radical scavenging activity, antimicrobial and antioxidant properties (Duda-Chodak, 2012). Polyphenols that are not absorbed in the stomach reach the colon and undergo hydrolysis in the small intestines; this process involves the release of aglyclones and oligomers by microbial glycosidases and esterases, resulting in an enhanced absorption (Duda-Chodak, 2012). This implies that polyphenolic compounds might have an influence on the gut microbiota.

2.4.1 The effect of diet on the gut microbiota

Research has shown that diet plays a significant role in the function and composition of the gut microbiota (Claesson *et al*., 2012; Yatsunenko *et al*., 2012). In return, the microbial populations extract energy from food; this includes fermentation of complex carbohydrates and the breaking down of proteins to fatty acids and other metabolites (Russell *et al*., 2013). Dietary intake of phenolic acids and flavonoids can modify the balance in the gut microbiota (Zhang *et al*., 2015). Furthermore, derivatives of tea phenolics suppress the growth of pathogenic bacteria such as *Bacteroides*, *Clostridium difficile,* and *Clostridium perfringens* and do not affect commensal anaerobic bacteria such as *Bifidobacterium, Clostridium,* and *Lactobacillus spp.* (Zhang *et al*., 2015).

2.4.2 The effect of antibiotics on the gut microbiota

Generally, the microbiome is stable but external factors can change its composition (Clemente *et al*., 2012). The use of a wide variety of antibiotics is a factor

that may alter the composition of the microbiome. The excessive use of antibiotics may result in the reshaping of the gut bacterial community, therefore enabling pathogens to invade and cause dysbiosis which is an imbalance in the gut microbiota that leads to diseases (Clemente *et al*., 2012).

The emergence of antibiotic-resistant pathogens is associated with the use of antibiotics excessively. Sommer *et al* (2009) hypothesized that the frequent use of antibiotics by human beings increases the risk of antibiotic-resistant genes in the microbiome. Furthermore, it was reported that reduction of the frequent intake of prescribed antibiotics led to the reduction of antibiotic-resistant pathogens in the gut (Clemente *et al.*, 2012).

2.4.3 GUT-associated microorganisms

Predominant colonic microbiota includes *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Peptococcus*, *Ruminococcus,* and *Peptostreptococcus* whereas *Escherichia*, *Enterococcus*, *Enterobacter*, *Lactobacillus*, *Proteus,* and *Klebsiella* are the subdominant group. They play a crucial role in hydrolyzing glycosides, amides, esters, sulfates, glucuronides, and lactones through enzymatic action. These enzymes include: β-glucuronidase, α-rhamnosidase, β-glucosidase, esterases and sulfatase (Hervert-Hernández and Goñi, 2011). Gut microbial enzymes also catalyze decarboxylation, dehydroxylation, isomerization, demethylation, and aromatic ring cleavage (Hervert-Hernández and Goñi, 2011).

2.4.3.1 *Enterococcus faecalis (E. faecalis)*

The human colonization of the gut by enterococci is initiated immediately after birth through gastrointestinal inoculation from maternal diet, sources, and environment (Keogh *et al*., 2018). *Enterococcus fecalis* (**Figure 2.6**) is a commensal gram-positive bacterium which is a member of the gastrointestinal flora (McBride *et al*., 2007). It is present in the lumen of the gastrointestinal tract as well as in the mucus epithelial layer and epithelial cysts of the small intestines (Keogh *et al*., 2018). It has been reported that the majority of enterococcal infections in humans are caused by *Enterococcus*

fecalis, this is associated with virulence factors such as cytolysin toxin, enterococcal surface protein Esp, bile salt hydrolase, aggregation substance, and gelatinase (McBride *et al*., 2007). These factors are known to play a vital role in the survival of the bacterium (McBride *et al*., 2007).

These microorganisms have been reported to produce reactive oxygen and nitrogen species (RONS) resulting in the breaking of DNA, point mutation, and instability of chromosomes. This has therefore demonstrated that this common colonic commensal has the potential to contribute to oncogenic transformation in the colon (Jahani-Sherafat *et al*., 2018).

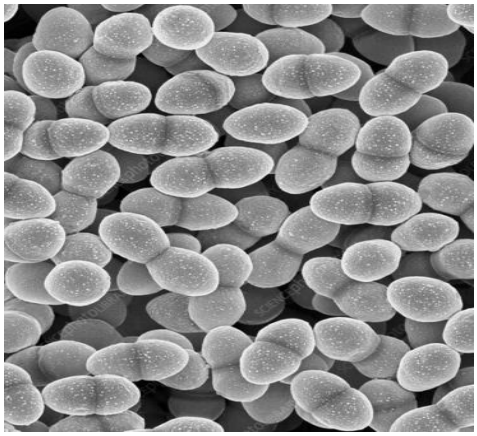


Figure 2.6: *Enterococcus fecalis* viewed under Scanning electron microscope (sciencephoto.com).

2.4.3.2 *Escherichia coli (E. coli)*

Escherichia coli (**Figure 2.7**) are gram-negative bacteria of the gut microbiota (Bonnet *et al*., 2014), classified as the pioneer bacterial species to colonize the intestines during infancy and initially originate from maternal fecal microbiota (Tenaillon *et al*., 2010). Commensal *E. coli* strains are found in the large intestine, mostly in the colon and caecum (Tenaillon *et al.,* 2010). *Escherichia coli* plays a crucial role in preventing pathogenic colonization in the gut microbiota and this is achieved through the production of bacteriocins to induce colonization resistance to the host (Tenaillon *et al*., 2010).

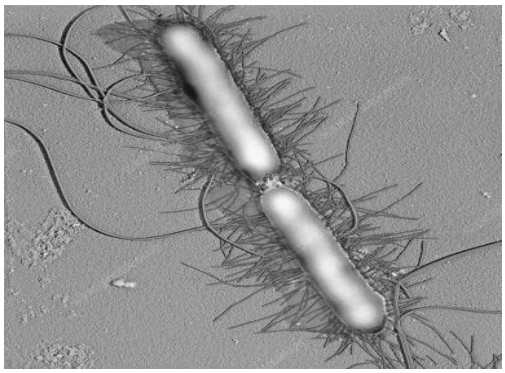


Figure 2.7: *Escherichia coli* viewed under Scanning electron microscope (sciencephoto.com).

2.4.3.3 *Proteus mirabilis (P. mirabilis)*

Proteus mirabilis (**Figure 2.8**) is a gram-negative bacterium (Wasfi *et al*., 2020) that has been reported as a frequent causative agent of human infections particularly in the gastrointestinal tract, respiratory tract, and urinary tract among others. It is an opportunistic pathogen characterized by a unique ability to form crystalline biofilms (organized structures of microbial communities) in the catheter surfaces leading to blockage. This results in reflux and retention of urine as well as painful distension of the bladder (Wasfi *et al*., 2020).

Two virulence factors play a pivotal role in the formation of the crystalline biofilms and these are capsule polysaccharides and urease enzyme (Jacobsen and Shirtliff, 2011). *Proteus mirabilis* clinical strains have been reported to produce the urease enzyme which serves as a catalyst in the hydrolysis of urea in urine into ammonia leading to tissue damage due to the toxicity of the alkaline ammonia manufactured by the enzyme (Wasfi *et al*., 2020).

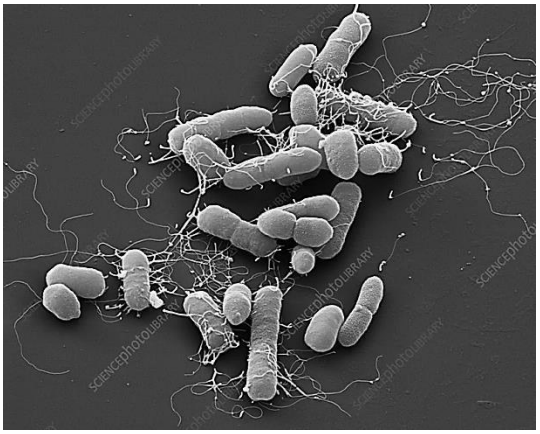


Figure 2.8: *Proteus mirabilis* viewed under Scanning electron microscope (sciencephoto.com).

2.5 *Momordica balsamina*

Momordica balsamina is characterized by a bitter taste attributed to containing myriad phytocompounds such as alkaloids and cucurbitacins (Madala *et al*., 2014; Nagarani *et al*., 2014; Madala *et al* 2016). Other phytochemical studies have reported that this plant may also contain flavonoids, phenols, sterols, and anthraquinones (Thakur *et al*., 2009; 2011). These have been reported to possess a plethora of significant bioactivities such as anti-inflammatory (Nagarani *et al*., 2014), antioxidant (Anokwuru *et al*., 2017; Farag *et al*., 2020), and cardiovascular activity (Madala *et al*., 2016).

The entire plant is used as a bitter gastrocolic and the concoction of the plant is employed in the management of fever as a wash (Uchegbu *et al.,* 2015). The roots and stems are used for the treatment of diarrhea (Otimenyin *et al.,* 2008; Uchegbu *et al.,* 2015). In another study carried out by Thakur *et al* (2009), it was found that *M. balsamina* contains a therapeutic agent called Momordicin which exhibits antiviral and anti-HIV activity*.*

Momordica balsamina leaves (**Figure 2.9**) are used as both food and medicine, they have been proven to be an essential source of nutrients and microelements such as zinc, calcium, and magnesium that act as co-enzymes in numerous metabolic activities (Thakur *et al*., 2009; 2011). These leaves were recommended to be used in cereal-based diets as they are frequently used as dietary requirements in most South

African villages due to their perceived “health benefits” (Hassan and Umar, 2006; Thakur *et al*., 2011). The leaves are also believed to be capable of regenerating lost blood during labour and induce milk production specifically for lactating mothers (Mshelia *et al*., 2017).

According to Sagor *et al* (2015), this species may prevent oxidative stress. Therefore, they may also exert cardioprotective activity (Raish, 2017) making them an excellent source in managing hypertension and other cardiovascular conditions (Thakur *et al*., 2011). Furthermore, in certain regions of South Africa, the leaves are utilized as a remedy for sugar diabetes and chronic hypertension however this has no scientific backing (Madala *et al*., 2016).

Figure 2.9: *Momordica balsamina* leaves during the summer season.

CHAPTER 3

Phytocompound profiling of *Momordica balsamina* leaf extracts

3.1 Introduction

Momordica species have been reported to have a wide spectrum of naturally occurring phytochemical compounds such as phenols and flavonoids with a plethora of health-promoting properties (Kubola and Siriamornpun, 2008; Madala *et al*., 2016; Gbashi *et al*., 2017). Research has proven these bioactive compounds to be liable for antioxidant (Masoko and Eloff, 2007), free radical scavenging (Anokwuru *et al*., 2015; Farag *et al*., 2020), and anti-inflammatory activities (Nagarani *et al*., 2014).

In order for herbal infusions to sustain quality, be reliable, reputable, and marketable, their safety status and effectiveness must meet the quality health standard (Matotoka and Masoko, 2017; 2018). A range of analytical methods can be used in assessing the degree of the chemical purity of phytomedicines (Matotoka and Masoko, 2018). Thin-layer chromatography (TLC) is a powerful and useful tool for analyzing phytochemical profiles in a complex mixture (Matotoka and Masoko, 2018). It is a quick resolution and time-efficient technique towards challenges that involve developing fingerprints for paramount chemical compounds that are present in plant mixtures (Matotoka and Masoko, 2018).

Fourier Transform Infrared (FTIR) spectroscopy is a powerful analytical technique that offers a rapid investigation to fingerprint plant extracts and detect bio-molecular composition (Kalaichelvi and Dhivya, 2017). It is used because myraid biomolecules such as lipids, proteins, and carbohydrates have vibrational fingerprints of molecular bonds that could be analyzed by IR spectroscopy (Kumar *et al*., 2015; Chandra, 2019). This effective tool is also used for chemical characterization of compounds (Patle *et al*., 2020), identification of chemical constituents (Kumar *et al*., 2015), and elucidation of structural compounds (Ashokkumar and Ramaswamy, 2014; Kalaichelvi and Dhivya, 2017; Chandra, 2019).

Analysis of complex media using Ultraviolet-Visible (UV-VIS) spectroscopy is a disadvantage due to limitation by inherent difficulties when it comes to assigning peaks

to any constituents in the system (Njoku *et al*., 2013). Therefore, the UV-VIS findings must be supplemented with other analytical techniques such as GC-MS or LC-MS for appropriate phytocompound profiling and constituent identification. Hence in the present study, extracts were further subjected to hyphenated chromatographic technique (UHPLC-qTOF-MS) to identify the phytochemical constituents present in *M. balsamina*.

Currently, the use of mass spectrometry (MS) in the development of plant metabolomics has made it a possibility to profile multiple compounds such as flavonoids (Madala *et al*., 2014; Rodriguez-Perez *et al*., 2015; Makita *et al*., 2016). Ultra-high-performance liquid chromatography hyphenated to high-resolution quadrupole time-of-flight mass spectrometer (UHPLC-qTOF-MS) was used in this study for profiling *M. balsamina* leaf extracts.

The main goal of this chapter was to profile phytochemical compounds in *M. balsamina* leaf extracts using TLC, phytochemical screening tests, UV-VIS, FTIR, and UHPLC-qTOF-MS.

