ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY ANALYSIS OF PHENOLIC COMPOUNDS FROM LEAF EXTRACTS OF *Ocimum gratissimum* AND *Rosmarinus officinalis*

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BSc

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Analytical Chemistry of the Department of Chemistry and Biochemistry, School of Sciences and Aerospace Studies, Moi University

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DECLARATION PAGE

Declaration by the Candidate

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the author and/or Moi University.

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DEDICATION

I dedicate this work to the Almighty God, for the strength, patience, grace and favor He has rendered to help me complete my studies.

ABSTRACT

Cancer remains a challenge in Africa despite the recent therapeutic advances. Due to the side effects associated with the use of clinical drugs, Lamiaceae plants such as *Ocimum gratissimum* and *Rosmarinus officinalis* have been used as anti-proliferatives against various cancer cell lines, and their leaves used as traditional medicine in conditions such as diabetes, cancer, diarrhea and typhoid. Even then, limited studies exist on the nature and variability of polyphenols in these plants and their efficacy as anti-proliferatives on prostate, colorectal and cervical cancer cell lines. The aim of this study was to isolate, characterize and determine the antioxidant and anti-proliferative properties of phenolic compounds of crude leaf extracts of *O. gratissimum* and *R. officinalis*. Specific objectives were; to determine the total phenolic content (TPC) and antioxidant(AO) activity of crude organic extracts, to evaluate the in vitro antiproliferative activity of crude organic extracts against human prostate (DU145), cervical (HeLa229) and colorectal (CT26) cancer cell lines, to isolate and characterize phenolic compounds in bioactive crude organic extracts, to evaluate in vitro antiproliferative activity of phenolic crude isolates against DU145, HeLa229 & CT26, and to evaluate the in vitro cytotoxic activity of phenolic crude compounds. Experimental design was used in the study. The plant leaf samples were obtained from their cultivated areas in Wakiso district, Uganda by judgemental sampling. Crude organic extracts were obtained by maceration method using n-hexane, dichloromethane, ethylacetate and methanol and their total phenolic content and antioxidant activity determined by Folin Ciocalteu and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) methods respectively. In vitro antiproliferative activity of seven different concentrations (1000 to 1.37 µg/ml) of crude organic extracts against cancer cell lines was evaluated by MTT assay. The Methanol extract showed the highest antiproliferative bioactivity. The extract was fractionated using n-hexane, dichloromethane, ethylacetate and methanol, in their order of increasing polarity. The fractions were further isolated into polyphenols using Solid phase extraction (SPE) and characterized by Liquid chromatography mass spectrometry (LC-MS/MS). The isolates antiproliferative activity against cancer cell lines and cytotoxicity activity on normal vero cells was evaluated by MTT method. Doxorubicin was used as the positive control drug in all bioassays. Crude methanol extracts yielded the highest Total phenolic content (*R. officinalis*: 476.80± 0.40 µg/ml; *O. gratissimum*: 401.00±6.47 µg/ml) and % inhibition of DPPH (*R. officinalis*: 69.76 ± 0.09%; *O. gratissimum*: 61.26 ± 0.09%), with significant differences between the two plants extracts ($p<0.05$). FTIR spectra confirmed presence of phenolic groups, alcohols, aromatics, nitro groups as well as carbonyl groups which is the reason for the various medicinal properties of these plants. Gallic acid, Rutin, Catechin & Quercetin compounds were found to be common in both plants' extracts. Procyanidin, Carboxystrictosinedine, Isoferullic acid, Psychotrin, hydroxyplorentin, cephalin, Isoquercetin, Diadzin and hyperin were reported for the first time in *O. gratissimum* while 8 compounds were reported for the first time in *R. officinalis* which include Procyanidin, hydroxyplorentin, cephalin, Isoquercetin, Latifoliamide, Diadzin, hyperin and emetine. Also, methanol crude extracts had the highest antiproliferative activity on all cancer cell lines, and whose minimum inhibitory concentration (µg/ml) that killed 50% of cells (IC_{50}) was DU145-147.378, CT26-301.992 and HeLa229-432.4745 for *R. officinalis* and DU145-104.839, CT26-586.683 and HeLa229-359.914 for *O. gratissimum*. The results obtained

show that all the extracts under investigation were not toxic to normal vero cells, compared to the positive control which was doxorubicin drug (6.36 ± 0.45 µg/ml) which is potentially very toxic. Therefore, results show selective action and potential use of these plants to generate lead compounds for use in developing drugs against prostate, colorectal and cervical cancers. Further antiproliferative activity studies in pure compounds of *O. gratissimum* & *R. officinalis* as well as vivo models are recommended.

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ANOVA	Analysis of Variance
ATR	Attenuated Total Reflection
CC_{50}	Cytotoxic Concentration of extract that kills 50% of normal cells
CTMDR	Centre for Traditional Medicine and Drug Research
DAD	Diode array detector
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EA	Ethylacetate
EA SPE	Ethylacetate extract of Solid Phase Extraction
FBS	Fetal bovine serum
FT-IR	Fourier transform Infrared
GC-MS	Gas Chromatography-Mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IC_{50}	Inhibition Concentration of extract that kills 50% of cancer cells
KEMRI	Kenya Medical Research Institute
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
MEOH	Methanol
MEOH SPE	Methanol extract of Solid Phase Extraction

MS	Mass Spectrum
MTT	Dimethylthiazol-2,5-diphenyl-tetrazolium bromide
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic Resonance spectroscopy
O. gratissimum	*Ocimum gratissimum*
PBS	Phosphate buffered saline
PVPP	Polyvinylpolypyrrolidine
ROS	Reactive oxygen species
R. officinalis	*Rosmarinus officinalis*
RPMI	Roswell park memorial institute
SD	Standard Deviation
SEM	Standard Error of Mean
SI	Selectivity Index
SPE	Solid Phase Extraction
TPC	Total phenolic content
UV	Ultraviolet
WHO	World Health Organization

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CHAPTER ONE: INTRODUCTION

1.1 Background

Cancer disease is one of the leading causes of death in humans worldwide. It is characterized by irregular proliferation of cells. Cancer is a complex disease where each stage involves different biochemical, molecular and cellular events which all contribute to malignant transformation (Akter, Uddin, Grice, & Tiralongo, 2014). During development of the malignant phenotype, reactive oxygen species (ROS) induce cellular damage. Increasing evidence suggests that anti-oxidants and natural product-based compounds with anti-oxidant activity can effectively neutralize oxidative stress (which is an imbalance between free radical production and opposing anti-oxidant defenses) and thus suppress reactive oxygen species-mediated tumorigenesis (Khalighi-Sigaroodi et al., 2012).

In 2018, International Association of Cancer Registries (IARC) reported that the incidence (newly diagnosed cases), mortality and prevalence of cancer cases worldwide was attributed to 36 different types of cancer. In 2018, 18.1 million new cancer cases and 9.6 million cancer deaths were reported (IARC, 2018). Lung cancer registered 11.6% of total cancer incidence and 18.4% of mortality. Lung cancer was followed by breast cancer (11.6%), prostate cancer (7.1%), and colorectal cancer (6.1%) in mortality. Colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) are among the top-4 deadliest tumors. Lung, prostate, liver and stomach cancers represent the top-4 deadliest among males while among women are breast, lung and colorectal cancers (GLOBOCAN, 2020).

In Africa, cancer accounts for over 1 million new cases yearly and the most common include cervix, breast, liver and prostate cancers as well as Kaposi's sarcoma and non-

Hodgin's lymphoma (WHO, 2017). Despite its increasing burden, cancer management is not a major priority in developing countries and this is largely attributed to limited resources and other pressing public health concerns like Human Immune Virus/ Acquired Immune Deficiency Syndrome (HIV/AIDS), malaria and tuberculosis (Omara et al., 2020a).

Cancer is the third leading cause of death in Kenya with a rate of 28,000 deaths per year of which 60% are of persons below 70 years of age (Bray et al., 2018). Cancer is caused by both internal factors such as mutations, hormones and immune conditions and external factors like chemicals, radiation and infectious organisms (Khalighi-Sigaroodi et al., 2012). This is attributed to changes in lifestyle such as smoking, unhealthy eating, lack of physical exercises and harmful use of alcohol (WHO, 2017). Chemicals which causes cancer can also be obtained from food aflatoxins (Omara et al., 2020 b).

A series of interventions such as surgery, chemotherapy, radiotherapy and hormone therapy are involved in the treatment of cancer (WHO, 2018) but most anti-cancer drugs kill both normal and cancer cells and have strong side effects (Mondal et al., 2012). As cancer incidences rise dramatically in developing countries, the need for radiotherapy in developing countries is much greater since most patients present themselves when cancer is at its later stages (WHO, 2008). Access to radiotherapy is however severely limited because facilities for radiotherapy are only accessible in 23 of Africa's 53 countries and accessible to only big cities, reaching less than 5% of the total African population (Abdel-Wahab et al., 2013).

Over many years, medicinal plants have been used in the treatment of cancer worldwide. Over 3000 plant species out of 250,000 have been reported to have anti-cancer properties

(Kaur, Kapoor, & Kaur, 2011). An example is an extract of *Camptotheca acuminate* from the Cornaceae family (Table 1.1) which showed anti-cancer activity against brain, rectal, liver, gastro-intestinal and breast tumors and this led to isolation of an anti-cancer drug, Camptothecin (Kaur et al., 2011). However, a number of those plants, including *Ocimum gratissimum* and *Rosmarinus officinalis* belonging to the mint family Lamiaceae, have not been fully investigated for the development of new anti-cancer drugs. These plants have however shown effectiveness against prostate, breast, colorectal and cervical cancer tumors (Agyare et al., 2018) but their safety to humans as well as isolation of their main phenolic compounds as reported as the source of anti-cancer activity (Cragg & Newman, 2013; Moein, 2015) has not been fully elucidated.