3.2 Materials and methods

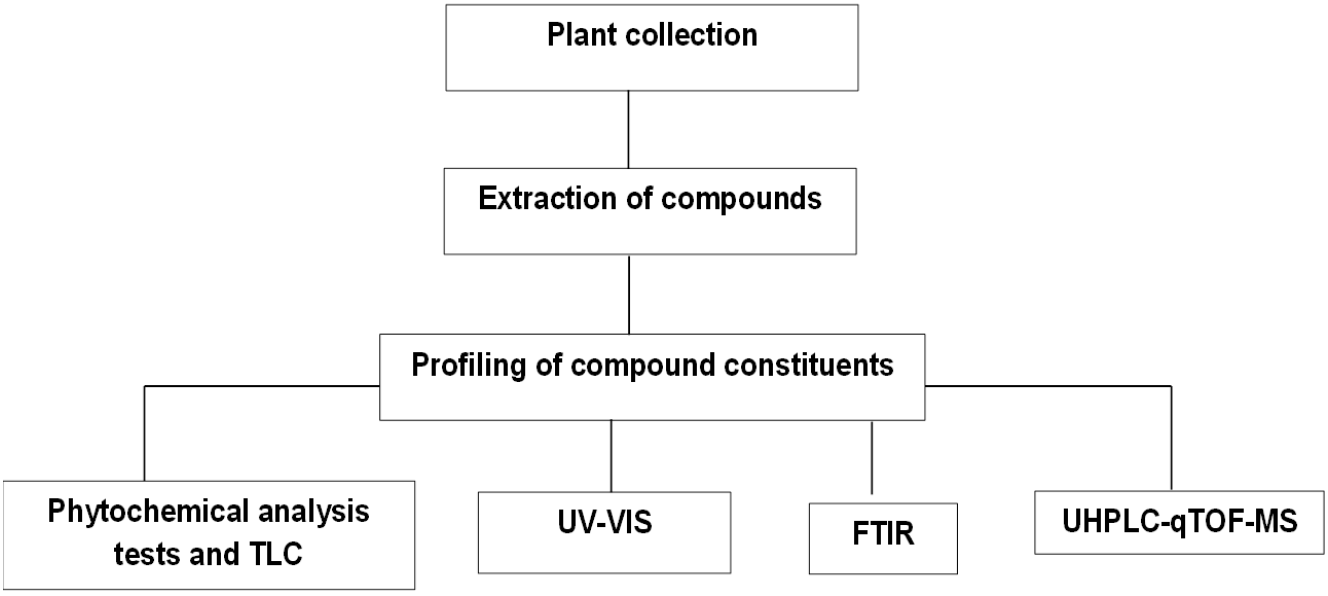


Figure 3.1: Schematic diagram of approaches used to achieve the objective in chapter 3.

The chemicals used in this chapter were obtained from different international suppliers. Briefly, analytical grade quality methanol (Romil, MicroSep, Milford, Massachusetts, USA) was used for extraction. All reagents and solvents were acquired from (Sigma Aldrich®, St Louis, MI, USA). ChemDraw Ultra 12.0.12 software was used to draw the chemical structures in this section.

3.2.1 Plant collection

Fresh *M. balsamina* leaves were gathered and transported in polyethylene bags to the University of Venda, Department of Microbiology laboratory.

3.2.2 Plant identification

The collected leaves were identified using the vernacular name and confirmation was later assessed by Prof Tshisikhawe MP (Department of Botany, University of Venda) using its International Plant Name Index (IPNI).

3.2.3 Plant preparation and metabolite extraction

The leaves were then separated from the twigs and dried at room temperature in a shade and ground into a fine powder using a mechanical grinder (Retsch cutting mill SM 100, Haan, Germany, Europe). The dried powdered sample was then sealed and kept in a dry area till used for further analysis (Jaradat, 2015).

In this study, two solvents were used for extraction namely: water and methanol. Methanol was used because it has been reported as the best extractant since it yields a massive mass of compounds (Lekganyane *et al*., 2012; Zininga *et al*., 2017), and water was used to mimic preparations of herbal portions and food used in traditional techniques (Gbashi *et al*., 2017).

Extraction was conducted according to a method described by Makita *et al* (2016). Briefly, a mass of 2 g of the powdered leaf sample was weighed and 20 ml of 80% methanol was used to extract and sonicated for 1 hour using an ultrasonic cleaning bath (SB-120DT, Loyal Key Group, Hong Kong). Centrifugation followed at 3000 rpm (Thermofisher, Waltham, MA; USA) for 10 minutes at room temperature (25

°C) to collect the supernatant or eradicate the debris from the homogenate. The supernatant was then dried to at least 2 ml of extract using a rotary evaporator under reduced pressure at 55 °C. The extracts were then poured into 2 ml Eppendorf tubes and further dried overnight at constant airflow in a fume hood at 40 °C. Reconstitution of the dried extracts was done in 1 ml of 50% MeOH and 0.22 µm nylon filters were used for filtration. Storage of extracts in a freezer at – 20 °C then followed to avoid degradation until they were used in other assays (Makita *et al*., 2016).

3.3 Phytochemical analysis

TLC, Phytochemical analysis tests, UV-VIS, FTIR, and UHPLC-qTOF-MS were used to profile phytochemical constituents in *M. balsamina* leaf extracts.

3.3.1 Thin-layer chromatography (TLC)

Thin-layer chromatography was done to isolate the compounds present in the extracts of *M. balsamina*; different solvent systems of varying polarities were used to determine which solvent system could reveal better resolution on TLC plates.

A method previously described by Biradar *et al* (2013) was used for thin-layer chromatography. Briefly, leaf extracts were applied on pre-coated aluminium-backed TLC plates using capillary tubes. A volume of 20 µl of each extract (10 mg/ml) was loaded on the TLC plates and development of the plates was conducted in saturated chambers using mobile phases of varying polarities [BEA: benzene/ethanol/ammonium hydroxide (non-polar/basic) (18:2:0.2), CEF: chloroform/ethyl acetate/formic acid (intermediate polarity/acidic) (10:8:2), EMW : ethyl acetate/methanol/water (polar/neutral) (10:5.4:4)] (Kotze and Eloff, 2002; Nemudzivhadi and Masoko, 2015).

The developed plates were then air-dried and visualized under ultraviolet light UV at both 254 nm and 366 nm. The plates were then later sprayed with vanillin and placed in an oven under 110 °C for a minute for the development of colour in separated bands (Biradar *et al*., 2013; Nemudzivhadi and Masoko, 2015). The movement of the compounds was analyzed, and expression was achieved by their retention factors (R_f). Values were calculated using the formulae below:

$$\text{Retention factor} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

3.3.2 Phytochemical screening tests

3.3.2.1 Test for tannins

The presence of tannins was tested by weighing 0.5 g of powdered sample in 5 ml of distilled water and boiling in a test tube; the mixture was then allowed to cool and filtered. Three drops of 0.1% w/v ferric chloride were added to 1 ml of the filtrate in a test tube and the formation of a blue-black or brownish-green colour was observed (Nemudzivhadi and Masoko, 2015).

3.3.2.2 Test for saponins

Saponins were tested by a persistent froth test as described by Nemudzivhadi and Masoko (2015). Briefly; 1 g of the leaf powder was weighed and 30 ml of tap water was added. This mixture was then strenuously shaken and heated at 100 °C, the formation of persistent froth was observed.

3.3.2.3 Test for steroids

Steroids were tested as described by Borokini and Omotayo (2012). This was achieved by adding 2 ml of acetic anhydride to 0.5 g of plant extracts, following that was the addition of 2 ml of sulphuric acid into the mixture. The appearance of a blue or green colour change was observed.

3.3.2.4 Test for Terpenoids

The Salkowski test was employed to check for the presence of terpenoids. Briefly; 0.5 g of extract was weighed and dissolved into 2 ml of chloroform and then, 3 ml concentrated sulphuric acid was cautiously added for a layer to form. The appearance of a reddish-brown colour of the interface was observed (Nemudzivhadi and Masoko, 2015).

3.3.2.5 Test for cardiac glycosides

The Keller-Killiani test was used to detect the presence of cardiac glycosides as highlighted in a study done by Borokini and Omotayo (2012). Briefly; 0.5 g of extracts were weighed and diluted in 5 ml of distilled water. A mixture of 2 ml glacial acetic acid and 0.1 % of ferric chloride was added into a diluted solution. This was followed by the addition of 1 ml concentrated sulphuric acid and the formation of a brown ring at the interface which serves as an indicator of a deoxysugar was observed.

3.3.2.6 Test for flavonoids

Flavonoids were tested as described by Borokini and Omotayo (2012). Briefly; 5 ml of diluted ammonia was added into aqueous extracts, 1 ml of concentrated sulphuric acid was then added into this mixture, and the formation of a yellow colour that disappears on standing was observed.

3.3.3 Ultraviolet and visible spectroscopy (UV-VIS)

The extracts were centrifuged at 3000 rpm for 10 minutes to collect supernatant or remove the debris from the homogenate (Makita *et al*., 2016). The supernatant liquid was then diluted to 1:10 with the same solvent. Dilutions were done in 2 ml Eppendorf tubes and extracts were then transferred into 96-well plates. The extracts were scanned in wavelengths ranging from 200-800 nm using a spectrophotometer (PerkinElmer, Waltham, Massachusetts, USA). The distinctive peaks of the UV-VIS were detected and their values were recorded. The table (**Table 3.1**) shows the wavelength ranges that are characteristic of specific secondary metabolites.

Table 3.1: Wavelength ranges representing specific secondary metabolites

Absorption maxima (wavelength ranges)	Phytochemical compounds (metabolites)	References
234 – 676 nm	Flavonoids, alkaloids, phenolic compounds	Karpagasundari and Kulothungan (2014) Patle *et al* (2020)
230 – 285 nm (band I)	Flavonoids and their derivatives	Kalaichelvi and Dhivya (2017)
230 – 290 nm (band I)	Flavonoids	Saxena and Saxena (2012) Renuka *et al* (2016) Johnson and Syed Ali Fathima (2018)
300 – 350 nm (band II)	Flavonoids and their derivatives	Saxena and Saxena (2012) Renuka *et al* (2016) Kalaichelvi and Dhivya (2017)
350 – 500 nm	Tannins	Patle *et al* (2020)
400 – 450 nm	Carotenoids	Patle *et al* (2020)
400 – 550 nm	Terpenoids	Saxena and Saxena (2012) Renuka *et al* (2016) Johnson and Syed Ali Fathima (2018)
600 – 700 nm	Chlorophyll	Saxena and Saxena (2012) Renuka *et al* (2016) Johnson and Syed Ali Fathima (2018)

3.3.4 Fourier Transform Infrared (FTIR) analysis

The extracts (2 g) were re-suspended in 200 µl of the same solvent; this was done in 2 ml Eppendorf tubes. A vortex (Thermofisher, Waltham, MA; USA) was used to allow extracts to solubilize and tubes were placed on a shaker for about an hour to allow further solubilization. The extracts were then analyzed using ATR-FTIR (Model/Make:IFS 25; Bruker, Germany, Europe). To obtain IR spectra, extracts were analyzed using KBr standard procedure in the scanning wave number ranging from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Interpretation of IR spectra obtained from extracts was achieved by comparing spectral data with references from the identification of functional groups existing in the leaf sample (Ashokkumar and Ramaswamy, 2014; Kumar *et al*., 2015; Alara *et al*., 2018).

3.3.5 Ultra-High-Performance Liquid Chromatography and Mass Spectroscopy (UHPLC-MS) analysis for phytochemical analysis

Ultra-High-Performance Liquid Chromatography and Mass Spectroscopy were employed for further profiling of phytoconstituents of *M. balsamina*. LC-QTOF-MS, model LC-MS 9030 instrument utilizing Shim Pack Velox C18 column (100 mm x 2.1 mm with a particle size of 2.7 µm) (Shimadzu, Kyoto, Japan) was used to analyze 1µl of extracts and placed in a column oven set at a temperature of 40 °C. A binary solvent system composed of solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile was utilized at a flow rate of 0.4 mL/min. Analytes were chromatographically separated through a 53-minute long gradient method composed of these steps: initially, 10% B for 3 minutes, following this was a step gradient to 60% B above 37 minutes and detained at 60% B for 3 minutes, following this was another gradient to 90% B for 2 minutes, an isocratic detain at 90% for 3 minutes and finally the initial conditions (10% B) were re-established conditions in 2 minutes and the column was re-equilibrated for a next run at 10% B for 3 minutes.

Mass spectrometry Detection Parameters

MS detection parameters were set in the following manner: Negative electrospray ionization (ESI) modes; an interface voltage of 3.5 kV; nebulizer gas flow at 3 L/min; heating gas flow at 10 L/min; the temperature of heat block at 400 °C; CDL temperature at 250 °C; voltage of detector at 1.70 kV and temperature of TOF tube at 42 °C. Acquisition of high accurate mass with a mass error below 1ppm was ensured by using sodium iodide (NaI) as a mass calibration. For both high-resolution MS and tandem MS (MS/MS) experiments, an *m/z* ranging from 100 – 1000 was employed. For MS/MS experiments, argon gas was utilized as collision gas, and to generate possible fragments, MS^E mode utilizing a collision energy ramp of 15 to 25 eV was required.

3.4 Results and discussion

3.4.1 Profiling of phytoconstituents of *Momordica balsamina* leaf extracts

3.4.1.1 Thin layer chromatography

TLC was conducted for the separation of compounds based on polarity and for establishing the phytochemical fingerprint profile of *M. balsamina*. A total of three solvent systems of different polarities namely BEA (non-polar), CEF (intermediate polar), and EMW (polar) were used and the results are presented in **Figure 3.2**.

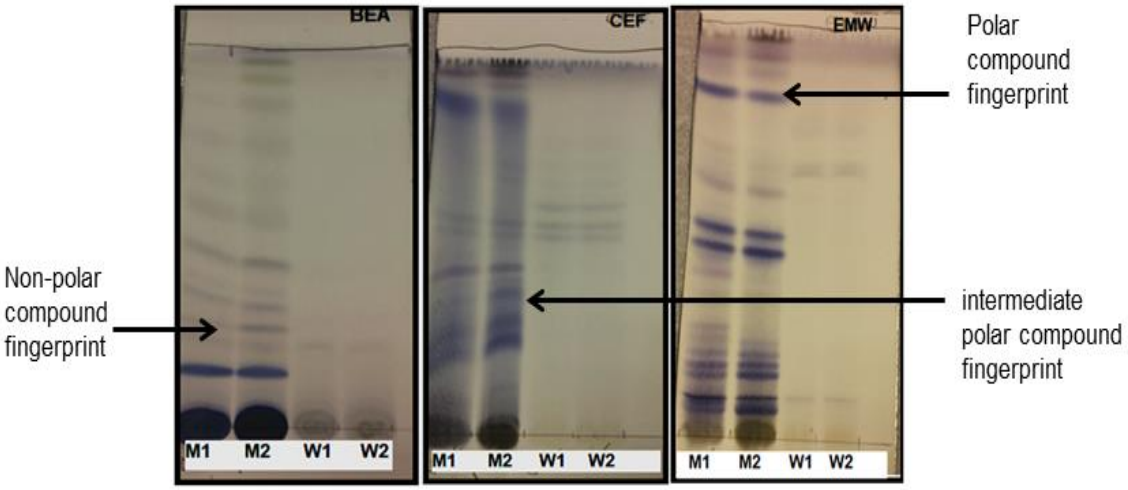


Figure 3.2: Chromatograms of *M. balsamina* extracts developed in 3 solvent systems based on polarity namely: BEA (non-polar: 18:2:0.2), CEF (intermediately polar: 10:8:2), EMW (polar: 10:5, 4:4) and sprayed with vanillin sulphuric acid to reveal phytocompounds present. The compounds were extracted with methanol (M1 and M2) and water (W1and W2) in lanes from left to right.

Based on visual inspection of the TLC plates (**Figure 3.2**), it is evident that more polar compounds were extracted. Furthermore methanol extracted more compounds as compared to water. Other studies revealed that methanol was the best extractant yielding to a greater quantity of compounds extracted (Masoko and Makgapeetja, 2015; Nemudzivhadi and Masoko, 2015) and this is in support of the current study.

The three solvent systems differed in separating the compounds with varying polarities. Non-polar compounds are best separated by the BEA solvent system, the CEF mobile system separates acidic and intermediate compounds and EMW

separates polar and neutral compounds best as described by Masoko and Eloff (2007).

Traditional healers use water to prepare decoctions due to availability and it is less harmful but a disadvantage when using water as an extractant is that water cannot extract non-polar bioactive compounds. The success of isolating compounds is determined by the extraction solvent. Thus, it is of great significance to extract with dissimilar solvents of varying polarity to cover the polarity range (Masoko and Makgapeetja, 2015).

TLC profile of methanolic extracts of *M. balsamina*, indicated the presence of 5 compounds with R_f values (**Table 3.2**) ranging from 0.14, 0.24, 0.40, 0.91, 0.95 respectively when BEA was used as a solvent system. When CEF was used, TLC revealed a total of 3 compounds having R_f values of 0.24, 0.41, 0.90 respectively. TLC also indicated the presence of 7 compounds having R_f values of 0.06, 0.10, 0.15, 0.18, 0.48, 0.50, 0.88 respectively when EMW was used.

Table 3.2: Phytochemical analysis of water and methanolic extracts of *M. balsamina* by TLC.

Solvent system	**Rf values**	
	Methanolic extract	**Water extract**
BEA	0.14	0.15
	0.24	
	0.40	
	0.91	
	0.95	
CEF	0.24	0.41
	0.41	0.60
	0.90	0.72
EMW	0.06	0.15
	0.10	0.48
	0.15	
	0.18	
	0.48	
	0.50	
	0.88	

BEA: benzene/ethanol/ammonium hydroxide; **CEF:** chloroform/ethyl acetate/formic acid; **EMW:** ethyl acetate/methanol/water

TLC of the water extract of *M. balsamina* revealed the presence of 1 compound having R_f values 0.15 when BEA was employed as a solvent system. When CEF was

used, TLC revealed a total of 3 compounds having R_f values (**Table 3.2**) of 0.41, 0.60, 0.72 respectively. TLC also depicted the presence of 2 compounds having R_f values of 0.15, 0.48 respectively when EMW was used.

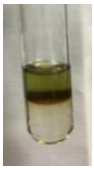
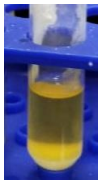
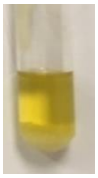
Thin layer chromatographic profiling provides impressive outcomes that direct the presence and number of phytochemicals present in extracts. In varying solvent systems, different phytochemical compounds provide different R_f values. This variation in R_f values serves as an important clue in comprehending the polarity of the phytochemicals (Biradar *et al*., 2013).

3.4.1.2 Phytochemical screening tests

The presence of phytocompounds in plants is beneficial to the host due to the improvement of health (Matotoka and Masoko, 2018). Phytochemical screening results revealed the presence of terpenoids, saponins, flavonoids, cardiac glycosides, tannins, except for steroids in both water and methanolic extracts of *M. balsamina* (**Table 3.3**).

Table 3.3: Phytochemical screening tests of water and methanolic extracts of *M. balsamina.*

Phytochemicals	Methanolic extract	Water extract

1. Terpenoids	 **Positive**	 **Positive**
2. Saponins	 **Positive**	 **Positive**
3. Flavonoids	 **Positive**	 **Positive**
4. Cardiac glycosides	 **Positive**	 **Positive**
5. Tannins	 **Positive**	 **Positive**
6. Steroids	 **Negative**	 **Negative**

Based on visual inspection of the extracts (**table 3.3: 1**), a reddish-brown color was observed. This is as a characteristic of terpenoids (Nemudzivhadi and Masoko, 2015) Terpenoids have been reported useful in treating various ailments such as cancer and have also been applied in the therapy of microbial infections (Watal *et al*., 2014; Shrestha *et al*., 2015; Matotoka and Masoko, 2018). They are also known to possess several bioactivities such as anti-inflammatory (Nthulane *et al*., 2020),

immunomodulatory and antimicrobial activities (Watal *et al*., 2014; Shrestha *et al*., 2015).

Formation of persistent froth was observed in the current study (**table 3.3: 2**) indicating the presence of saponins as described by Nemudzivhadi and Masoko (2015). Saponins are known to possess a significant ability to precipitate and coagulate red blood cells (Watal *et al*., 2014), possess anti-tumor (Vanjala and Kavitha, 2016) and anti-inflammatory activity (Matotoka and Masoko, 2018) by inhibiting COX-2 and 5-LOX enzymes (Nthulane *et al*., 2020). They have been reported as inhibitors of inflammation mediators such as histamine, prostaglandin, and serotonin (Desai *et al*., 2009). The antioxidant property of saponins is attributed to the inhibition of ROS (Reactive Oxygen Species) formation which also plays a major role in inflammation (Desai *et al*., 2009). These secondary metabolites have also been reported to exhibit antimicrobial activity (Matotoka and Masoko, 2018) by inhibiting the growth of Gram-positive and negative microbes. However, some saponins are less effective against gram-negative microbes because of the failure to penetrate the cell membrane of these microbes (Desai *et al*., 2009).

The results (**table 3.3: 3**) in current study also revealed the formation of a yellow colour that vanishes on standing. This is a characteristic of flavonoids (Borokini and Omotayo, 2012). Flavonoids have been reported to contain several therapeutic properties (Madala *et al*., 2016) such as the ability to scavenge free radicals thereby preventing cell damage due to induced oxidative stress. They have also been reported to possess robust anticancer activity (Masoko and Eloff 2007; Biradar *et al*., 2013; Matotoka and Masoko, 2018). Gbashi *et al.* (2017) reported that the medicinal and pharmaceutical activities of flavonoids depend on their structural configuration. The functionality of these compounds in human health has been supported by their ability to enhance protective effects against several diseases (Khoza *et al*., 2016).

From visual inspection of the extracts **(table 3.3: 4)** the development of a brown ring at the interface was observed. This is an indicator of a deoxysugar, a feature of cardenolides in cardiac glycosides (Borokini and Omotayo, 2012). Their presence could mean that the plant is capable of lowering blood pressure (Watal *et al*., 2014; Shrestha *et al*., 2015). They have also been reported as treatment agents for diabetic disorders and ulcers (Matotoka and Masoko, 2018).

A brownish-green colour was observed in both extracts (**table 3.3: 5)** of *M. balsamina* leaves. This is a characteristic of tannins (Nemudzivhadi and Masoko, 2015). Tannins have been reported as free radical scavenging molecules (Masoko and Eloff, 2007; Biradar *et al*., 2013; Matotoka and Masoko, 2018). Biradar *et al* (2013) highlighted that these antioxidant compounds possess anti-inflammatory activity. Biological activities associated with tannins include epithelial function improvement, cardiovascular protection, and antimicrobial activities (Masoko and Eloff, 2007; Shrestha *et al*., 2015). These have also been reported to inhibit viral reverse transcriptase, complex with proteins and polysaccharides (Bot *et al*., 2007).

The findings of this study correspond to the results obtained in another study that was done by Adamu *et al* (2015) who also detected the presence of the same phytochemicals in methanolic extract of *M. balsamina* leaves.

In the current study, steroids were not detected in either extracts (**table 3.3: 6).** The presence of steroids is represented by the appearance of a blue or green colour change as described by Borokini and Omotayo (2012). The results in this study were different from other studies that detected steroids in the fruit pulp of *M. balsamina* (Bot *et al*., 2007; Thakur *et al*., 2009). This dissimilarity could be an outcome of geographical and ecological differences (Sudi *et al*., 2011; Adamu *et al*., 2015).

3.4.1.3 UV-VIS

The UV-VIS profile of methanolic (**Figure 3.3**) and water extracts (**Figure 3.4**) of *M. balsamina* were selected from 200 nm to 700 nm due to the sharpness of distinctive peaks and proper baseline. This technique was used in detecting the presence of phytochemicals by identifying compounds containing π-bonds, lone pairs of electrons, σ-bonds, aromatic rings, and chromophores in extracts (Njoku *et al*., 2013).

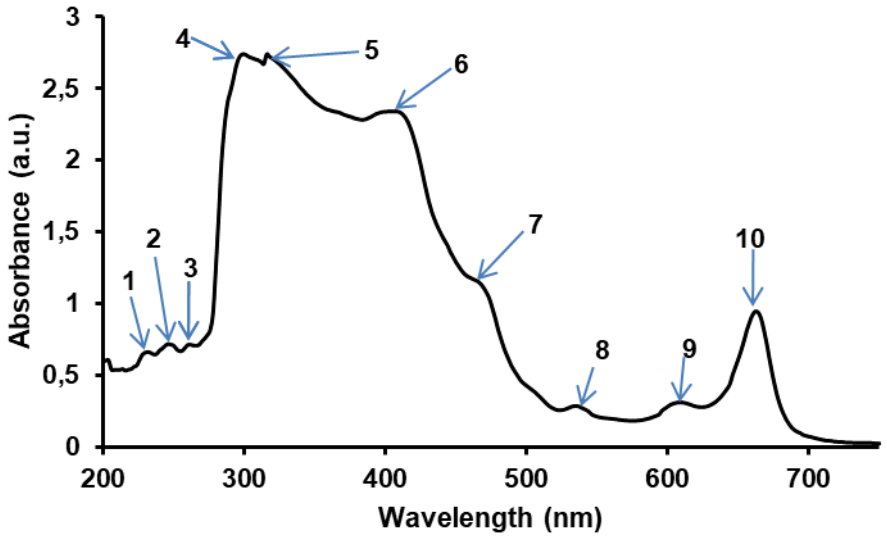


Figure 3.3: UV-visible spectral analysis of a methanolic extract of *M. balsamina* selected from 200 nm to 700 nm due to sharpness of distinctive peaks and proper baseline.

The UV-VIS profile of methanolic extract (**Figure 3.3**) of *M. balsamina* revealed peaks as displayed in **Table 3.4**. The presence of alkaloids, flavonoids, and phenolic compounds in the methanolic extract of *M. balsamina* is indicated by peaks that occur at 234 – 676 nm (Karpagasundari and Kulothungan, 2014; Patle *et al*., 2020). The presence of aromatic rings and other rings is the reason behind the two absorption spectra for flavonoids and phenolic compounds (Patle *et al*., 2020).

The spectrum of flavonoids consist of two absorption maxima ranging from 230 – 285 (band I) nm and 300 – 350 (band II) nm. Thus, the occurrence of peaks at these absorption maxima is characteristic of flavonoids and their derivatives (Kalaichelvi and Dhivya, 2017). The spectrum also showed an absorption band at 400 – 450 nm which indicates the presence of carotenoids (Patle *et al*., 2020). The occurrence of peaks at 350 – 500 nm indicates the presence of tannins and flavonoids (Patle *et al*., 2020). Flavonoids and their derivatives are also indicated by the occurrence of peaks at 300 – 350 nm (band II) (Saxena and Saxena, 2012; Renuka *et al*., 2016; Kalaichelvi and Dhivya, 2017).

Terpenoid and flavonoid spectra normally consist of the first two absorption maxima ranging from 230 – 290 nm (band I) and 400 – 550 nm (band II) (Saxena and Saxena *et al*., 2012; Renuka *et al*., 2016), peaks occurring at those wavelengths were

detected in this UV-VIS spectrum profile of the methanolic extract. Peaks detected at a wavelength of 600 – 700 nm indicate the presence of chlorophyll (Saxena and Saxena *et al*., 2012; Renuka *et al*., 2016).

Based on visual inspection, the UV-VIS spectrum profile of the methanolic extract of *M. balsamina* above revealed more distinctive peaks as compared to the water extract (**Figure 3.4**) profile as shown below. This could be a strong indication that methanol extract had more phytocompounds as compared to the water extract.

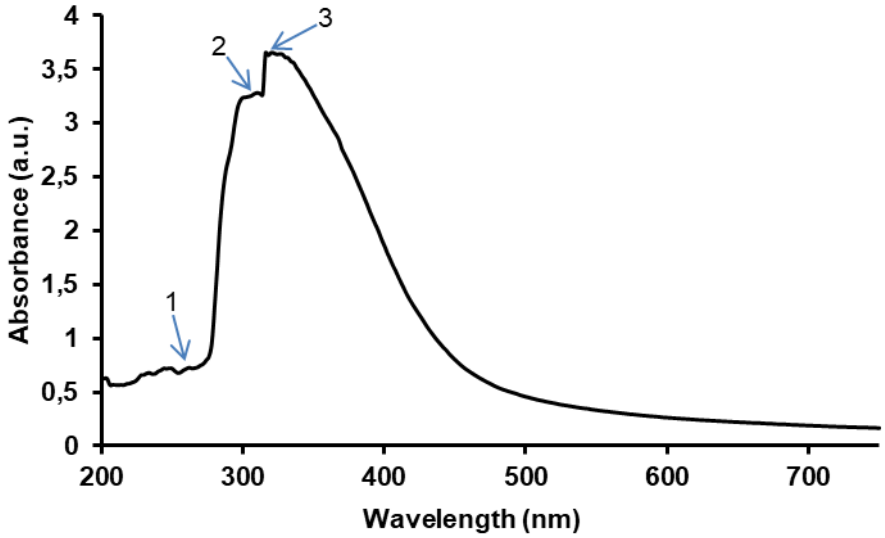


Figure 3.4: Ultraviolet-Visible spectral analysis of water extract of *M. balsamina* selected from 200 nm to 700 nm due to sharpness of distinctive peaks and proper baseline.