Table 1.1 Some plant derived anticancer compounds in clinical use (Akter et al., 2014).

Compound	**Drug class**	**Plant source**	**Type of cancer**	**Plant part used**	**Reference**
Vinblastine, vincristine	Vinca alkaloids	*Catharanthus roseus*	Breast, liver, leukemia	Leaves	Iqbal et al. (2017)
Etoposide	Lignans	*Podophyllum* species	Colorectal	seeds	Kaur et al. (2011)
Topotecan	Camptothecins	*Camptotheca acuminate*	Ovarian & lung	Leaves & bark	Iqbal et al. (2017)
Flavopiridol	Flavones	*Dysoxylum binectariferum*	Breast	Leaves	Osafo et al. (2017)
Docetaxel & paclitaxel	Taxanes	*Taxus baccata*	Ovarian, breast & lung	Bark & leaves	Iqbal et al. (2017)
Harringtonine & isoharringtonine	Cephalotaxus alkaloids	*Cephalotaxus harringtonia*	Lung, cervical & gastric	Leaves	Iqbal et al. (2017)
Maytansine	Ansamycin macrolide	*Maytenus serrate*	Leukemia & lung	Roots & bark	Osafo et al. (2017)

Extracts of *O. gratissimum* and *R. officinalis* leaves from Lamiaceae family are widely used in traditional medicine for treating conditions such as breast, cervical and prostate cancers, diabetes, wound healing, cholera, dysentery and diarrhea (Akshay, Swathi, Bakshi, & Boggula, 2019). There is limited research about the effectiveness of the extracts against prostate, cervical and colorectal cancer and therefore, there is need to study the biochemical and cytotoxic properties of their isolated phenolic groups on biological systems as this could enable the development of new chemotherapeutic drugs against prostate, cervical and colorectal cancers.

1.2 Problem statement

In the recent years, Africa has had a great problem of various cancers which has led to an increased number of deaths (WHO, 2017). Prostate cancer is the first among the top four deadliest cancers among males. Current treatments include prostatectomy, chemotherapy, and radiotherapy which often leave patients with several complications due to their side effects such as urinary dissoluteness, bowel problems and erectile dysfunction (S. I. N. Ekunwe et al., 2010). Colorectal cancer is also fourth among the top four deadliest cancers in humans worldwide. Cervical cancer is the second among cancer prevalence in women, after breast cancer (Bourhia et al., 2019; Mesquita et al., 2019). The treatment therapies are also of high prices, cells also tend to resist the drugs and these drugs such as Vincristine sulphate, Cisplatin and doxorubicin also have side effects such as vomiting, nausea, diarrhea, constipation, weight loss, as well as teratogenesis (Roy, Attre, & Bharadvaja, 2017).

Studies carried out confirm the effectiveness of *O. gratissimum* and *R. officinalis* against prostate cancer (Petiwala, Puthenveetil, & Johnson, 2013). Flavopiridol, a natural phenol from *Dysoxylum binectariferum* was found to have significant growth inhibitory and cytotoxic properties in prostate cancer cell lines, DU-145 and PC3 (Petiwala et al., 2013). Also, fractions of *O. gratissimum* have been shown to exhibit better antiproliferative activity against prostate cancer cells than crude extracts (S. I. N. Ekunwe et al., 2010). However, studies have not been carried out to isolate the phenolic compounds of *O. gratissimum* and *R. officinalis* as these are responsible for the anti-cancer activity (Tai, Cheung, Wu, & Hasman, 2012) and also comparing their effectiveness against various cancer cells as well as their selectivity on normal cells. Therefore, there is need to isolate and characterize phenolic compounds from *R. officinalis* and *O. gratissimum* crude extracts of their leaves and study their effectiveness against selected cancer cell lines as pure phenolic compounds have been reported to be the responsible for the antiproliferative activity yet not toxic to normal cells.

1.3 Research questions

1. Does *Ocimum gratissimum* and *Rosmarinus officinalis* leaves crude organic extracts contain phenols and antioxidants?
2. Does the *Ocimum gratissimum* and *Rosmarinus officinalis* leaves crude extract contain different polyphenols?
3. Does *Ocimum gratissimum* and *Rosmarinus officinalis* leaves crude organic extracts and isolated polyphenols have antiproliferative activity against human prostate, cervical and colorectal cancer cells?

4. Are the *Ocimum gratissimum* and *Rosmarinus officinalis* crude extracts and isolated polyphenols cytotoxic to normal cells?

1.4 Hypothesis of the study

1. *Ocimum gratissimum* and *Rosmarinus officinalis* leaves crude organic extracts contain phenols and antioxidants.
2. *Ocimum gratissimum* and *Rosmarinus officinalis* leaves crude extract contain different phenolic compounds.
3. *Ocimum gratissimum* and *Rosmarinus officinalis* leaves crude organic extracts and isolated polyphenols have antiproliferative activity against human prostate, cervical and colorectal cancer cells.
4. *Ocimum gratissimum* and *Rosmarinus officinalis* crude extracts and isolated polyphenols are not toxic to normal cells.

1.5 Significance of the study

The findings of the study are expected to.

1. Provide data on efficacy of polyphenols in *O. gratissimum* and *R. officinalis* leaf extracts against human prostate, cervical and colorectal cancer cells.
2. Provide toxicity data on efficacy of polyphenols of these plants against the human normal cells.
3. Provide information as to which of *O. gratissimum* and *R. officinalis* in the Lamiaceae family is highly effective against human prostate, cervical and colorectal cancer cells and recommend for which to use under a given cancer condition.

1.6 Justification of the study

The increased cases of cancer prevalence today due to increased use of synthetic components like drugs, cosmetics and poor feeding diets among people (WHO, 2017) is a great concern to public health. The currently used treatment therapies are very expensive and unavailable to a large population, they are also toxic and do not distinguish between the normal and cancer cells hence causing side effects such as vomiting, nausea, diarrhea, constipation, weight loss, as well as teratogenesis (Roy et al., 2017). Another major challenge is drug resistance and therefore rendering some drugs ineffective to treat the intended cancer. Therefore, the need of urgent search for more drugs/or drug leads for treatment of cancer. This study was taken with this in mind and it aimed at isolating and characterizing phenolic compounds from the leaves of commonly used herbs in the community (*Ocimum gratissimum* and *Rosmarinus officinalis*) and evaluating their antioxidant and antiproliferative activity against human prostate, cervical and colorectal cancer cell lines. This study in the end will justify the use of these plants extracts as anticancer drugs in pharmaceuticals against the above mentioned cancer cells without causing harm to human normal cells.

1.7 Objectives of the study

1.7.1 General objective

To isolate, characterize and evaluate the antioxidant and antiproliferative activities of phenolic compounds of crude extracts of *Ocimum gratissimum* (African Basil) and *Rosmarinus officinalis* (Rosemary).

1.7.2 Specific objectives

1. To determine the total phenolic content and antioxidant activity of crude organic extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves.
2. To isolate and characterize phenolic compounds in the active crude extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves.
3. To evaluate the in vitro antiproliferative activity of crude organic extracts and phenolic isolates of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves against human prostate, cervical and colorectal cancer cell lines.
4. To evaluate the *in vitro* cytotoxic activity of crude organic extracts and isolated phenolic compounds of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves.

CHAPTER TWO: LITERATURE REVIEW

2.1 Cancer disease

Cancer is when abnormal cells divide in an uncontrollable way and this may eventually lead to the patient's death if untreated (Hejmadi, 2013). It arises due to a defect in one or more genes responsible for cell division which makes the cells divide uncontrollably to form a tumor. Normally, when the body's cells grow old or become damaged, they die and new ones take their place. When cancer develops, this order is broken and old or damaged cells survive when they should die yet new cells are also being formed when they are not needed which results into tumors (Pudata, Subrahmanyam, & Jhansi, 2011). Cancers may form solid tumors while others may not be solid for example cancer of the blood. Cancerous tumors can also be malignant which can spread into nearby tissues, while others are benign that don't spread into nearby tissues and when these ones are removed, they don't usually grow back while the malignant ones sometimes do (Mazalovska & Kouokam, 2020).

2.2 Cancer burden

About 90.5 million people had cancer in 2017 (WHO, 2018) and about 14.1 million arise per year causing about 15.7% deaths per year. The most common types of cancers in females are lung, breast and human cervical cancers whereas prostate, colorectal and stomach cancers are common in males (WHO, 2018). In 2012, about 165, 000 children were diagnosed with cancer while the risk of cancer increases significantly with age (WHO, 2018).

Approximately 70% of global cancer burden is in low- and middle-income countries like Kenya (Kenyan Network of cancer organizations, 2010). Cancer is the third leading cause

of death in Kenya with a rate of 28000 deaths per year and 60% are below 70 years of age amongst those affected (WHO, 2018). According to Nairobi Cancer Registry, 23.3% of all registered cases were of human cervical cancer, cervical cancer as 20% and prostate as 9.4% and health systems in Kenya are putting much emphasis on prevention of communicable diseases yet non-communicable diseases like cancer are also on the rise and this is as a result of poor health and diagnostic facilities, lack of financial resources as well as lack of awareness (Bray et al., 2018). (WHO, 2020). In 2015, deaths due to non-communicable diseases cancer had risen to 72% globally. 85% of these were due to cancers (Juma et al., 2019).

A series of interventions such as surgery, chemotherapy, radiotherapy and hormone therapy are involved in the treatment of cancer (WHO, 2008) and most anti-cancer drugs kill both normal and cancer cells and have various side effects on the patients such as hair loss and anemia.

2.3 Plant use in treatment of cancer

Over many years, plants have been used in the treatment of cancer and over 3000 plant species out of 250,000 have been reported to have anti-cancer properties (Kaur et al., 2011) for example an extract of *Camptotheca acuminata* showed ant-cancer activity which led to isolation of an anti-cancer drug, Camptothecin (Kaur et al., 2011). However, there a number of plants identified with ant-cancer properties that have not yet been investigated for the isolation and development of new anti-cancer drugs with *Ocimum gratissimum* and *Rosmarinus officinalis* among the list.

2.3.1 *Ocimum gratissimum* L.

2.3.1.1 Description of *Ocimum gratissimum* L.

Ocimum gratissimum is a small herb (Figure 2.1) belonging to the Lamiaceae family and also known for its scented leaves. In Uganda, it is commonly called "*Mujaaja*", while the Maasai of Kenya know it as "*Olemoran*" and Nigerians call it "*Nchanwu*". It is found in the wild or cultivated throughout the tropics and subtropics (Okoli, Ezike, Agwagah, & Akah, 2010). The leaves have a strong aromatic odor and is among the plants in the group known as spices. It's also known as African basil from the genus of *Ocimum*. It's a perennial herb and un-endangered in the tropics and subtropics. It is 1-3m tall, stem is erect and much branched. When fully ready for consumption, the plant is 3m tall and the leaves tend to fall off with a woody and branched stem (Nweze & Eze, 2009). Information is lacking on plant yields between cultivated and wild species.

Figure 2.1 *Ocimum gratissimum* leaves

Ocimum gratissimum leaves prevent bacterial growth and therefore used as a preservative (Philippe, Guy, Paulin, & Issaka, 2012). Its leaves are traditionally used as digestive, carminative, aromatic and tonic agent. It is also known for its antimicrobial activity (Monga, Dhanwal, Kumar, & Kumar, 2017), antimalarial, antiviral (Mahapatra,

Chakraborty, Das, & Roy, 2009), anesthetic, antidiabetic, antifertility, anti-inflammatory and anti-stress activity (Usunomena & Eseosa, 2016). These effects are due to free phenolic compounds that are easily extracted by organic solvents (Quatrin et al., 2019).

Rosmarinic acid was found to be the most abundant phenolic acid in Ocimum species according to the following order *O. basilicum* L., *O. gratissimum* L. and *O. canum* Sims when phytochemical analysis was done by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) (Tshilanda et al., 2016). Strong antioxidant activities of ethanolic extracts of *O. gratissimum* was reported and therefore its potential as an anti-cancer agent (Usunomena & Eseosa, 2016).

Antiproliferative and chemo-preventive activity of *O. gratissimum* aqueous leaf extract on HeLa cells was reported (Usunomena & Eseosa, 2016). Anti-cancer activity of Eugenol isolated from essential oils of *O. gratissimum* was reported (Raja, Srinivasan, Selvaraj, Mahapatra, & Rose, 2015) .

It was shown that aqueous crude extract of *O. gratissimum* leaves and some of its partially purified fractions inhibit proliferation of several cancer cell lines including prostate cancer (PC- 3) cells. *O. gratissimum* fractionated leaf extracts also showed increased activity (S. I. N. Ekunwe et al., 2010) and therefore the basis of this study to isolate phenolic compounds from the crude extracts of *O. gratissimum* leaves and test for its effectiveness on Prostate, colorectal and cervical cancer cells.

2.3.2 *Rosmarinus officinalis* L.

2.3.2.1 Description of *Rosmarinus officinalis* L.

Rosmarinus officinalis L. is a medicinal plant that belongs to the Lamiaceae family. It is commonly known as Rosemary. It has a characteristic aroma and a perennial herb with

pleasant smelling, ever-green, needle like leaves (figure 2.2) with white, pink, purple or blue flowers. It originates from the Mediterranean region. The Latin commonly know it as "*Ros marinus*" while the Greek commonly know it as "*Anthos*" (Petiwala et al., 2013). It is normally cultivated and propagated by means of seeds, cuttings, layering and division of roots. It is suited in both temperate and subtropical areas with most varieties thriving at temperatures between 6-24^{0}C, in a well moisture loamy soil of a neutral pH. The herb is highly thriving in Meru, at the shores of Lake Nakuru in the rift valley, Kiambu and Central Province in Kenya.

Figure 2.2 *Rosmarinus officinalis* leaves

2.3.2.2 Biological activity of *R. officinalis*

The herb has reported anti-inflammatory, anti-microbial, anti-cancer, anti-diabetic, anti-depressant, anti-obesity and ant-oxidant properties (Petiwala et al., 2013). The use of rosemary extracts for food preservation has been approved and adopted into the European Union (EU) food additive legislation by the EU (Petiwala et al., 2013). Rosemary extracts standardized to carnosic acid and carnosol, which are also phenolic compounds, due to

their strong anti-oxidant activity were used in the study where food additives , flavorings , processing aids and materials in contact with food were studied (Aguilar et al., 2009).

Phenolic compounds from Rosemary such as carnosic acid, carnosol and rosmarinic acid were found to be responsible for its high anti-oxidant activity and therefore having better anti-cancer properties (Hamidpour, Hamidpour, & Elias, 2017; Segura-carretero & Fernández-gutiérrez, 2011). Rosemary essential oils have been used to treat minor wounds and headache. They also showed anti-proliferative, anti-bacterial and anti-oxidant properties (Bhalla, Gupta, & Jaitak, 2013).

Rosemary crude ethanolic extracts have been reported to show anti-proliferative activity against breast and leukemia carcinoma cells (Borrás-Linares et al., 2014). The major compounds in the plant's extract, such as carnosic acid, carnosol, and rosmarinic acid, have been shown to induce apoptosis within cancer cells, possibly through the production of nitric oxide (Moore, Megaly, MacNeil, Klentrou, & Tsiani, 2016).

Rosemary extracts have been studied and showed chemo-prevention and tumor reduction of prostate cancer cells by phenolic carnosol and therefore the researcher recommended further studies to be conducted on purified phenolic compounds (Kar, Palit, Ball, & Das, 2012). Modern techniques of analysis have been used to isolate and identify many components of rosemary extracts and thereby prompting studies on pharmacological effects of pure compounds and investigation of their underlying metabolomics (Cattaneo et al., 2015).

2.4 Plant derived anticancer compounds

Africa is believed to have one of the oldest and diverse systems of traditional medicine. It has rich botanical and cultural diversity with various approaches in using traditional

medicine for disease curing purposes (Mbele, Hull, & Dlamini, 2017). Traditional healers are depended on by 90% of the population in Africa to meet their healthcare needs since synthetic anti-cancer drugs are beyond reach to the common people due to costs (Iqbal et al., 2017).

Only 30% of the population is catered for by the conventional system in Kenya. Meaning more than two thirds rely on traditional medicine for their healthcare needs (National Coordinating Agency for Population and Development, 2007).

Plants contain a mixture of various classes of compounds which have shown different anticancer activities (Dehelean et al., 2021). The isolation of Vinca alkaloids, vincristine and vinblastine from *Catharansus roseus* led to the introduction of a new era of use of plant extracts as anticancer agents. Taxanes from Taxus species are being used against breast and ovarian cancers (Dehelean et al., 2021). Colchicine from *Colchicum autunnale* for treatment of colorectal, breast and leukemia. Ellipticine from *Ochrosia elliptica* against breast, prostate and colorectal cancer (Iqbal et al., 2017) as shown in Table 2.1.

Table 2.1 Some plant derived phytochemicals that are used to treat different cancers with their dosages.

Natural compound	**Cancer type**	**Active concentrations (IC_{50} µm), dosage time**	**References**
Rutin	Colon cancer	100 & 200, 24 hrs	Dehelean et al. (2021)
Curcumin	Breast Cervical Colon	1-50, 72 hrs 12-14, 48 hrs 1-50, 24 hrs	Iqbal et al. (2017)
Quercetin	Pancreatic Cervical Colorectal	40, 24 hrs 100, 24 hrs 35, 24 hrs	Dehelean et al. (2021)
Betullinic acid	Ovarian Colorectal	44.5, 24 hrs 5, 72 hrs	Dehelean et al. (2021)

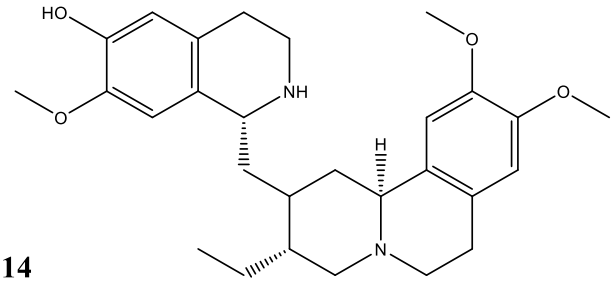
2.5 Phytochemicals

Phytochemicals are naturally occurring chemicals produced by plants through primary or secondary metabolism. They give plants colors, distinctive aromas and can help plants to attract pollinators (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017), they also help them retard predators and competitors. As pigments, Anthocyanin are widely spread through the plants fruit, vegetables, leaves and flowers and are responsible for most of the colors in plants. They are very efficient in absorbing and dissipating light energy via excited state proton transfer or charge transfer mediated internal conversion without formation of appreciable excited triplet state (C. P. Silva et al., 2020). Phytochemicals can be classified into major categories such as.