The spectral analysis of water extract of *M. balsamina* revealed various peaks as displayed in **Table 3.4.** The absorption bands detected are characteristic of alkaloids, flavonoids and phenolic compounds, these occur at 234 – 676 nm (Karpagasundari and Kulothungan, 2014; Patle *et al*., 2020). The occurrence of peaks ranging from 230 – 285 nm and 300 – 350 nm in the spectrum indicates the presence of flavonoids and their derivatives (Kalaichelvi and Dhivya, 2017). According to Johnson and Syed Ali Fathima (2018) occurrence of peaks ranging from 280 – 330 nm indicate the presence of phenolic derivatives suggesting the presence of phenolic compounds in *M. balsamina* leaf extracts. More peaks were detected in the methanolic

extract as compared to the water extract (**Table 3.4**) and this could be a clear indication that the methanolic extract contained more compounds than water extract.

Table 3.4: UV-VIS peak values of methanolic and water extracts of *M. balsamina.*

Extracts	Peak no.	Wavelength (nm)	Absorption (a.u)
Methanolic extract	1	226 (Flavonoids)	0.61
	2	240 (Flavonoids, alkaloids, phenolic compounds)	0.67
	3	256 (Flavonoids, alkaloids, phenolic compounds)	0.67
	4	296 (Flavonoids, alkaloids, phenolic compounds)	2.71
	5	312 (Flavonoids)	2.69
	6	404 (Terpenoids, carotenoids, tannins)	2.34
	7	462 (Terpenoids, tannins)	1.17
	8	530 (Terpenoids)	0.27
	9	604 (chlorophyll)	0.30
	10	658 (chlorophyll)	0.87
	Peak no.	**Wavelength (nm)**	**Absorption (a.u)**
Water extract	1	240 (Flavonoids, alkaloids, phenolic compounds)	0.69
	2	304 (Flavonoids, alkaloids, phenolic compounds)	3.25
	3	316 (Flavonoids, alkaloids, phenolic compounds)	3.65

3.4.1.4 FTIR

FTIR spectral analysis was used to detect functional groups of compounds present in *M. balsamina* and this was based on peak values in the region of infrared radiation. When extracts passed into FTIR, the functional groups of the compounds were separated based on their peak ratios (Saxena and Saxena, 2012). The FTIR spectral analysis of water (**Figure 3.5**) and methanolic extracts (**Figure 3.6**) as well as peak values and functional groups are displayed in **Table 3.5 and 3.6**.

3.4.1.4.1 FTIR analysis of water extract of *M. balsamina*

The FTIR spectral analysis (**Figure 3.5**) of the water extract revealed the presence of functional groups in 4 various frequency ranges (**Table 3.5**). The FTIR spectrum showed 4 major peaks ranging from 3363.59 cm^{-1}, 2154.12 cm^{-1}, 1645.68 cm^{-1} and 1087.68 cm^{-1} (Figure **3.5** and Table **3.5**).

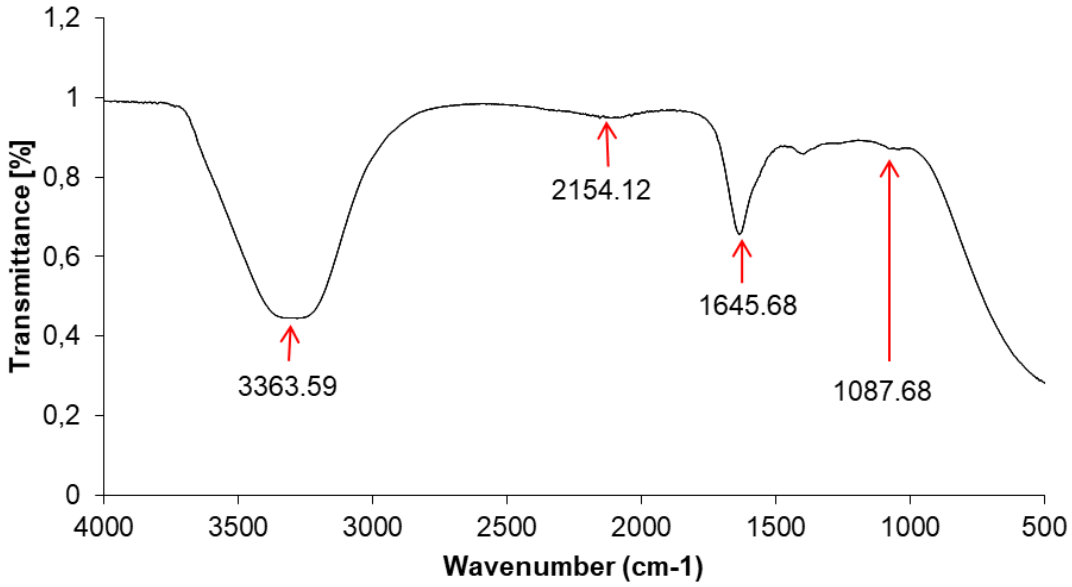


Figure 3.5: FTIR spectral analysis of water extract of *M. balsamina* with each arrow showing distinctive peaks characteristic for various functional groups indicating specific phytochemical compounds.

The analysis indicated the presence of 9 different functional groups, namely, phenolic group, amines, amides, alkenes, alkynes, carboxylic acids, esters, ethers, and alcohols. These functional groups belong to 6 different compounds which are aromatic, amines, amides, aliphatic, acid, and alcohol (**Table 3.5**).

Table 3.5: FTIR peak values and functional groups in water extract of *M. balsamina*.

No.	Frequency ranges (cm^{-1})	Frequency peak values (cm^{-1})	Vibration/ bond	Specific functional group	Chemical compound
1	3600 – 3200	3363.59	O-H stretch	Alcohols, phenols (hydrogen bonding)	Aromatic
	3400 – 3250	3363.59	N-H stretch	1°, 2° amines and amides	Amines and amides
2	2260 – 2100	2154.12	C≡C stretch	Alkynes	Aliphatic
3	1680 – 1620	1645.68	C=C stretch	Alkenes	Aliphatic
4	1320 – 1000	1087.68	C-O stretch	Carboxylic acids, esters, ethers, alcohols	Acid, alcohol

The observed peak at 3363.59 cm^{-1} may be due to stretching vibration of an O-H group or O-H wagging of phenolic compounds, strongly suggesting the presence of phenolic compounds (Oliveira *et al*., 2016) and flavonoids (Kalaichelvi and Dhivya, 2017). These have been reported as free radical scavenging molecules (Masoko and Eloff, 2007; Biradar *et al*., 2013). Hence this suggests the presence of antioxidant activity in *M. balsamina* leaves. The occurrence of a band at 3363.59 cm^{-1} may also be attributed to an N-H stretching vibration, indicating the presence of primary and secondary amides and amines. According to Were *et al* (2015), an N-H stretch depicts the presence of alkaloids in extracts.

The identified band at 2154.12 cm^{-1} could be due to the presence of alkynes attributed to C≡C stretching vibrations (Were *et al*., 2015). The band at 1645.85 cm^{-1} could be ascribed to the stretching vibration of C=C groups, deformation of an aromatic ring, presence of amino acids and flavonoids (Alara *et al*., 2018). The observed band at 1087.68 cm^{-} could be due to the presence of C-O stretching vibration attributed to an ester group or secondary alcohol (Johnson and Syed Ali Fathima, 2018), indicating the presence of anthraquinones (Were *et al*., 2015).

The presence of flavonoids demonstrates different biological and pharmacological effects such as anti-oxidation, anti-inflammation, and anti-allergic effects (Alara *et al*., 2018). Makita *et al* (2016) highlighted that it has been scientifically confirmed that frequent intake of dietary flavonoids from plants decreases the

consequences of oxidative damage such as diabetes and cardiovascular associated ailments.

3.4.1.4.2 FTIR analysis of methanolic extract of *M. balsamina*

The FTIR spectral analysis of methanolic extract of *M. balsamina* detected the presence of functional groups in 6 different frequency ranges (**Table 3.6**). The FTIR spectrum (**Figure 3.6**) showed 6 major peaks ranging from 3384.84 cm^{-1}, 2938.72 cm^{-1}, 2039.40 cm^{-1}, 1637.18 cm^{-1}, 1084.85 cm^{-1} and 1029.61 cm^{-1}.

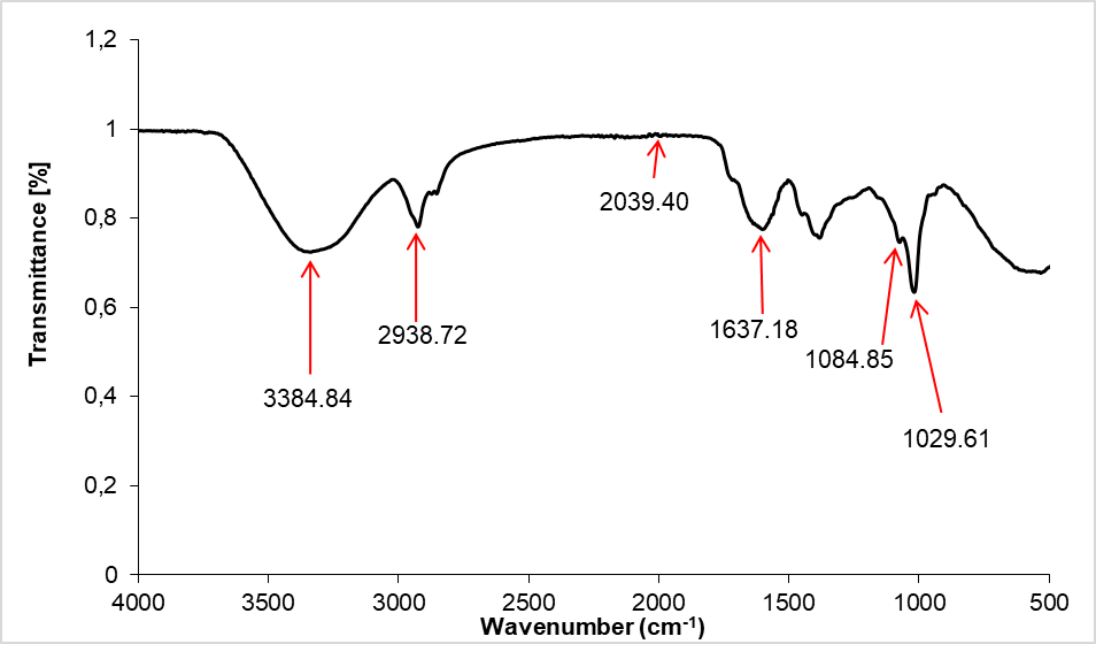


Figure 3.6: FTIR spectrum analysis of methanolic extract of *M. balsamina* with each arrow showing distinctive peaks characteristic for various functional groups indicating specific phytochemical compounds.

Since aqueous methanol was used to extract the sample, there could be a methanol band in the spectrum (**Figure 3.6**). Thus, the characteristic methanol band would be 3384.84 cm^{-1} showing stretching vibration of O-H group or O-H wagging of phenolic compounds (Oliveira *et al.*, 2016). This therefore strongly suggests the presence of phenolic compounds which have been reported to exhibit antioxidant activities (Masoko and Eloff, 2007).

The analysis depicted the presence of 10 various functional groups, namely, phenolic group, amides, amines, alkanes, alkenes, alkynes, alcohols, carboxylic acids, esters, and ethers. These functional groups belong to 6 different compounds which are aromatic, amines, amides, aliphatic, acid, and alcohol (**Table 3.6**).

Table 3.6: FTIR peak values and functional groups in methanolic extracts of *M. balsamina.*

No.	Frequency ranges (cm^{-1})	frequency peak values (cm^{-1})	Vibration/ bond	Specific functional group	Chemical compound
1	3600 – 3200	3384.84	O-H stretch	Alcohols, phenols (hydrogen bonding)	Aromatic
	3400 – 3 250	3384.84	N-H stretch	1° , 2° amines and amides	Amines and amides
2	3000 – 2850	2938.72	C-H stretch	Alkanes	Aliphatic
3	2270 – 1940	2039.40	C≡C stretch	Alkynes	Aliphatic
4	1680 – 1620	1637.18	C=C stretch	Alkene	Aliphatic
5	1320 – 1000	1084.85	C-O stretch	Alcohols, Carboxylic acids, esters, ethers	Acid, Alcohol
6	1320 – 1000	1029.61	C-O	Alcohols, Carboxylic acids, esters, ethers	Acid, Alcohol

The band occurring at 3384.84 cm^{-1} indicates that alkaloids may be present which could be due to the N-H stretch (Were *et al*., 2015; Kalaichelvi and Dhivya, 2017). The identified band at 2039.40 cm^{-1} could be due to the presence of alkynes and this is attributed to the C≡C stretching vibrations. The stretching vibration of the C-H band at 2938.72 cm^{-1} could be ascribed to the presence of CH_2 and CH_3 groups (Oliveira *et al*., 2016) which indicates the presence of terpenes (Were *et al*., 2015; Kalaichelvi and Dhivya, 2017).

The band at 1637.18 cm^{-1} could be ascribed to the presence of a deformed aromatic ring, amino acids, flavonoids, and stretching vibrations of C=C groups (Alara *et al*., 2018). The identified bands at 1084.85 cm^{-1} and 1029.61 cm^{-1} could be due to

the presence of C-O stretching vibration due to an ester group or secondary alcohol (Johnson and Syed Ali Fathima, 2018).

Fourier transform infrared transmission is very useful in plant characterization because it reveals the presence of inorganic and organic compounds in plants. The presence of these functional groups such as phenolic group, ether, alcohols, carboxylic acids, and aliphatic amines serves as an indicator of different medicinal properties or biological activities of *M. balsamina* leaves.

3.4.1.5 UHPLC-qTOF-MS

Metabolite extraction and profiling of *M. balsamina* leaf extracts were carried out subsequently. Analyses were done using an LC-qTOF-MS operating in negative electrospray ionization (ESI) mode. From the UHPLC-qTOF-MS chromatograms (**Figure 3.7** and **3.8**) a total of 12 chromatographic peaks showing various metabolites as displayed in **Figure 3.7** were identified and tabulated in **Table 3.7**.

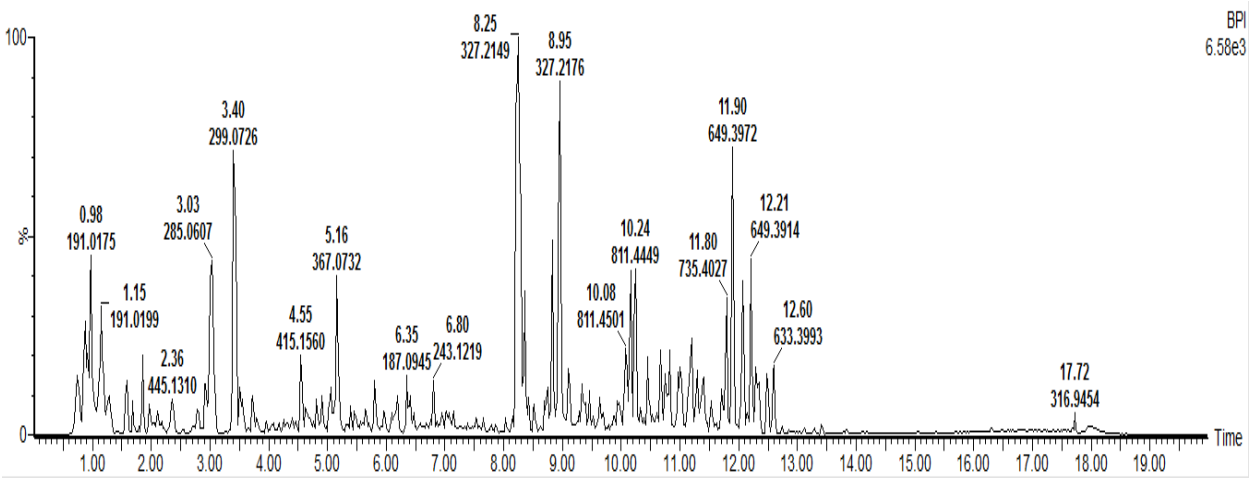


Figure 3.7: Representative UHPLC-qTOF-MS chromatogram showing water extract of *M. balsamina.*

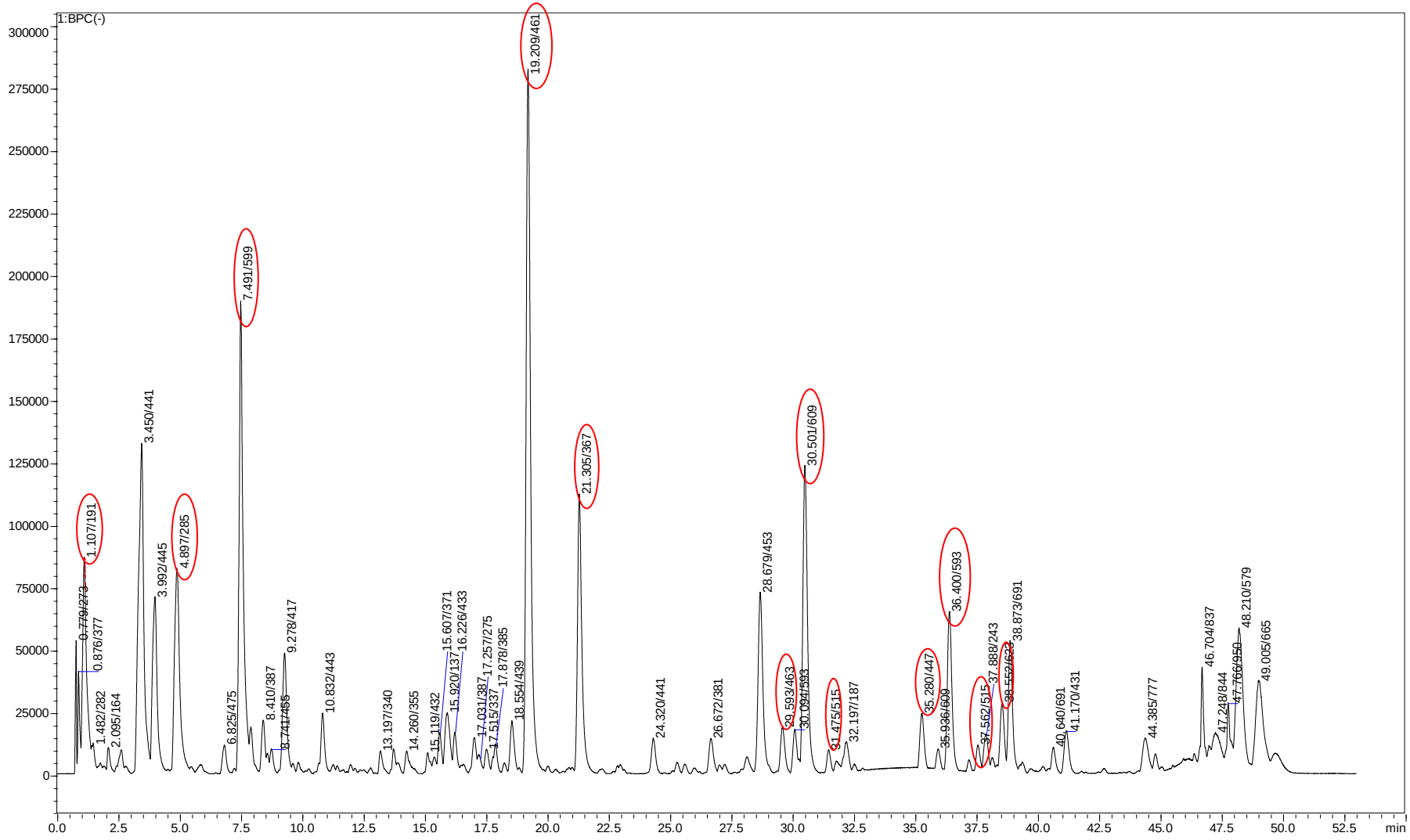


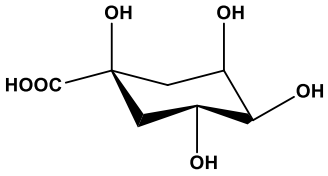
Figure 3.8: Representative UHPLC-qTOF-MS chromatogram showing metabolites present in methanol extract of *M. balsamina.*

From visual inspection of chromatograms (**Figure 3.7** and **3.8**), it is evident that water extracted less active compounds as compared to methanol. The water extract had a lot of signal/noise peaks thus there were no defined peaks detected. However, this does not mean that compounds present in water extract are not active; there are other compounds that can exhibit an activity of their own. The chemical structures of the secondary metabolites isolated from *M. balsamina* extracts are displayed in **Figure 3.9.**

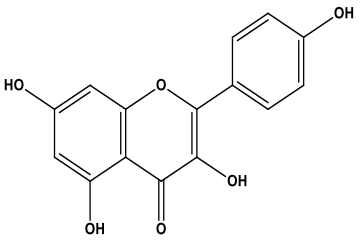
Table 3.7: UHPLC-qTOF-MS profile of metabolites isolated from leaf extracts of *M. balsamina*.

	Metabolite	**Elemental composition**	**Rt (min)**	**$[M-H]^-$**	***Leaf extracts***	
					Water	**Methanol**
1	Quinic acid	$C_7H_{12}O_6$	1.107	191	O	✓
2	Kaempferol	$C_{15}H_{10}O_6$	4.897	285	O	✓
3	Pseudolaroside A (dimer)	$C_{26}H_{32}O_{16}$ Dimer: $C_{13}H_{16}O_8$	7.491	599 Dimer: 299	O	✓
4	Kaempferol glucuronide	$C_{21}H_{18}O_{12}$	19.209	461	O	✓
5	Feruloyl isocitric acid	$C_{16}H_{16}O_{10}$	21.305	367	O	✓
6	Quercetin hexose	$C_{21}H_{20}O_{12}$	29.5923	463	O	✓
7	Quercetin rutinoside	$C_{27}H_{30}O_{16}$	30.501	609	O	✓
8	Dicaffeoylquinic acid isomer I	$C_{25}H_{24}O_{12}$	31.475	515	O	✓
9	Kaempferol hexose	$C_{21}H_{20}O_{11}$	35.936	447	O	✓
10	Kaempferol rutinoside	$C_{27}H_{30}O_{15}$	36.400	593	O	✓
11	Dicaffeoylquinic acid isomer II	$C_{25}H_{24}O_{12}$	37.562	515	O	✓
12	Isorhamnetin rutinoside	$C_{27}H_{30}O_{16}$	38.552	623	O	✓

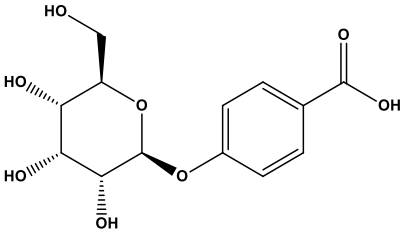
✓ **: Presence; o: absence of metabolites**



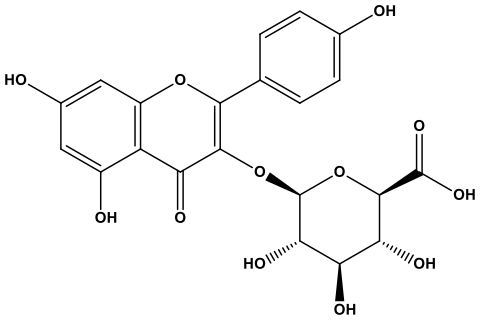
1. Quinic acid



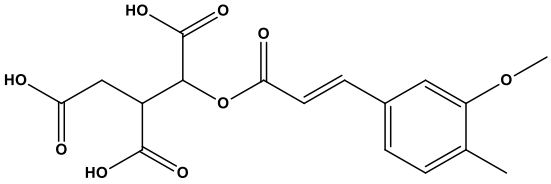
2. Kaempferol



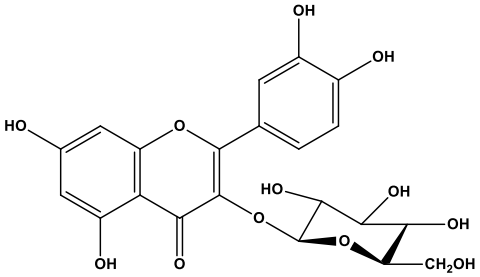
3. Pseudolaroside A acid



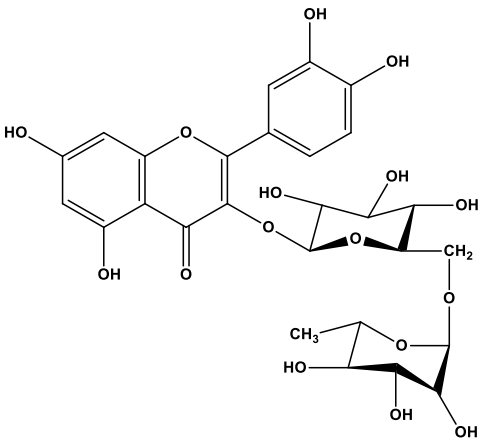
4. Kaempferol glucuronide



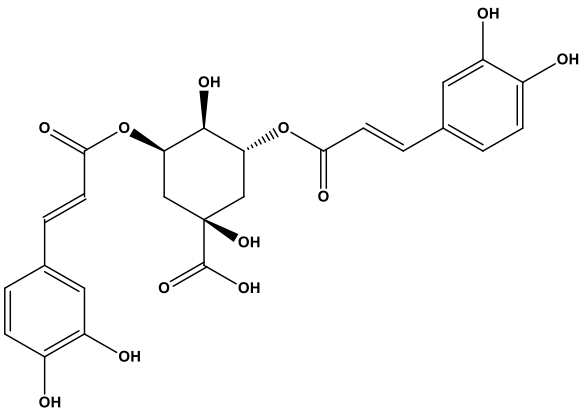
5. Feruloyl isocitric acid



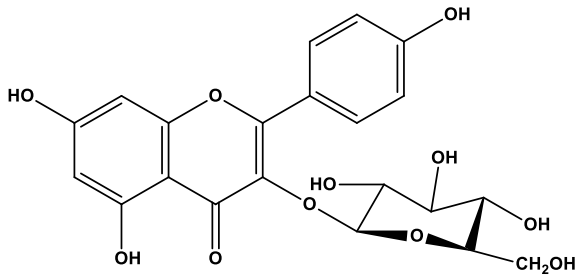
6. Quercetin hexose



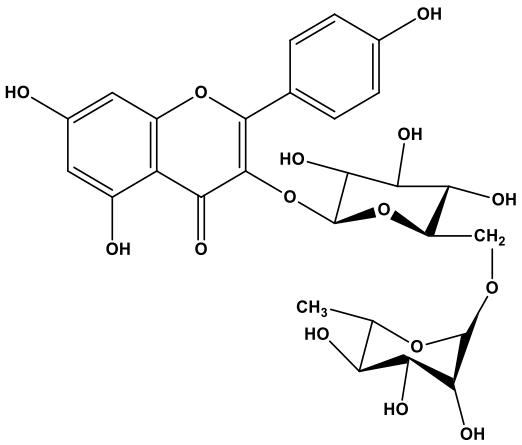
7. Quercetin rutinoside



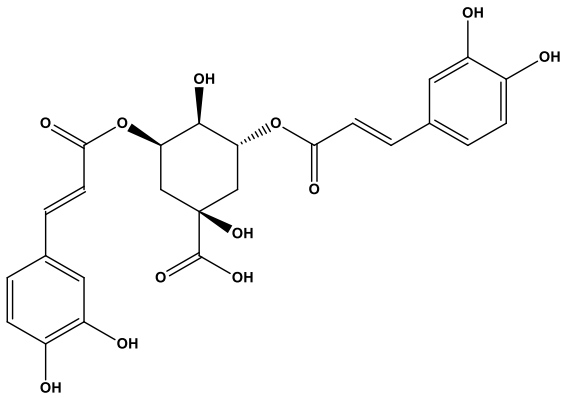
8. 3.5- dicaffeoylquinic acid



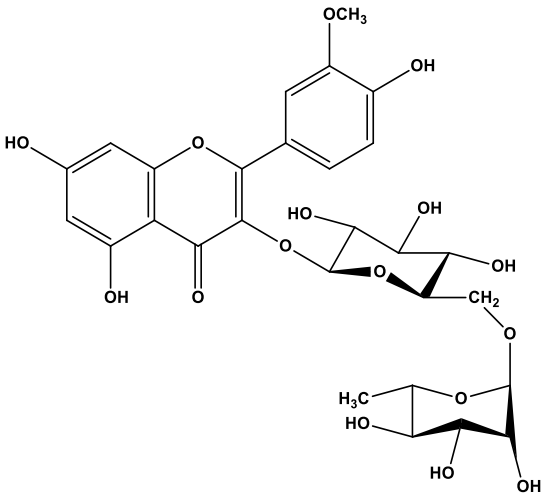
9. Kaempferol hexose



10. Kaempferol rutinoside



11. 3.5- dicaffeoylquinic acid



12. Isorhamnetin rutinoside

Figure 3.9: Chemical structures of metabolites isolated from methanolic extract of *M. balsamina* leaves indicating flavonoid aglyclones and acids.