2.5.1 Alkaloids

These are made of ammonia compounds comprising of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen. Examples of some plant derived alkaloids include sanguinarine, indole, nicotine, isoquinoline, cephalin (14). A few of these alkaloids have been discovered in both *O. gratissimum* and *R.* officinalis extracts. The compounds have basic properties and are alkaline in reaction, turning red litmus paper blue. Depending on the structure of the molecule and location of the functional groups, the degree of basicity varies (Galtung, 2004). The solutions of alkaloids are intensely bitter and these nitrogenous compounds function in the defense of plants against herbivores and pathogens, and are widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities (Altemimi et al., 2017). In nature, the alkaloids

exist in large proportions in the seeds and roots of plants (Madziga, Sanni, & Sandabe, 2010).



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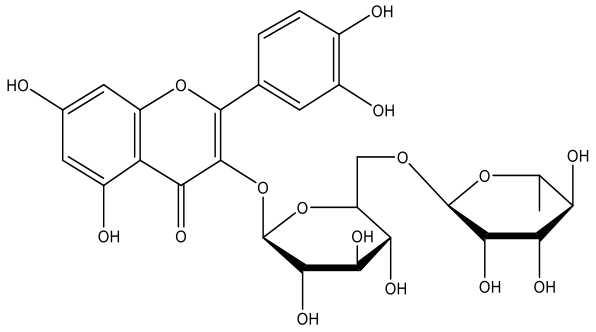
2.5.2 Glycosides

Glycosides are the condensation products of sugars. They are colorless, crystalline carbon, hydrogen and oxygen-containing though some contain nitrogen and sulfur and are water-soluble phytoconstituents, found in the cell sap. Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) (Raja et al., 2015). Alcohol, glycerol or phenol represents aglycones. Examples of some plant derived glycosides include beta-glycosides, aglycone, glucoside, quercetin. Only quercetin compound of these has been discovered in both *O. gratissimum and R. officinalis* extracts (Baskaran, Pullencheri, & Somasundaram, 2016).

2.5.3 Flavonoids

Flavonoids belong to the group of polyphenols widely distributed among the plant flora. They are made of more than one benzene ring in their structure and numerous reports support their use as antioxidants (Karimi, Jaafar, & Ahmad, 2011). Examples of some

plant derived flavonoids include dihydroflavanol, catechin, Quercetin, rutin (5). A number of flavonoid compounds have been discovered in both the two plants under study.



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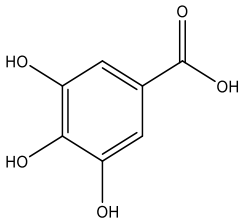
2.5.4 Saponins

The term saponin is derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant, which abounds in saponins and was once used as soap. Saponins therefore possess a 'soap like' behavior in water that they produce foam. The two major groups of saponins include: steroid saponins and triterpene saponins. They are soluble in water and insoluble in ether. Saponins are extremely poisonous, as they cause heamolysis of blood and are known to cause cattle poisoning (Kontogianni, Tomic, Nikolic, Nerantzaki, & Sayyad, 2013). They are mostly amorphous in nature, soluble in alcohol and water, but insoluble in non-polar organic solvents like benzene and n-hexane.

2.5.6 Tannins

These are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, that is, to convert things into leather. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or carboxylic

group (Kontogianni et al., 2013). Examples of some plant derived tannins include gallic acid (2), Procyanidin, castalagin. Tannins are divided into hydrolysable tannins and condensed tannins. They are used as antiseptic and this is due to presence of the phenolic group. Tannin rich medicinal plants are used as healing agents in a variety of diseases like leucorrhoea, rhinorrhea and diarrhea.



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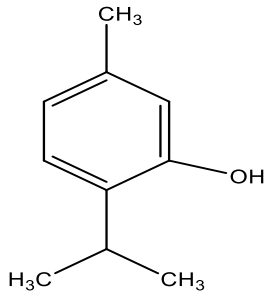
2.5.7 Terpenes

They are flammable unsaturated hydrocarbons, existing in liquid form found commonly in essential oils, resins or oleoresins (Raja et al., 2015). They are of the general formula $(C_5H_8)_n$ and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. Examples of commonly important monoterpenes include terpinen-4-ol, thujone, camphor, eugenol and menthol. Diterpenes are known as anticancer agents and the triterpenes are known for anti-inflammatory, sedative, insecticidal or cytotoxic activities and are major components of many essential oils (Chen, 2013).

2.5.8 Essential oils

Essential oils are the odorous and volatile products of various plants. Essential oils have a tendency to evaporate on exposure to air even at ambient conditions and are therefore also referred to as volatile oils (Aziz et al., 2018). They are used in enhancing the aroma of some spices (Martinez, Lazaro, Olmo, & Benito, 2008). Essential oils are either secreted

directly by the plant protoplasm or by the hydrolysis of some glycosides and structures. Essential oils have been associated with different plant parts including leaves, stems, flowers, roots or rhizomes. Examples of some plant derived essential oils include Eugenol, thymol (39), eucalyptol, menthol, limonene. They are anti-cancer agents and therefore being cytotoxic (Bunrathep, Palanuvej, & Ruangrungsi, 2007). Chemically, a single volatile oil comprises of more than 200 different chemical components, and mostly the trace components are solely responsible for its characteristic flavor and odor (Chen, 2013). Essential oils can be prepared from various plant sources either by direct steam distillation, expression, extraction or by enzymatic hydrolysis. Direct steam distillation involves the boiling of plant part in a distillation flask and passing the generated steam and volatile oil through a water condenser and subsequently collecting the oil in florentine flasks. Depending on the nature of the plant source the distillation process can be either water distillation, water and steam distillation or direct distillation. Expression or extrusion of volatile oils is accomplished by either by sponge method, scarification, rasping or by a mechanical process.



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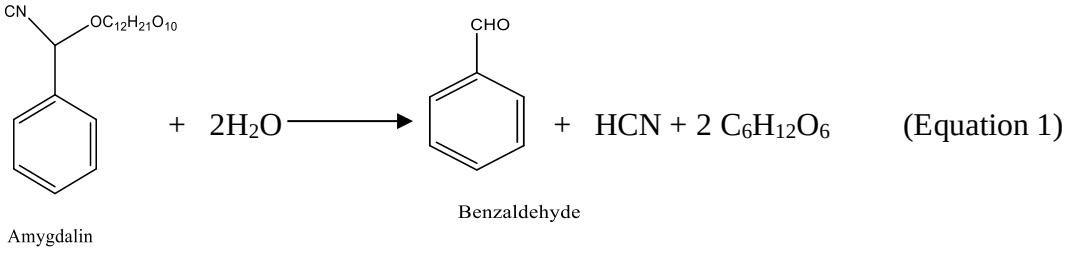


Phytochemicals can therefore be used as preservative, anti-microbial, anti-inflammatory, anti-bacterial, anti-viral as well as chemotherapeutic or chemo-preventive agents with chemoprevention referring to the use of agents to inhibit, reverse, or retard tumorigenesis (Javed et al., 2014; Yesil-Celiktas, Sevimli, Bedir, & Vardar-Sukan, 2010).

2.5.9 Antioxidants

Antioxidants protect cells against the damaging effects of reactive oxygen species otherwise called, free radicals such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite which results in oxidative stress leading to cellular damage by inhibiting oxidation (Langowski et al., 2006). These oxidants can damage cells by starting chemical reactions such as oxidizing DNA or proteins which can cause mutations and cancer while damage to proteins causes enzyme inhibition and denaturation. Natural antioxidants such as vitamin C, carotenes, Glutathione, uric acid play a key role in health maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing by donating electrons to free radicals, which neutralizes them and prevents them from causing harm (Jayasri, Lazar, & Radha, 2009). Various studies have indicated the presence of a high antioxidant activity in both *O. gratissimum* and *R. officinalis* (Meenakshi et al., 2012; Ashokkumar & Ramaswamy, 2014; Singh, 2016).

The generation of reactive oxygen species begins with rapid uptake of oxygen, activation of NADPH oxidase and production of superoxide anion radical as shown in Equation 2 (Nimse & Pal, 2015).

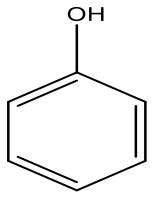
$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$$

$$GSH + \cdot OH \rightarrow H_2O + GS\cdot \qquad \text{(Equation 2)}$$

Here, a hydroxyl radical seizing a hydrogen atom from glutathione molecule results in the formation of a water molecule and a glutathione radical.

2.5.10 Phenolics

Phenolics are chemical compounds that bear at least one phenolic ring in their molecule. They occur as natural color pigments responsible for the color of fruits of plants. They are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PAL) in plants. They play a major role in plant defense against pathogens and herbivore predators, and thus are applied in the control of human pathogenic infections (Doughari, 2012). They are classified into (i) phenolic acids (40) and (ii) flavonoid polyphenolics (41) (flavonones, flavones, xanthones and catechins). Phenolics are natural antioxidants, used as nutraceuticals, and found in apples, green-tea, and red-wine for their enormous ability to combat cancer and are also thought to prevent heart ailments and sometimes are anti-inflammatory agent (Karimi et al., 2011). Their anti-oxidant behavior depends on the position of the hydroxyl groups and the number of the hydroxyl groups (Trivellini et al., 2016).