The most common flavonoids are kaempferol, quercetin, and Isorhamnetin and these exist as glycosides abundantly in plant tissue (Makita *et al*., 2016). Flavonoids with these aglyclones have been reported to exhibit a wide range of health-promoting activities such as inhibition of inflammation (Xiao *et al*., 2012, Khoza *et al*., 2016). Hence, two Quercetin-O-glycosides, four Kaempferol-O-glycosides, and one Isorhamnetin-O-glycoside were identified in this study.

Quercetin has been reported as a crucial dietary flavonoid that is associated with a plethora of properties capable of suppressing certain ailments linked to chronic diseases (Makita *et al*., 2016). According to Gbashi *et al* (2017), this flavonoid aglycone has been suggested as a hepatoprotective agent. Quercetin rutinoside (Molecule **7**) with a precursor ion at *m/z* 609 $[M-H]^-$ was identified in this study. Other studies highlighted that quercetin rutinoside known as rutin has high antioxidant activity potential attributing to biological activities such as protection of liver cells and suppression of haemoglobin oxidation (Rodriguez-Perez *et al*., 2015; Makita *et al*., 2016). Furthermore, the molecule has also been reported as an anti-inflammatory agent making it useful in the treatment of chronic diseases (Rodriguez-Perez *et al*., 2015; Makita *et al*., 2016). Quercetin hexose (Molecule **6**) with a precursor ion at *m/z* 463 $[M-H]^-$ was also detected in the current study.

Research has shown that kaempferol is of great significance in managing cancer-associated ailments (Chen and Chen, 2013; Makita *et al*., 2016) as well as inhibiting oxidative stress (Gbashi *et al*., 2017). Molecule **10** with a precursor ion at *m/z* 593.1496 $[M-H]^-$ was identified as kaempferol rutinoside (Madala *et al*., 2016; Makita *et al*., 2016). Molecule **9** was identified as kaempferol hexose (Khoza *et al*., 2016; Madala *et al*., 2016; Makita *et al*., 2016; Gbashi *et al*., 2017) with a precursor ion at *m/z* 447 $[M-H]^{-\cdot}$. Molecule **2** with a precursor ion at *m/z* 285 $[M-H]^-$ was identified as kaempferol. Molecule **4** was identified as kaempferol glucuronide (Gbashi *et al*., 2017) with a precursor ion at *m/z* 461.

Isorhamnetin is a methylated form of quercetin (Rodriguez-Perez *et al*., 2015; Makita *et al*., 2016), and has been reported to exhibit anti-inflammatory activity (Antunes-Ricardo *et al*., 2015). This flavonoid aglyclone has been proven as an antioxidant agent (Kim *et al*., 2011; Xu *et al*., 2011; Khoza *et al*., 2016). Molecule **12** with a precursor ion at *m/z* 623 $[M-H]^-$ was identified as Isorhamnetin rutinoside (Makita *et al*., 2016).

Chlorogenic acids (CGA) are secondary metabolites found in plants and of great significance because they possess a variety of health benefits which include anti-inflammatory and antidiabetic activities (Madala *et al*., 2014; Makola *et al*., 2016). Madala *et al* (2014) defined CGA as a molecule that is formed from an ester bond between single or multiple cinnamic acids (*p*-coumaric, caffeic, and ferulic acid) and

quinic acid, therefore resulting in *p*-coumaroylquinic acid, caffeoylquinic acid, and feruloylquinic acid. A total of three chlorogenic acids were identified namely, quinic acid with a precursor ion at *m/z* 191 $[M-H]^-$ and 2 isomers of dicaffeoylquinic acids (molecule **8** and **11)** with a precursor ion at *m/z* 515 $[M-H]^-$ eluted at different retention times 31.475 and 37.562 respectively (**Table 3.7**; **Figure 3.8**).

Hydroxyl-cinnamic acids (HCAs) have been reported to exist as positional and geometric isomers conjugated to various organic acids namely quinic and isocitric acid (Parveen *et al*., 2008; Masike *et al*., 2017). The formation of hydroxycinnamoyl-isocitric acid is due to the formation of a conjugate between HCA derivatives and organic acids and this includes the esterification between one of the derivatives of HCA and an isocitric acid, this can occur at position 2 (C2) (Masike *et al*., 2017). Masike *et al* (2017) further highlighted that derivatives of hydroxycinnamoyl-isocitric acid are not well documented and this is ascribed to the misidentification of these compounds with mono-acyl chlorogenic acids because they have the same molecular mass of conjugates namely, caffeoyl- (354 Da), p- coumaroyl- (338 Da) and feruloyl- (368 Da). In this study, Feruloyl isocitric acid (Masike *et al*., 2017) was identified at precursor ion *m/z* 367 $[M-H]^-$ (molecule **5**) as predicted by the accurate high-resolution mass spectrometer (LC-QTOF-MS model LC-MS 9030 instrument). To the best of our knowledge, this is the first report on the presence of feruloyl isocitric acid in *M. balsamina* leaves.

A peculiar compound that has not yet been documented in relation to compounds isolated in *M. balsamina* was identified in the current study. Pseudolaroside A acid (Molecule **3:** $C_{13}H_{16}O_8$) of precursor ion *m/z* 299 $[M-H]^-$ was identified as a dimer of ($C_{26}H_{32}O_{16}$) at precursor ion *m/z* 599 $[M-H]^-$. This benzoic acid allopyranoside was isolated from the bark of *Pseudolarix kaempferi* as a colourless amorphous solid by Lui *et al* (2006). The structure of this compound was determined by analysis of high-resolution electrospray ionization mass spectrum (HR-ESI-MS) identified at a precursor ion *m/z* 299.0766 $[M-H]^-$. The structure was also determined by 1D and 2D NMR spectroscopic data which displayed a structure to be benzoic acid 4-*O*-*β*-*D*-allopyranoside or 4-(beta-D-glucosyloxyl) benzoic acid (Lui *et al*., 2006). Kim *et al* (2016) isolated this compound from the roots of *Coix lachrymal-jobi var. mayuen.* To the best of our knowledge, only a few studies have been done on this compound and its significance is yet to be documented.

Nagarani *et al* (2014) reported other flavonoid molecules in other *Momordica* species such as catechin, chlorogenic acid, caffeic acid, and ferulic acid. Previous studies highlighted that these species namely: *M. balsamina, M. charantia,* and *M. foetida* are abundant sources of flavonoids of different forms and these include a variety of isomers of the quercetin-, kaempferol- and isorhamnetin- *O*- glycosides (Madala *et al*., 2016; Khoza *et al*., 2016; Guarniz *et al*., 2019). In another study conducted by Madala *et al* (2014), the results revealed that *Momordica* species are comprised of all forms of common cinnamic acids such as caffeic, *p*-coumaric, and ferulic acid. In addition, 4-acylated quinic acids were identified: 4-*pCo*QA, 4-CQA, and 4-FQA.

The results demonstrated that methanol extracted more compounds as compared to water. This agrees with previous studies which highlighted that methanol extracts contain a variety of compounds (Masoko and Makgateetja, 2015; Nemudzivadi and Masoko, 2015; Zininga *et al*., 2017).

CHAPTER 4

Assessment of antioxidant, antibacterial, anti-inflammatory, and cytotoxic activities of *Momordica balsamina* leaf extracts

4.1 Introduction

Throughout evolution, plants developed the capability to produce secondary metabolites which play a pivotal role in the plant's environmental interactions, strategies of reproduction, and mechanisms of defence (Chandra, 2019). The presence of secondary metabolites with different biopotency namely, flavonoids, tannins, terpenoids, and phenolic compounds provide specific properties and distinctiveness to plants (Johnson and Syed Ali Fathima, 2018). Therefore, analysis of phytochemical constituents can help in determining the biological activities of plants (Johnson and Syed Ali Fathima, 2018).

Medicinal plants contain a broad spectrum of free radical scavenging molecules, such as phenolic compounds (flavonoids, phenolic acids, tannins), vitamins, alkaloids, and terpenoids, which are rich in antioxidant activity (Masoko and Eloff *et al*., 2007; Alara *et al*., 2018). Studies have revealed that these antioxidant compounds exhibit other bioactivities such as anti-inflammatory and antibacterial activities (Masoko and Eloff, 2007). *Momordica balsamina* is a significant plant used as a wound healing or anti-inflammatory agent (Msheila *et al*., 2017). The biological activities of *Momordica* species may be linked to the presence of health-promoting secondary metabolites (Nagarani *et al*., 2014).

Plants contain bioactive compounds with healing abilities (Masoko and Makgapeetja, 2015) and act synergistically to combat microbial growth (Nthulane *et al*., 2020). They have been routed as new sources of antimicrobial agents consisting of novel modes of action and represent an alternative to treat infections resulting from resistant microorganisms (Nthulane *et al*., 2020).

Multiple synthetic drugs have been used to treat infectious diseases; however, these are associated with side effects such as immunosuppression and hypersensitivity in the human body (Tamokou *et al*., 2011; Nagarani *et al*., 2014). Therefore, this has led to the use of natural resources by researchers for the discovery

of anti-inflammatory agents as highlighted by Nagarani *et al* (2014). Plant extracts with anti-inflammatory activity have been reported to have active therapeutic effects that promote healing and repair cell tissue (Nthulane *et al*., 2020).

This chapter focused on the assessment of antioxidant (DPPH-TLC), anti-bacterial (MIC), anti-inflammatory (NO production), and cytotoxic activities (MTT) of *M. balsamina* leaf extracts.

4.2 Materials and methods

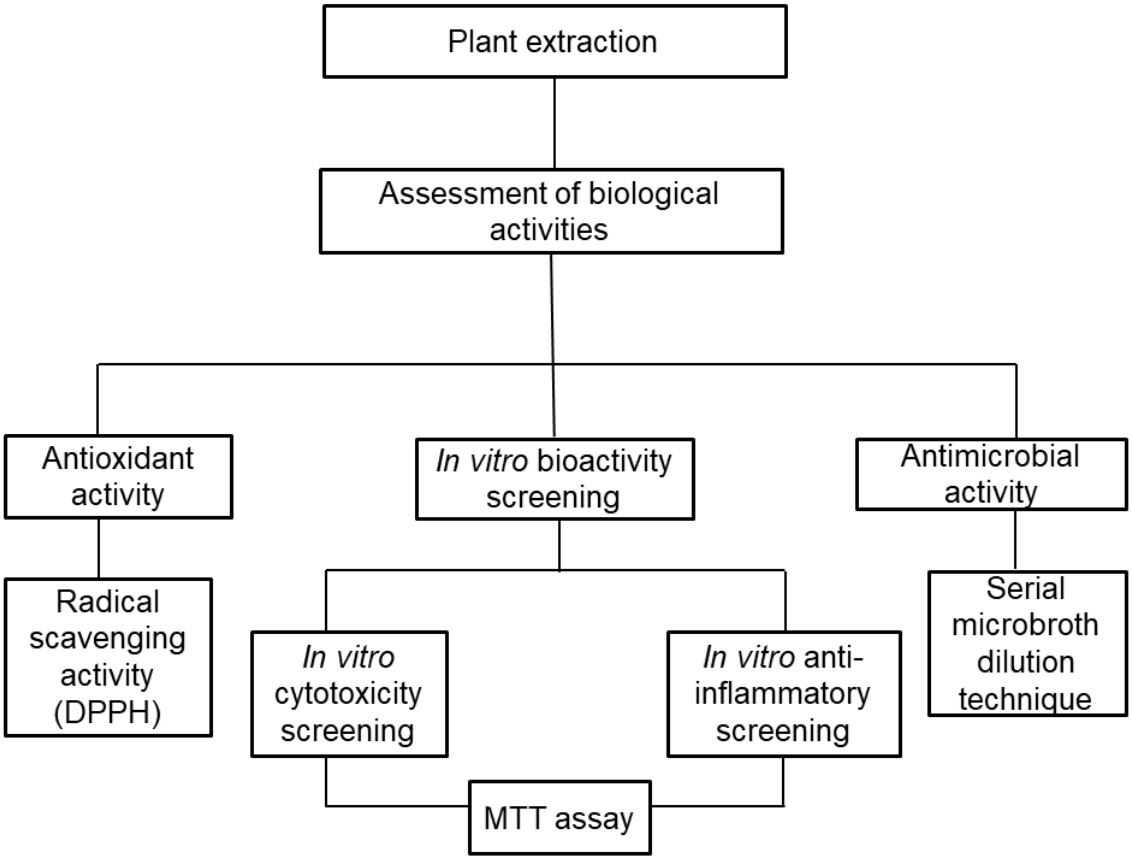


Figure 4.1: Schematic diagram of approaches used to achieve the objectives in chapter 4.

Solvents and reference strains used in the current study were purchased from (Sigma Aldrich®, St Louis, MI, USA). Melphalan was purchased from (GlaxoSmithKline, Brentford, UK) and Griess reagent from (Roche Diagnostics, Basel, Switzerland). Anti-inflammatory and cytotoxicity assays were carried out in Prof Van Venter's laboratory (Bioassaix, Nelson Mandela Metropolitan University, PE, South Africa).

4.2.1 Plant preparation and metabolite extraction

Plant preparation and extraction were conducted according to the method described in chapter 3 (**3.2.3**).

4.2.2 Antioxidant activity assays

A stable free radical (1.1 Diphenyl 2-Picryl Hydrazyl) with a red colour that is absorbs at 517 nm and turns yellow when scavenged was used in this study. This character is used by the DPPH assay to depict free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be represented as:

(DPPH) + (H-A) → DPPH – H + (A)

Antioxidants react with DPPH and reduce it to DPPH – H, resulting in a decrease in absorbance. The scavenging potential of antioxidant compounds or extracts in terms of hydrogen donating ability is indicated by the degree of discoloration as reviewed by Alam *et al* (2013).

DPPH (2,2-diphenyl- 1-picrylhydrazyl) assay was used to determine free radical scavenging activity for the crude extracts on aluminium-backed TLC plates. This method was previously described by Nemudzivhadi and Masoko (2015). Briefly, thin layer chromatography was conducted using TLC plates to separate compounds in extracts. For antioxidant analysis, TLC plates were loaded with 20 µl of each extract (10 mg/ml) and development of the plates was carried out in saturated chambers using mobile phases of varying polarities [BEA: benzene/ethanol/ammonium hydroxide (non-polar/basic) (18:2:0.2), CEF: chloroform/ethyl acetate/formic acid (intermediate polarity/acidic) (10:8:2) and EMW: ethyl acetate/methanol/water (polar/neutral) (10:5.4:4)] (Kotze and Eloff, 2002; Nemudzivhadi and Masoko, 2015).