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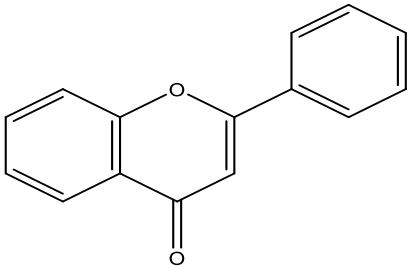

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They can be isolated purely from crude extracts by use of supercritical fluid extraction, pressurized liquid extraction, solid phase extraction and solid-liquid extraction (Segura-carretero & Fernández-gutiérrez, 2011) and by use of polyvinylpolypyrrolidone (PVPP) as proven by Mitchell, Hong, May, Wright, and Bamforth (2005) where PVPP, silica gel and PVP were compared for their adsorption capacities of phenolics (Ranatunge, Adikary, Dasanayake, Fernando, & Soysa, 2017).

Phenolic compounds identification in plant matrices is not easy because of limited standards commercially available but separation techniques have been used such as gas chromatography (GC), capillary electrophoresis (CE) and High performance liquid chromatography (HPLC) (Segura-carretero & Fernández-gutiérrez, 2011). GC requires derivatization procedures; CE has a problem of low concentration sensitivity and therefore HPLC being the most commonly used method.

A number of phenolics has been found in Lamiacea species (Trivellini et al., 2016). A number of phenolic compounds including Catechin, cinnamic acid, gallic acid, epigallocatechin, Rutin were found in HPLC isolates of *R. officinalis* leaf extracts (Segura-carretero & Fernández-gutiérrez, 2011) though not yet studied for their antiproliferative activities.

LC-MS/MS analysis of *O. gratissimum* ethanolic extract showed a greatest percentage of Rosmarinic acid, followed by other polyphenols (Venuprasad, Kandikattu, Razack, & Khanum, 2014). The results obtained agreed with results of Tshilanda et al. (2016) where extracts were analyzed by HPLC.

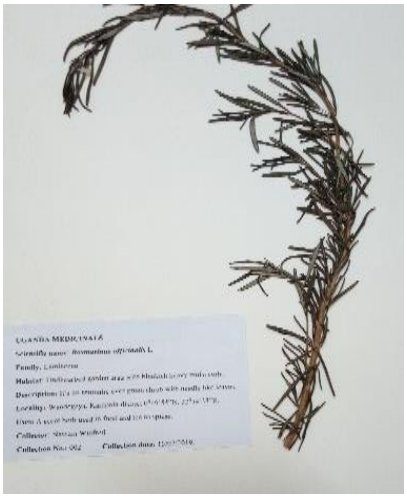
CHAPTER THREE: MATERIALS AND METHODS

3.1 Research design

The research design used was experimental design. This was mainly composed of classical scientific experiments where independent variables (solvent of extraction, concentration) were manipulated and applied to measure their effect on the dependent variables (yield, antioxidant activity, total phenolic content, antiproliferative and cytotoxicity activities).

3.2 Collection and identification of plant materials

Leaves of *O. gratissimum* and *R. officinalis* were collected on 29th July 2019 from cultivated plants in Wakiso district, Uganda ($0^0$23'36" N, $33^0$0'9" E). The area is subtropical, with hot and humid summers, with cold to mild winters with loamy soils of pH 6.40. The temperatures were 23.4^{0}C and permission was sought from Uganda Natural Chemotherapeutics Research Institute, Kampala, Uganda. The plants leaves were identified by Kyoshabire Medius (taxonomist, Natural Chemotherapeutics Research Institute, Kampala-Uganda, department of botany) and then transported to Directorate of Government Analytical Laboratory, Kampala, Uganda. Voucher samples (voucher numbers: 50908 [Plate (a)] and 50907 [Plate (b)]) were deposited at Makerere University Herbarium, Kampala, Uganda on 7th August 2019.



(a)

(b)

Plate 3.1 Herbarium specimen of (a) *O. gratissimum*, (b) *R. officinalis*

3.3 Experimental methods

3.3.1 Preparation of experimental materials

3.3.1.1 Preparation of plant materials

This was done at the Directorate of Government Analytical Laboratories, Uganda. The leaves (1000 g) of *O. gratissimum* and *R. officinalis* were separately air-dried under shade for 14 days. Using a laboratory mill, the dry material was individually ground into powder. Successful extraction was done by soaking 150 g (A_0) of *R. officinalis* and *O. gratissimum* powder separately in 775 ml of organic solvent in the 1000 ml conical flasks starting with the least polar solvent hexane, dichloromethane, ethyl acetate and then methanol. Soaking was done separately for each of the solvents and plant samples for 96 hours under room temperature. The contents were filtered using a cheese cloth. The residue was then rewashed with 100 ml of subsequent solvent and the filtrates were then combined and further filtered using Whatman filter paper followed by concentration using rotary

evaporator (Rotavapor B·U·CHI R-100, Heating bath B-100, Vacuum pump V-100, Switzerland) to dryness (Azwanida, 2015). The weight of extract obtained after dryness was recorded (A). Yield of extract was then obtained using Equation 3.

$$\% \text{ Yield} = \left(\frac{\mathbf{A}}{\mathbf{A0}}\right) \times \mathbf{100} \qquad \text{(Equation 3)}$$

Where.

A is the amount of crude extract obtained after drying

A_0 is the weight of leaves used for extraction.

The anti-proliferative bioactivity of all the crude extracts obtained from each solvents were determined and the extract that gave the highest activity was divided into two parts; one portion (1.5 g) was kept to be tested as the crude extract and the other portion (8 g) was used for further fractionation using column chromatography

3.3.1.2 Preparation of plant extracts

After complete drying of the plant extracts using a rotavapor, 10 mg of each dried extract was weighed. To each 10 mg of extract in an Eppendorf tube, 100 µl of Dimethyl sulfoxide (DMSO) was added to dissolve the extract. The solution was then vortexed for 1 minute and 900 µl of 0.01M Phosphate Buffered Saline (PBS) was added. The solution was sonicated for 20 minutes at 100% frequency. This made a stock solution of 10,000 µg/ml of extract with 1% DMSO content. DMSO content must not have exceeded 2% because it's toxic to cells (Njuguna et al., 2018).

3.3.1.3. Preparation of media and solutions for cell culturing

Media for cell culturing included both maintenance and growth media and both consisted of the following; 200 mM L-Glutamine, 3.7 g/L Sodium hydrogen carbonate ($NaHCO_3$) which was made by dissolving 3.7 g of $NaHCO_3$ in 1000ml distilled water, 9.4 g/L Minimum Earls Essential Media (MEM) made by dissolving 9.4 g of MEM in 1000 ml distilled water, 1M 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) made by dissolving 4.7660 g of HEPES in 20ml Distilled water, Phosphate Buffered Saline (PBS) made by dissolving 5 tablets of PBS in 1000ml distilled water. Then 0.2 g EDTA was added to the solution to make 0.02% EDTA/PBS and pH maintained at 7.4, 10%Fetal Buffered Saline (FBS) made by dissolving 1 g of FBS in 10 ml Distilled water, 0.4% Phenol red made by dissolving 0.4 g in 100 ml distilled water and 100 µg/ml Penicillin made by dissolving 100 µg of penicillin in 1 ml of Distilled water. The growth media was prepared by adding 5 ml of L-Glutamine to 426.5 ml MEM. This was followed by addition of 0.2 ml Phenol red, 5 ml Penicillin, 5 ml HEPES, 7.5 ml $NaHCO_3$ and finally 50 ml FBS. The solution was then filtered through a 500 ml sterile filter unit into the filter unit container and solution thoroughly mixed. Maintenance media was prepared by mixing 5 ml L-Glutamine, 465 ml MEM, 0.2 ml Phenol red, 5 ml Penicillin, 5 ml HEPES, 7.5 ml $NaHCO_3$ and then 12.5 ml FBS. The solution was then filtered through a 500 ml sterile filter unit into the filter unit container and solution thoroughly mixed.

3.3.1.4 Cell culturing

The normal Vero (normal cells), human prostate cancer cell line (DU145), Human cervical cancer cell line (HeLa229) and human colorectal cancer cell line (CT26) were obtained

from Centre for Traditional Medicine and Drug Research (CTMDR), KEMRI. The normal Vero, prostate, colorectal and cervical cancer cells were separately thawed in a water bath at 37^0C. Growth media (20 ml) was added to 1 ml of each of the cell lines in T-75 culture flasks and incubated at 5% CO_2 and 37 ^{0}C in order to revive the cells. Culturing was done for 3 days until when the cells obtained at least 80% confluence (Plate 3.2). Thereafter, the cells were passaged in order to extend the colon. The excess media was poured off, leaving the cells attached to the surface of the flask and the flask was washed 3 times with PBS. Excess PBS was poured off and then 500 µL of Trypsin-EDTA was added into the flask having cells attached to the surface. This was spread evenly on the inner surface of the flask by tilting the flask back and forth and then incubated for 3 minutes. Trypsin was added to detach the cells off the surface of the flask. Growth media (10 ml) was added immediately to stop action of Trypsin. Growth media was purged gently to allow breaking of clumps between cells.

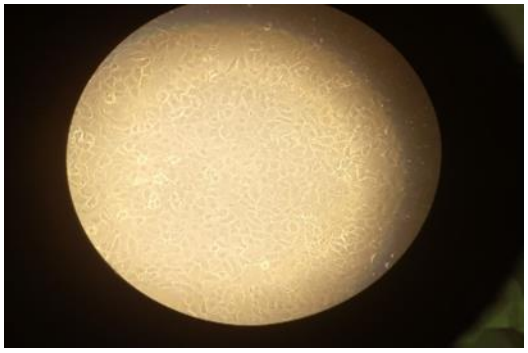


Plate 3.2 Prostate cancer cell lines at 80% confluence

3.3.1.5 Cell density determination using Trypan blue

Fifty µl of each of the cell suspension (normal Vero cells, human prostate cancer cells (DU145), human cervical cancer cells (HeLa229) and human colorectal cancer cells (CT26)) in the T-75 flask was pipetted and individually introduced into an Eppendorf flask. The cells were further diluted with 100 µl of 0.4% Trypan blue in an Eppendorf flask to make a dilution factor of 3. The mixture was then loaded onto a Hemocytometer which was fixed onto an electronic microscope to count the number of visible viable cells. The viable cells could fluoresce (Plate 3.3) while the nonviable were stained blue under the microscope. From the hemocytometer, viable cells were counted in four quarters which gave an average of 8.375 DU145 cells, 18.500 CT26 cells, 13.625 normal Vero cells and 8.750 HeLa cells. Cell density/ml was obtained using Equation 4.

$$\text{Cell density/mL} = \mathbf{A} \times \mathbf{D.F} \times \mathbf{CONSTANT} \qquad \text{(Equation 4)}$$

Where

A is the average number of viable cells obtained from 50 µl of cell suspension.

D.F is the dilution factor, CONSTANT is 10^4.

Cell density/ml of DU145 cells = $8.375 \times 3 \times 10^4 = 2.5125 \times 10^5$ cells/ml. Therefore, the total number of viable DU145 cells in the 10 ml growth media was 2.5125×10^6 cells. Total number of viable cells in CT26 was 5.5500×10^6 cells, 4.0875×10^6 cells of Normal Vero and 2.6250×10^6 cells of HeLa in 10 ml growth media. Cells of each cell line in the respective 10 ml growth media were then separately transferred to T-175 culture flask and 40 ml growth media was added so as to increase number of cells in order to obtain cells

enough for seeding on 96 well plates. The cells were incubated at 5% CO_2 and 37 ^{0}C for 4 days until when they obtained 100% confluence.

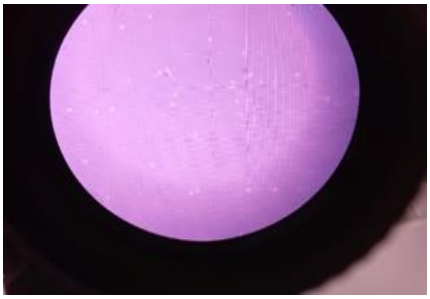


Plate 3.3 Prostate cancer cells that fluoresced under the microscope at x500000

3.3.2 Determination of the total phenolic content and antioxidant activity of the crude organic extracts of *O. gratissimum* and *R. officinalis* leaves

3.3.2.1 Quantitative analysis of the total phenolic content

Total phenols were determined using Folin-Ciocalteu reagent as described by (Baliga et al., 2013). Measured 0.5 ml of the dry crude extract (crude extracts of each of the plants extracted by the four different solvents separately) dissolved in methanol (1 mg/L) was added into falcon tubes. Gallic acid solutions (positive control) of 0, 20, 40, 80 and 100 µg/ml (dilutions from 1mg/ml made by dissolving 0.1 g of gallic acid in 100 ml distilled water) were also added separately and respectively into different tubes containing methanol : water (50:50 v/v) were mixed with 0.5 ml of Folin-Ciocalteu reagent diluted 10-fold in distilled water in falcon tubes and allowed to stand at room temperature for 5 min. 1.5 ml of sodium carbonate (Na_2CO_3) (20g in 100 ml distilled water) solution was then added and then 8.5 ml distilled water was added to the mixture. The experiment was done in

triplicates. The solutions were kept under darkness for 90 min and then the absorbance was measured at 755 nm using Gallic acid as the standard solution (Krishnaiah, Devi, Sarbatly, Bono, & Sarbatly, 2009).

3.3.2.2 Determination of antioxidant activity of crude extracts of *Ocimum gratissimum* and *R. officinalis* leaves

This was determined by use of DPPH as described by Baliga et al. (2013). Measured 8.5 ml of methanol was added to 0.1 g of the dried crude extracts (*O. gratissimum* and *R. officinalis* methanol, ethyl acetate, dichloromethane, hexane extracts) as well as Ascorbic acid (positive control) to make a stock solution. From these concentrations of 0 to 10 mg/ml (0, 2, 4, 8, 10 mg/ml) were made. The solutions were then mixed with 1ml of 0.1 mM DPPH in methanol (made by dissolving 3.94 mg of DPPH in 100 ml methanol). The mixtures were shaken vigorously and allowed to stand at room temperature in the dark for 25 min. Blank solutions (negative control) were prepared with 1ml methanol while the negative control was 1ml of 0.1 mM DPPH solution plus 2 ml of methanol. The experiment was done in triplicates and thereafter, the absorbance of the assay mixtures was measured at 517 nm with a UV-visible spectrophotometer to measure the decolorization to yellow diphenylpicrylhydrazine which is the radical scavenging of DPPH by the extracts (Awah & Verla, 2010). Mean absorbances and standard deviations were obtained, then the crude extract concentration that inhibited the free radical by 50% was considered as effective. DPPH radical inhibition was calculated using Equation 5 (Awah & Verla, 2010).

$$\text{Percent inhibition} = \left(\frac{A_S - A_O}{A_o}\right) \times \mathbf{100} \qquad \text{(Equation 5)}$$

Where

A_o is the absorbance of blank

A_S is absorbance of the sample.

From the results of % inhibition, minimum inhibitory concentration (mg/ml) of extracts that caused 50% (IC_{50}) inhibition of DPPH was obtained using linear regression analysis.

3.3.3 Evaluation of the *in vitro* antiproliferative activity of the crude organic extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves against human prostate, cervical and colorectal cancer cell lines

The experiment was done on only the crude extracts (*O. gratissimum* and *R. officinalis* n-hexane, dichloromethane, ethyl acetate and methanol extracts) and doxorubicin drug (which acted as the positive control) on all the cancer cells to check their antiproliferative bioactivity. Anti-proliferative activity of the crude organic extracts of *O. gratissimum* and *R. officinalis* leaves was measured using MTT assay (Torres et al., 2018).

After attainment of 100% confluence, the cancer cells were washed 3 times with 5 ml PBS to clear wastes and dead cells off the flask. The cells were then trypsinized as described in section 3.3.1.4 above to determine their concentration. From each of the cell lines in T-175 culture flasks, 50 µl containing $2x10^4$ cells were seeded into the 96 well plates in each of the wells apart from the wells of the blank. The plates were then incubated for 24 hours for the cells to attach themselves onto the surface of the wells.

Maintenance media 135 µl was added to each of the wells of the 96-well plate. 15 µl of the drug was added to row H of each of the plates. This led to a total volume of 150 µl of solution in row H which led to a concentration of 1000 µg/ml of drug in row H. 15 µl of solution was then picked from row H to row G which led to a serial dilution of 3. Dilution

factor 3 was obtained by dividing the final volume of row G which had come to 150 µl by the initial volume 50 µl. This gave a new concentration in row G which was obtained by dividing 1000 µg/ml which was in H by dilution factor 3 to give 333.33 µg/ml. Serial dilution from row H went on to row B and this led to 7 different concentrations (1000, 333.33, 111.11, 37.03, 12.34, 4.11 and 1.37 µg/ml).

Row A acted as the negative control. This is because drugs were not added to row A and therefore the concentration here was 0. The drugs used were the plant extracts and the commercial doxorubicin drug. The experiment was done in triplicates. The plates were incubated for 48 hours and then cell viability was determined using MTT dye.

MTT dye solution was made by dissolving 5 mg of MTT dye in 1ml PBS. After 48 hour of incubation, 10 µl of MTT dye solution was added to each of the wells in the plates and incubated for 4 hours. The media was then poured off from the wells of the plates leaving cells alone attached to the surface. 50 µl of DMSO was added to solubilize the formazan crystal formed by viable cells. Absorbance was then read on a scanning multi-well spectrophotometer at 562 nm (Nenavath & Darling, 2019). MTT is based on the ability of the mitochondria of living cells to reduce the yellow MTT to a purple formazan product as shown in Plate 3.4 (Njuguna et al., 2018).

Data was then analyzed to obtain the minimum inhibitory concentration of 50% cells, the concentrations needed to reduce absorbance of Formazan by 50% (IC_{50}) on the cancer cells (El-Attar et al., 2019). Absorbance values higher than the control cells indicate an increase in rate of cell proliferation and vice versa (Horn, Pieters, & Bezuidenhout, 2013). The % viability data was then evaluated by determining absorbance with the corresponding chemical concentrations. Linear regression analysis with 95% confidence limits and R^2

were used to define dose response curves of percentage viability of cells against concentration and to determine the IC_{50} values of the extract concentrations. Percentage cell viability was calculated using Equation 6 and Equation 7 following formula (Njuguna et al., 2018).

$$\text{Percent cell viability} = \mathbf{100} - \%\ \mathbf{cytotoxicity} \qquad \text{(Equation 6)}$$

$$\text{Percent cytotoxicity} = \frac{\mathbf{A-B}}{\mathbf{A}} \times \mathbf{100} \qquad \text{(Equation 7)}$$

Where

A is optical density of negative control

B is optical density of test drug.