For detection of antioxidant activity, 0.2% (w/v) 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was used as an indicator and was sprayed on the chromatograms. The appearance of a yellow colour on a purple background showed antioxidant potential (Nemudzivhadi and Masoko, 2015).

4.2.3 Antibacterial screening of *M. balsamina* leaf extracts

Nutrient broth was prepared and bacterial inocula obtained from ATCC reference strains were sub-cultured and incubated at 37 °C for 24 hours, then standardized to 0.5 Mc-Farland Scale (10^8 cfu/mL) as highlighted by Andrews (2003).

Table 4.1: Reference microorganisms and their sources.

Microorganism	**Classification**	***ATCC reference no.***
Escherichia coli	Gram-negative	35218 and 25918
Enterococcus faecalis	Gram-positive	14913
Proteus mirabilis	Gram-negative	7002

ATCC American Type Culture Collection

Serial microbroth dilution technique was used as described by Songca *et al* (2013) with minor modifications. This assay was used to determine the minimum inhibitory concentration (the lowest concentration of compounds that inhibits microbial growth). Acetone was used to reconstitute the dried crude extracts to a concentration of 10 mg/ml. Acetone was used because it serves as a very efficient solvent for components of the plant and when compared to other solvents, it is not toxic to microorganisms (Eloff, 1998).

Serial dilution was carried out at 50% using water in 96 well microtiter plates. This was followed by the transfer of sub-cultured bacterial strains into fresh nutrient broth. A hundred microliters of the culture were then added into each well and acetone was included for control purposes. Similar dilutions of ampicillin were used as a positive control and acetone was used as a negative control. The microtiter plate was then incubated at 37 °C for 24 hours. After the incubation period, 20 µl of 2 mg/ml *p*-

iodonitrotetrazolium violet was dissolved in water and then added to each microtiter plate well as an indicator of growth. The covered microtiter plates were then incubated for 30 minutes at 37 °C and 100% relative humidity. All determinations were carried out in triplicate (Nemudzivhadi and Masoko, 2015). Microbial growth leads to the appearance of a purple-red colour which results from INT (*p*-iodonitrotetrazolium) reduced into formazon. The presence of clear wells serves as an indication of compounds in the extracts that inhibit microbial growth. The minimum concentration of the extract that inhibited microbial growth (MIC) after 24 hours will be determined as previously done by Songca *et al* (2013); Nemudzivhadi and Masoko (2015).

4.2.4 *In vitro* cytotoxicity screening of *M. balsamina* leaf extracts

A mass of 20 mg of extracts was weighed and dimethyl sulfoxide (DMSO) was used as a reconstitution solvent to give a final concentration of 100 mg/mL, followed by sonication of samples to completely dissolve the extracts and then stored at 4 °C for further use. The human colorectal adenocarcinoma cell lines (HT29 and Caco2) and African green monkey kidney cells (Vero cells) were used for cytotoxicity screening. These were maintained in 10 cm culture dishes inside a humidified incubator (Thermofisher, Waltham, MA; USA) with 5% CO_2 at 37 °C. The constituents used for the complete growth medium were Dubelco's modified Eagle Media (DMEM) supplemented with 10% Foetal bovine serum (FBS) and 10% penicillin-streptomycin for the 3 cell lines.

Cells were seeded into 96 well microtiter plates at a density of 4000 cells/well using a volume of 100 µl in each well. For cell attachment, the cells were left overnight at 37 °C, 5% CO_2, and 100% relative humidity. Cells were treated with 50, 100, and 200 µg/ml of each extract. Melphalan, a toxic agent as highlighted by Sigidi *et al* (2017) was used as a positive control, and volumes of 10, 20, and 40 µM were diluted in the culture medium. Cells were then further treated with 100 µL aliquots of the diluted extracts in the fresh medium and incubated again for 48 hours. The treatment medium was aspirated from all wells and 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) was added into each well and incubation followed for 20 minutes at room temperature (25 °C).

Propidium iodide (PI) was used at 100 µg/mL to stain the cells and this was done for enumeration of the proportion of dead cells within the population. An image of the cell was then captured immediately after PI was added using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices San Jose, California, USA) with a 10x Plan Fluor objective and DAPI and Texas Red filters cubes. For each well, nine images were acquired as a representative of 75% of the surface area of the well. For quantifying viable and dead cells, a screening assay was performed and acquired images were analyzed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module.

4.2.5 Anti-inflammatory activity of *M. balsamina* leaf extracts

Momordica balsamina extracts were dissolved in DMSO to give a final concentration of 100 mg/ml and diluted further into the culture medium. A total of 100 µM of aminoguanidine was used as a positive control since it is known as an inhibitor of nitric oxide as highlighted by Sigidi *et al* (2017); hence it was employed as an indicator for anti-inflammatory activity.

RAW 264.7 cells were seeded at a density of 1 x 10^5 cells / well into 96-well plates and cell attachment took place overnight in a Heracell VIOS CO_2 Incubator (Thermofisher, Waltham, MA; USA). Samples were then diluted in DMEM after removal of the spent culture medium. These samples were added in volumes of 50 µl in each well to give final concentrations of 25, 50, 100, and 200 µg/mL. The corresponding wells were filled with 50 µl of LPS containing medium to give a final concentration of 500 µg/mL and this was done for assessment of anti-inflammatory activity.

Aminoguanidine was utilized as a positive control and cells were further incubated for 18 hours. To quantify NO production, a new 96-well plate was used and 50 µl of spent culture medium was transferred and 50 µl Griess reagent was also added. Measurement of absorbance (VersaMax ELISA Microplate Reader, Sunnyvale, CA; USA) was done at 540 nm and results were expressed relative to the appropriate untreated control. For the determination of NO concentration in each sample, a standard curve using sodium nitrite dissolved in culture medium was used.

Assessment of cell viability was conducted using MTT to confirm the absence of toxicity as a contributory factor. This was done by removing the remaining medium and treating each well using a medium comprising of 0.5 mg/ml MTT as a replacement and incubating for 30 minutes at 37 °C. MTT was then eradicated and 200 µL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 540 nm using a spectrophotometer (BioTek® PowerWave XS, Winooski, VT, USA).

4.2.6 Statistical analysis

All results were conducted in triplicates and expressed as means. The differences between test extracts in these experiments were examined for significance using analysis of variance (ANOVA) and student t-test, where probability (**$p \leq 0.05$**) was considered significant.

4.3 Results and discussion

4.3.1 Antioxidant activity

The TLC-DPPH screening method was carried out to evaluate the antioxidant activity of *M. balsamina* extracts (**Figure 4.2**). The DPPH method measures the electron-donating activity of compounds in a mixture and provides an evaluation of antioxidant activity due to free radical scavenging. Any electron or hydrogen donating molecule will react with and bleach DPPH. Oxidant compounds use electrons to

reduce DPPH from a purple compound to a light-yellow compound. Therefore, the degree of antioxidant activity of the tested extracts was determined from observation of the yellow intensity as adopted from Masoko and Eloff (2007).

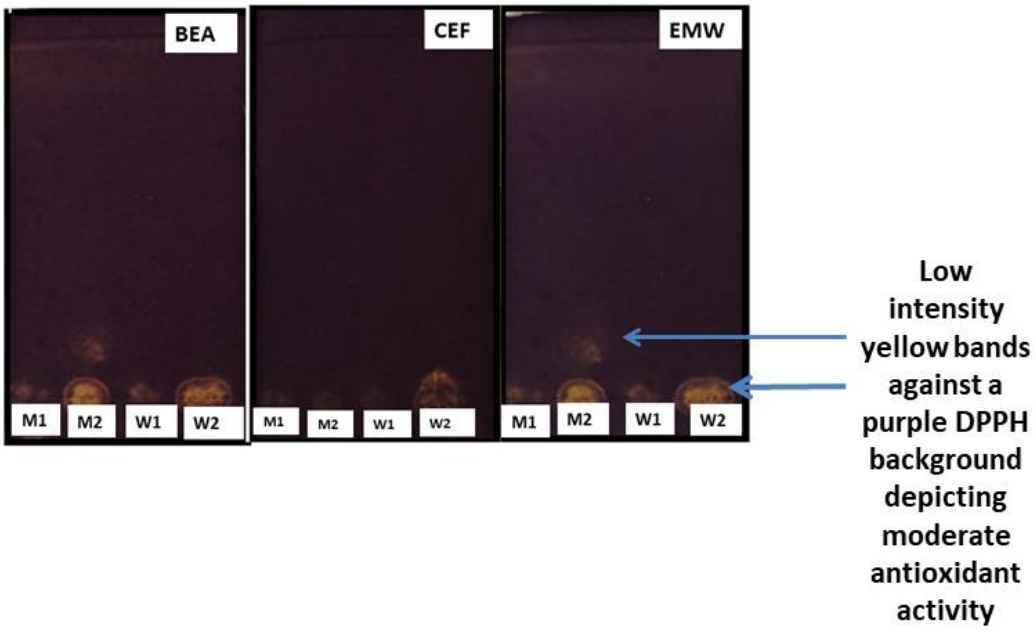


Figure 4.2: Chromatograms of *M. balsamina* extracts developed in 3 solvent systems based on polarity namely: **BEA** (non-polar: 18:2:0.2), **CEF** (intermediately polar: 10:8:2), **EMW** (polar: 10:5, 4:4) and sprayed with 0.2% DPPH to visualize the antioxidant activity of extracts. The compounds were extracted with methanol (M1 and M2) and water (W1and W2) in lanes from left to right.

In the current study, moderate antioxidant activity was detected in both extracts of *M. balsamina* (**Figure 4.2**) as depicted by low intensity of yellow colour on chromatograms or yellow band against a purple DPPH background. The results correspond with a study done by Matotoka and Mafoko (2018) which suggested that these results could be a clear indication that antioxidant activity does not depend on single compounds but the collective interaction of the compounds in the crude extract.

Radical scavenging has been reported as a significant antioxidant activity where a stable free radical (DPPH) is used and its decolourization suggests the presence of an electron or hydrogen donor in extracts (Meenakshim *et al*., 2012). This strongly supports that *M. balsamina* leaf extracts have potential antioxidant activity

since the results above revealed that these extracts are capable of decolourizing DPPH.

According to Nagarani *et al* (2014), the presence of flavonoids, tannins, and phenolic compounds could be correlated with the antioxidant activity of *Momordica* species. Anokwuru *et al* (2018) further highlighted that phenolic compounds are excellent antioxidants attributed to their capability to scavenge free radicals and reactive oxygen. The results obtained in this study correspond with many studies that are in agreement with the fact that tannins, phenolic compounds (Vanjala and Kavitha, 2016), and flavonoids are contributors to the antioxidant activity of many vegetables (Meenakshim *et al*., 2011; Nagarani *et al*., 2014; Anokwuru *et al*., 2018).

A study done by Ajji *et al* (2016) reported that the *M. balsamina* protein (balsamin) exhibited antioxidant activity. According to Abdulfatai and Aduwamai (2018), *M. balsamina* leaves are effective against oxidative stress and this is associated with the presence of antioxidants and bioactive compounds in the plant, these studies are in agreement with the current study.

TLC-DPPH analysis is used to assess total antioxidant activity and this technique may be useful in screening medicinal herbs and dietary plants for their relative antioxidant content (Masoko and Eloff, 2007). Antioxidants play a crucial role in controlling levels of free radicals in the human body by thus reducing oxidative damage and inhibiting lipid peroxidation (Masoko and Eloff, 2007; Nemudzhivhadi and Masoko, 2015; Anokwuru *et al*., 2018). Nagarani *et al* (2014) highlighted that an oxidation reaction stimulates the production of free radicals and these are known as causative agents for many degenerative disorders by abducting biomolecules such as DNA, lipids, and proteins. Hence, living organisms maintain complex systems of multitudinous antioxidants in their body (Nagarani *et al*., 2014).

4.3.2 Antibacterial activity of *M. balsamina* extracts

According to Ramalhete *et al* (2011), the search for antimicrobial agents from herbs is a promising approach as shown by numerous papers published recently. It is therefore pivotal to investigate the use of plant products to treat diseases associated with bacterial infections. The antibacterial activity of *M. balsamina* extracts on gut-associated bacterial species is tabulated in **Table 4.2**.

Table 4.2: Minimum Inhibitory Concentration (MIC) values (mg/mL) of methanolic and water extracts of *M. balsamina* against 3 selected gut-associated bacterial species.

Bacterial species	**MIC values (mg/mL)**	
	Methanol extract	**Water extract**
Escherichia coli (35218), (25918)	0.0	0.0
Enterococcus faecalis (14913)	0.0	0.0
Proteus mirabilis (7002)	0.0	0.0

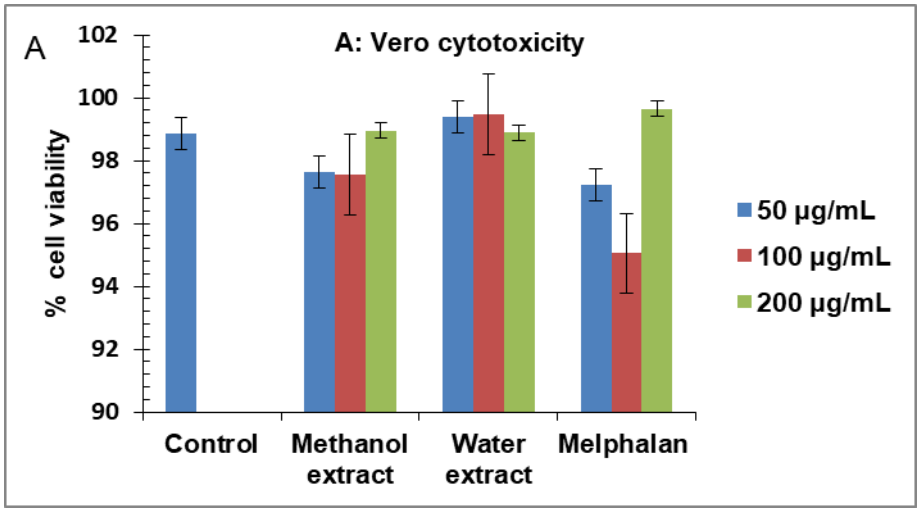
Momordica balsamina extracts were screened for antibacterial activity against three gut-associated bacteria namely, *Escherichia coli* (35218), (25918), *Enterococcus fecalis* (14913), and *Proteus mirabilis* (7002). Results showed that both extracts exhibited no antibacterial activity against gut-associated bacteria (**Table 4.2**).

In other studies, methanolic extract of *M. balsamina* leaves was reported to exhibit inhibitory activity against a pathogenic strain of *Escherichia coli, Proteus mirabilis, Bacillus subtilis,* and *Pseudomonas auruginosa* (Otimenyin *et al*., 2008, Thakur *et al*., 2009). However, on the contrary, the methanolic extract of *M. balsamina* in the current study exhibited no activity. This could be due to the failure of extract to diffuse into the agar or fewer diffusion properties of extract in agar. This may also be associated with the extraction step either destroying or altering with active metabolites that are present in fresh plants (El Astal *et al*., 2005).

A previous study reported that methanolic extract of *M. balsamina* has antibacterial activity against gram-positive bacteria as compared to the gram-negative bacteria (Souda *et al*., 2018) and these results are contradictory to the results obtained in the current study. However, a study conducted by Shamsuddeen *et al* (2010) where aqueous extracts exhibited no antimicrobial activity against *E. coli* and these results are in agreement with the results obtained in the current study. However, this is not surprising since Sigidi *et al* (2016) also reported that water extracts barely exhibit any biological activities.

4.3.3 *In vitro* cytotoxicity screening of *M. balsamina* extracts against human colorectal adenocarcinoma (Caco2 and HT29), Vero, and RAW 264.7 cell lines.

The cytotoxic effects of *M. balsamina* extracts against CAC02, HT29, and Vero cell lines were assessed and evaluated using MTT assay. In all the 3 cell lines tested, no cytotoxic effect was observed. *M. balsamina* extracts exhibited no cytotoxicity in CACO2, HT29, RAW 264.7, and Vero cell lines (**Figures 4.3 a, b,** and **c** respectively). Both water and methanol extracts showed less toxicity as compared to the Melphalan standard as presented in **figure 4.3 a**. Cell viability at the highest concentration (200 µg/ml) for both extracts was above 98% which is comparable to cells only control. Based on visual inspection of cell viability of both extracts against all cell lines (**Figures 4.3 a-c)**, cell viability was higher with water extract as compared to the methanol extract and this could be due to the solvent or alcohol residues that might have remained in the extracts after drying. Overall, cell viability was not affected or did not decrease even in the presence of the extracts, strongly suggesting that these leaves are safe for consumption.



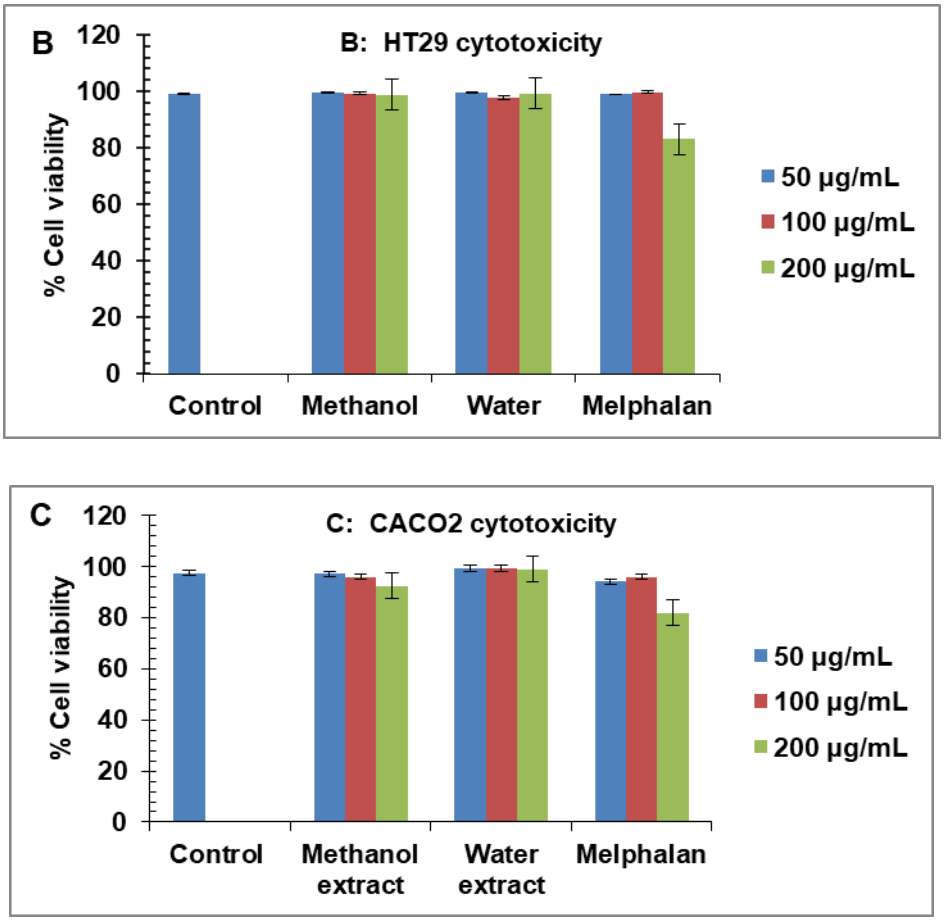


Figure 4.3: Cytotoxicity analysis of *M. balsamina* extracts against three cell lines, namely Vero cytotoxicity (**a**); HT29 cytotoxicity (**b**) and CaCo2 cytotoxicity (**c**) at 3 concentrations. Melphalan was employed as a positive control.

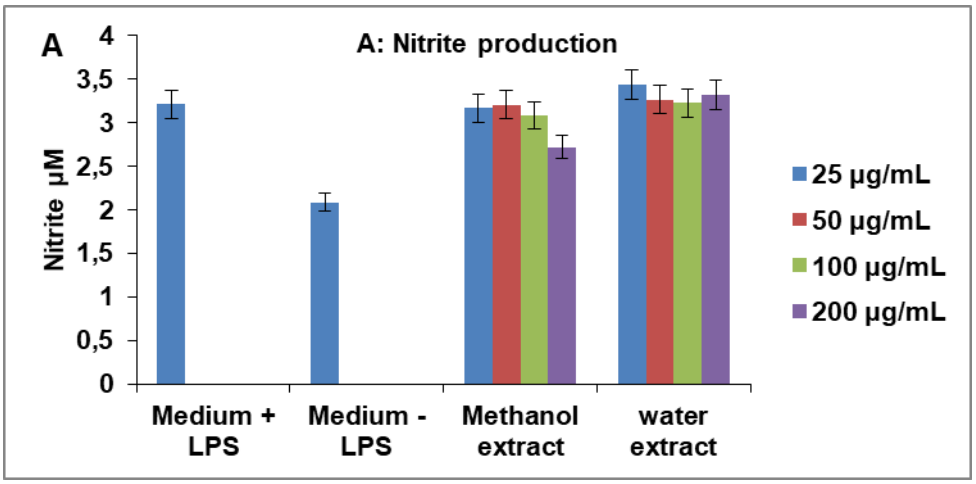
Medicinal plants may be considered effective in clinical applications provided if the preparations show selective toxicity to the targeted microorganism (Sigidi *et al*., 2016). The findings confirmed that *M. balsamina* leaves have no cytotoxic activity against human colorectal adenocarcinoma cell lines HT29 and CACO2.

According to a study done by Ramalhete *et al* (2010), *M. balsamina* compounds showed no cytotoxicity against human breast cancer (MCF-7) cell lines. Furthermore, *in vivo* studies have demonstrated that *M. balsamina* extracts have shown an extremely weak or inactive toxicity (Benoit-Vical *et al*., 2006; Ramalhete *et al*., 2010). In another study done by Ramalhete *et al* (2011), on the activity of *M. balsamina* against bacterial efflux pumps, the extracts showed no toxicity against human lymphocytes (Ramalhete *et al*., 2011). These findings support the observed lack of toxicity of *M. balsamina* extracts despite the diversity of the phytochemicals detected.

4.3.4 *In vitro* anti-inflammatory screening of *Momordica balsamina* extracts on RAW 264.7 cell lines.

The *in vitro* anti-inflammatory potential of *M. balsamina* extracts was evaluated on RAW 264.7 cells. The observed results (**Figure 4.4 a and b**) indicated that the methanol extract at the highest concentration (200 µg/ml) exhibited potential anti-inflammatory activity this is therefore due to the presence of anti-inflammatory exhibiting compounds such as flavonoids and phenolic compounds, as identified by phytochemical analysis assays conducted in this study. In support of the results obtained in this study, Nagarani *et al* (2014) highlighted that *Momordica* species have potential anti-inflammatory activity and it is possible that anti-inflammatory effects may be correlated to the phytochemical composition of the plant.

Macrophages have been reported to generate myraid pro-inflammatory signals which alter the functionality of surrounding cells (Sigidi *et al*., 2016). These signals include the production of nitric oxide which participates in multiple cytotoxic and regulatory activities as well as plays an essential role in mediating the human innate immunity (Sigidi *et al*., 2016).



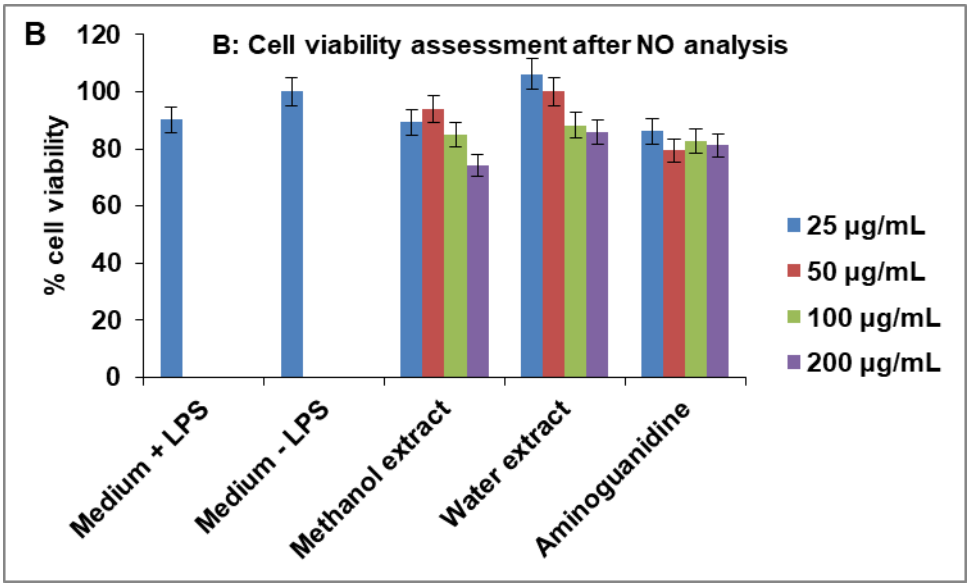


Figure 4.4: Anti-inflammatory analysis of *M. balsamina* extracts in RAW 264.7 cell lines; (**A**): Effect of plant extracts on the production of nitrate and cell viability in LPS-stimulated and unstimulated RAW macrophages. (**B**): Aminoguanidine, an inhibitor of iNOS expression serves as a positive control to confirm the functionality of the assay.

A study done by Thakur *et al* (2009) revealed that methanolic extract of *M. balsamina* exhibited anti-inflammatory activity and this is in agreement with the current study. Sigidi *et al* (2017) defined NO as an intracellular free radical that can be generated in different mammalian cells. This molecule plays a role in neurotransmission, both acute and chronic inflammation as well as host defense mechanisms against different pathogenic microbes. However, it may induce a toxic reaction against host tissue if produced in higher levels (Sigidi *et al*., 2016; 2017).

The results obtained in the current study revealed that the water extract exhibited neither anti-inflammatory nor cytotoxicity potential. This is in correspondence with Sigidi *et al* (2016) who highlighted that this outcome is not shocking because as reported by previous studies, some water extracts do not exhibit any bioactivity. In this study, flavonoids were detected in extracts and these have been reported as naturally occurring anti-inflammatory agents (Read, 1995). In support of that statement, a few studies have reported these bioactive compounds to have anti-inflammatory properties through inhibition of transcription factors and regulatory enzymes that play a crucial role in controlling inflammatory mediators (Kumar and Pandey, 2013; Maleki *et al*., 2019).

Flavonoids may inhibit enzymes such as tyrosine and serine-threonine protein kinases that are crucial in inflammatory processes; inhibition is ascribed to competitive binding of flavonoids with ATP at catalytic sites on the enzymes (Kumar and Pandey, 2013). Protein kinases are involved in signal transduction and cell activation during an inflammatory response (Maleki *et al*., 2019). Thus, flavonoids also target several central kinases that participate in several signalling pathways (Kumar and Pandey, 2013).

Flavonoids have also been reported as potent inhibitors of phosphodiesterases that play a role in cell activation and thus the anti-inflammatory impact of flavonoids is on the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Kumar and Pandey (2013) further highlighted that flavonoids can inhibit the expression of isoforms of inducible cyclooxygenase, lipooxygenase, and nitric oxygen synthase which are crucial to produce plethora nitric oxide and other mediators of inflammation such as cytokines and chemokines. Therefore, flavonoids exhibit anti-inflammatory activity hence it is important to highlight that the anti-inflammatory activity of *M. balsamina* extracts may be linked to the presence of flavonoids in the extracts.

Qualitative and quantitative analysis of the phytochemical composition and toxicological effects of *Momordica* leaves are pivotal because these leaves can be suitable for incorporation into functional food in consideration of traditional and scientific knowledge of diverse assessments (Nagarani *et al*., 2014).

CHAPTER 5

General conclusion and recommendations

5.1 Conclusion

The phytocompound profiling of *M. balsamina* leaf extracts demonstrated a variety of secondary metabolites such as flavonoids, alkaloids, tannins, cardiac glycosides, terpenoids, and acids. The assessment of bioactivities confirmed that the compounds present in the leaves were biologically active due to the potential antioxidant and anti-inflammatory activities exhibited by the methanolic extract.

However, the extracts showed neither antibacterial activity against gut-related bacteria nor cytotoxic potential against adenocarcinoma cell lines. In conclusion, this study has confirmed that *M. balsamina* leaves contain myriad health-promoting compounds.

5.2 Recommendations

Further antioxidant and anti-inflammatory assays need to be conducted to further prove that *M. balsamina* leaves may indeed be used as antioxidant and anti-inflammatory agents. Further studies may also investigate the biotransformation events of polyphenolic compounds by gut microbiota and evaluate how polyphenolic compounds in *M. balsamina* leaves are transformed by the gut bacteria.

CHAPTER 6

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