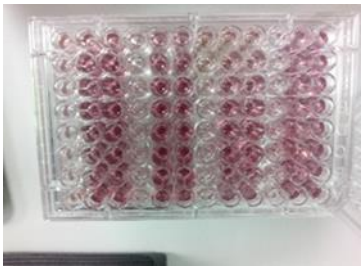


Plate 3.4. Purple color of formazan crystals after dissolving in DMSO.

3.4.4 Isolation and characterization of phenolic compounds in the active crude extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves

3.3.4.1 Column chromatographic fractionation

This was done on the methanol dried crude extract since this showed greater antiproliferative activity. 15 g of Alumina was packed into the column, followed by 1.5 g of Silica (EC-C18 50 µm particle diameter). Measured 20 ml of dichloromethane were added to the column to condition it. 8 g of dried methanol extract was added into the column

followed by 50 ml of hexane to 50 ml of dichloromethane, to 50 ml of ethyl acetate and lastly 50 ml of methanol. Each of the fractions was collected separately in a clean beaker and stored in an airtight container at 4^0C till further use (Billah et al., 2013).

3.3.4.2 FT-IR Analysis of plants extracts

The functional groups in the methanol fractions of the plants were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) (Shimadzu FTIR, Japan). Leaf extracts (0.1 g) of the two plants were dissolved in 10 ml of methanol to form a solution. 0.6 ml of sample solution was poured on ATR crystal and spectra read spectrophotometrically (4500-400 cm^{-1}) and frequencies of different components present in each sample were recorded. The resolution was 4 cm^{-1} for 20 scans on each sample. The analysis was repeated twice for confirmation of spectra. The peaks obtained were compared with published data of functional groups (Ashokkumar & Ramaswamy, 2014; Silva, Feliciano, Boas, & Bronze, 2014).

3.3.4.3 Isolation of polyphenols from crude extracts by Solid Phase Extraction and clean-up

This was done on the fraction that showed greater activity on cancer cells which was methanol and Ethyl acetate fractions**.** The end-capped C18 cartridge (sorbent mass, 500 mg; particle size, 50 µm; pore diameter, 48Å; surface area, 526 m^2/g) was conditioned with 5 ml of 10% methanol in acidified water. Measured 20 ml containing 15 mg of each fraction solution was loaded into a C-18 (Supelco, Sigma-Aldrich Germany) SPE column and allowed to flow through under gravity. The co-extracted substances were eluted from the sorbent with 100 ml of aqueous acetic acid (2% v/v). The column was dried by use of

pressure pump in the vacuum manifold for 5 minutes and total retained phenols were eluted with 1.2 ml of acidified methanol (0.1% formic acid) (Rajauria, 2018). Purified extracts were filtered through a 0.1 µm filter prior to LC-MS/MS analysis (Quatrin et al., 2019).

3.3.4.4 Characterization of phenolic isolates by Liquid Chromatography-Mass Spectrometry/Mass spectrometry

LC-MS/MS was used for characterization of the isolated phenolic compounds. The solvents used were of LC grade and obtained from Sigma Aldrich. The auto-sampler LC system was coupled to MS (Agilent 6530 Q-TOF Mass spectrometer) detector (Agilent Technologies, 6420 Triple Quad (QQQ), USA). Sample solutions of 5 µL were injected into C-18 reverse phase column (Poroshell 120 EC-C18 3X50 mm, 2.7 µm, USA) at 40°C. Data acquisition software was 6400 Series Triple Quadrupole, Version B.08.00, Qualitative analysis software used was Version B.07.00 Service Pack 1. Solvent A was made of a mixture of 0.1% formic acid in water and 0.1% ammonium formate in water. This was made by; adding 1ml of formic acid (LC-MS HiperSolv Chromanorm from VWR chemicals, Belgium) to 1000 ml of water (LC-MS Ultra ChromaSolv, Germany) and then a solution of 1g of ammonium formate dissolved in 1000 ml water and the two solutions were mixed together to form solvent A.

Solvent B was made of 0.1% formic acid in methanol which was made by adding 0.6 ml formic acid to 600 ml methanol. The elution was conducted at column flow rate 0.5 ml/min, pressure of 350 bar, column temperature 40^0C at gradient elution for 35 minutes (Zhong et al., 2018). From 0-0.5 min, elution was 95% solvent A and 5% solvent B, 0.5-12 min was 58%A and 42%B, at 12-15min was 40%A and 60%B, 15-20 min was 5%A and 95%B, 20-

25 min was 5%A and 95%B, 25-25.5 min was 90%A and 10%B and then 25-35 min was 95% A and 5% B. The eluent was monitored by Electron spray ionization connected to an ion trap MS (ESI-MS) under negative ion mode at full scan mode of 55-500 m/z (Ren et al., 2019). Identification of phenolic compounds was based on retention time in reversed phase LC and MS spectra features (Zhong et al., 2019).

3.3.5 Evaluation of *in vitro* anti-proliferative activity of phenolic crude isolates of *O. gratissimum* and *R. officinalis* leaves against selected cancer cell lines

This was done on cancer cell lines of colorectal, prostate and cervical. The treatments used here were the *O. gratissimum* and *R. officinalis* fractions (methanol, ethyl acetate, dichloromethane and hexane fractions) obtained from the methanol crude extracts and the pure phenolic isolates of SPE (SPE methanol and ethyl acetate) as well as the reference drug doxorubicin. The procedure as described in section 3.4.3 above was used.

3.3.6 Evaluation of the cytotoxic activity of phenolic crude compounds of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves.

This was tested for all the plant crude extracts, fractions and SPE phenolic isolates (crude extracts and fractions of methanol, ethylacetate, dichloromethane and n-hexane and SPE phenolic isolates of methanol and ethylacetate for both *O. gratissimum* and *R. officinalis*) on the normal vero cells (Njuguna et al., 2018). The same procedure as described in section 3.4.3 above was followed. The experiment was done in triplicates. The plates were incubated for 48 hours and then cell viability was determined using MTT. MTT solution was made by dissolving 5 mg of dye in 1 ml PBS. After 48 hours of incubation, 10 µl of MTT was added to the plates and incubated for 4 hours. The media was then removed from

the plates and 50 µl of DMSO was added to solubilize the formazan formed. Absorbance was then read on a scanning multi-well spectrophotometer at 562 nm (Nenavath & Darling, 2019). The cytotoxic concentration, which is the concentration required to kill 50% of the normal cells (CC_{50}) on normal Vero cells was calculated as well as Selectivity index of the treatments. Selectivity index is the ability of an extract to inhibit the growth of cancer cells more than it does to the normal cells. This was calculated from the ratio of CC_{50} values on normal Vero cells to IC_{50} values on cancer cells ($S.I = CC_{50}/IC_{50}$) for each of the extracts.

3.4 Data analysis

All experiments were performed in triplicates and results stored in excel data sheets. Data was analyzed and presented as mean ± standard deviation / standard error of mean. The results of yields, antioxidant activity and total phenolic content were statistically analyzed by use of Minitab 17 and presented on bar chats and tables. Differences between mean antioxidant and total phenolic content between extracts were statistically tested using independent students T-test and one-way ANOVA in Minitab 17 ($P < 0.05$). Results of antiproliferative activity were statistically analyzed using Cruzi7 Drug Cytotoxicity software version 7 and Minitab 17. The differences between treatments and controls were tested for their statistical significance using one-way analysis of variance. A value of $P < 0.05$ was considered significant. The IC_{50} and CC_{50} values were expressed as mean ± standard deviation. The presentation of results was enhanced by use of tables.

CHAPTER FOUR: RESULTS

4.1 Percentage yield of crude extracts

The percentage yields were obtained by dividing the weight of extract obtained after drying by weight of powder used for extraction in the solvent and then multiplied by 100. The results obtained show significant differences in yield of crude extracts of methanol, ethylacetate, dichloromethane and hexane of *O. gratissimum* and *R. officinalis* ($P<0.05$). Methanol crude extract of *R. officinalis* showed the highest percentage yield (54.14±0.01%) while hexane crude extract of *O. gratissimum* showed the lowest percentage yield (3.19±0.03%). The methanol extracts showed the highest yield (*R. officinalis* 81.21 g; *O. gratissimum* 41.49 g) while n-hexane extracts showed the lowest (*R. officinalis* 32.03 g; *O. gratissimum* 4.79 g) (Table 4.1). *R. officinalis* (Rosemary) extracts showed highest percentage yields with all organic solvents compared to those of *O. gratissimum* (Table 4.1). Significant differences between the means were determined by use of ANOVA.

Table 4.1 *O. gratissimum* and *R. officinalis* Percentage yields of Methanol, Ethyl acetate, Dichloromethane and hexane extracts.

Crude organic extracts	***O. gratissimum***		***R. officinalis***	
	Yield (g)	**Percentage yield**	**Yield (g)**	**Percentage yield**
Methanol	41.49	27.66 ± 0.01^{A}	81.21	54.14 ± 0.01^{A}
Ethyl acetate	30.95	20.63 ± 0.07^{B}	65.12	43.41 ± 0.02^{B}
Dichloromethane	20.61	13.74 ± 0.04^{C}	58.01	38.67 ± 0.01^{C}
n-hexane	4.79	3.19 ± 0.03^{D}	32.03	21.35 ± 0.02^{D}

Different letters in the same column show significant differences in means at 95% confidence level (P<0.05). Results of percentage mean are presented as (Mean ± SEM, n = 3).

4.2 Total phenolic content and antioxidant activity of the crude organic extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves

4.2.1 Total phenolic content of crude organic extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves

From the graph (Figure 4.1), concentrations of total phenolic content present in the plants' crude extracts were determined and presented in the Figure 4.2. The crude extracts' concentrations were obtained by use of the equation of the line (y = 0.0025x) obtained in Figure (4.1) which gave the concentration of extract. Y was the value of absorbance obtained by the sample well as X was the value of concentration to be obtained from the equation.

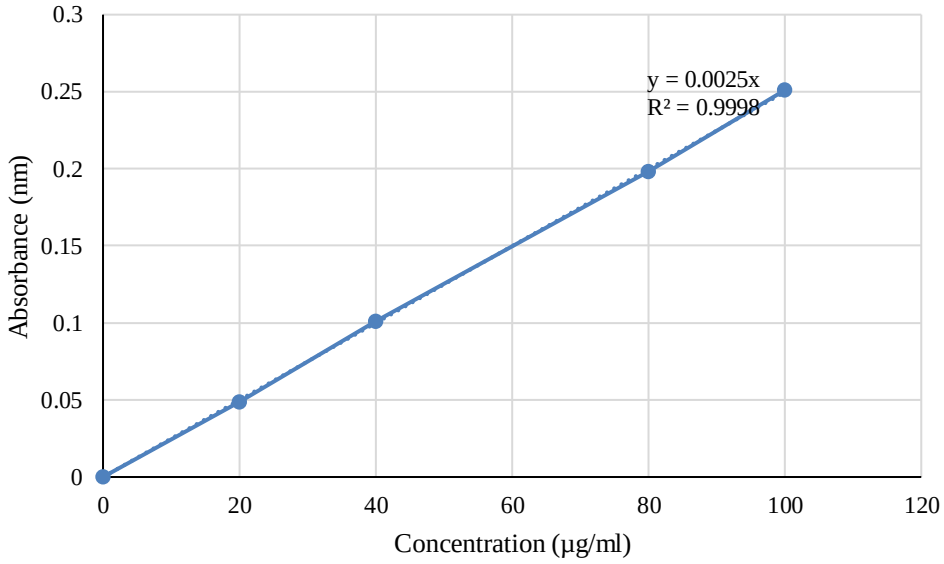


Figure 4.1. A graph showing absorbance (nm) against concentration (µg/ml) of Gallic acid standard.

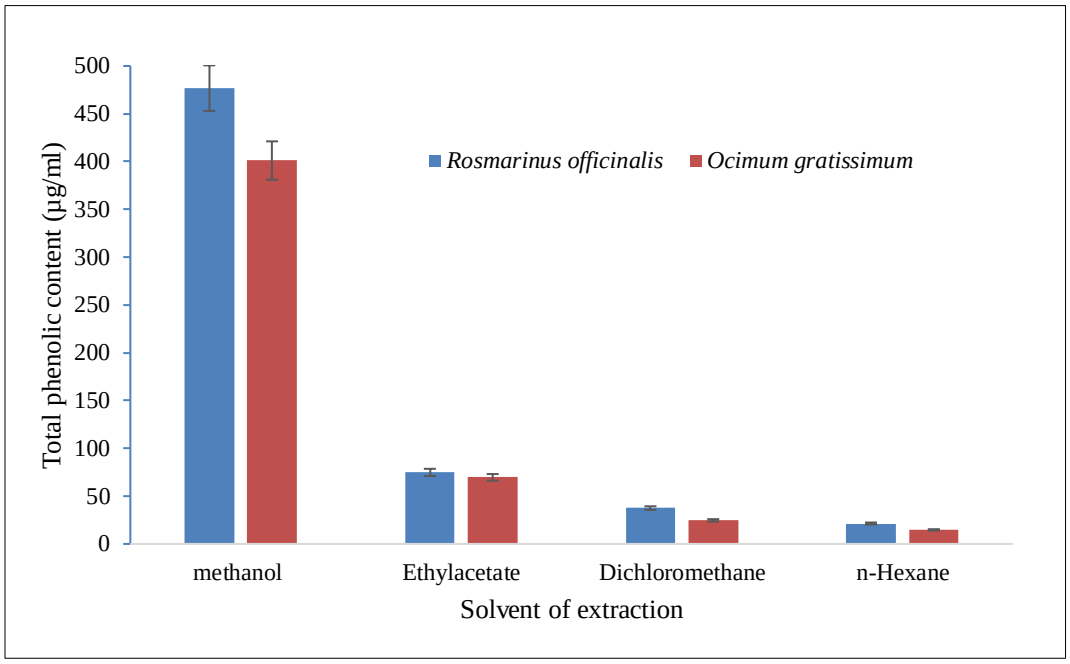


Figure 4.2 Total phenolic contents in crude extracts of *R. officinalis* and *O. gratissimum* obtained using different organic solvents.

The total phenolic content of methanolic extract of *R. officinalis* (476.80 ± 0.40 µg/ml Gallic acid equivalent) has been observed to be the highest of all organic extracts of both plants. *O. gratissimum* crude hexane extract has been shown to have the lowest total phenolic content (14.67 ± 0.58 µg/ml Gallic acid equivalent). *R. officinalis* crude organic extracts exhibited a higher total phenolic content than the *O. gratissimum* crude organic extracts. There was atleast one mean that showed significant difference ($P<0.05$) in total phenolic content mean values of both plants for methanol, dichloromethane, ethylacetate and n-hexane in ANOVA.

4.2.2 Total antioxidant activity of crude organic extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves.

From the results of percent (%) inhibition, the minimum inhibitory concentration (mg/ml) of extracts that caused 50% (IC_{50}) inhibition of DPPH was obtained using linear regression analysis. Significant differences between the mean IC_{50} values were determined by use of ANOVA and are presented in table (4.2) below. The IC_{50} results of DPPH caused by the crude extracts of *O. gratissimum* and *R. officinalis* are shown in the table (4.2) below.

Table 4.2. IC_{50} values of DPPH caused by the positive control ascorbic acid and crude extracts of *O. gratissimum* and *R. officinalis* after extraction by different organic solvents.

Extract	Ascorbic acid IC_{50} (mg/ml)	*O. gratissimum* IC_{50} (mg/ml)	*R. officinalis* IC_{50} (mg/ml)
Crude methanol	0.06 ± 0.01	5.79 ± 0.09^{D}	5.39 ± 0.09^{D}
Crude ethyl acetate	0.06 ± 0.01	6.59 ± 0.09^{C}	6.25 ± 0.33^{C}
Crude dichloromethane	0.06 ± 0.01	8.16 ± 0.24^{B}	7.50 ± 0.18^{B}
Crude hexane	0.06 ± 0.01	11.30 ± 0.0^{A}	10.10 ± 0.36^{A}

Different letters in the same column represent significant difference in means at 95% confidence level ($P<0.05$), n=3. (Mean ± S.D).

The results in the table above show that ascorbic acid has a very low IC_{50} value (0.06±0.01 mg/ml) meaning it had a very high % inhibition on DPPH and therefore the concentration of ascorbic acid required to reduce DPPH by 50% was very low compared to all the extracts. Among the extracts, methanol crude extracts of *R. officinalis* (5.39 ± 0.09) and *O. gratissimum* (5.79 ± 0.09) showed the least IC_{50} value meaning it required a lower concentration to reduce 50% of DPPH while hexane crude extracts of *R. officinalis* (10.10

± 0.36) and *O. gratissimum* (11.30 ± 0.0) showed the highest IC_{50} value and therefore a very high concentration was required to reduce DPPH by 50%. There was a significant difference in mean IC_{50} values of hexane crude extracts of the two plants ($P<0.05$), ethylacetate extracts of *R. officinalis* and methanol and ethylacetate extracts of *O. gratissimum* ($P<0.05$). There was no significant difference in IC_{50} values of methanol crude extracts ($P>0.05$) of the two plants and ethylacetate crude extracts ($P>0.05$) of *R. officinalis* and *O. gratissimum*. The results show that antioxidant activity is directly proportional to polarity of solvent used since the most polar (methanol) showed a greater activity compared to the least polar (hexane).

4.3 *In vitro* anti-proliferative activity of the crude organic extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves against selected cancer cell lines.

4.3.1 IC_{50} results of crude organic extracts with the cancer cells

Minimum inhibitory concentrations (µg/ml) required to give 50% of cell death (IC_{50}) of plant extracts on the prostate, colorectal and cervical cancer cells are shown in table (4.3) below. Doxorubicin drug showed high activity on all cancer cell lines compared to all the plant extracts ($P<0.05$). This is evidenced by Doxorubicin showing the least IC_{50} values on all cell lines (prostate 4.36 ± 0.22 µg/ml; colorectal 6.39 ± 0.47 µg/ml; cervical 3.64 ± 0.33 µg/ml) compared to the extracts. Hexane crude extracts of both plants showed the least antiproliferative activity in all organic crude extracts. In addition, hexane crude extract of *R. officinalis* did not show activity on prostate cancer cell lines while hexane crude extract of *O. gratissimum* did not show activity on colorectal cancer cell lines (Table 4.3). Methanol crude extracts showed the highest activity amongst all extracts with methanol

crude extract of *O. gratissimum* showing the highest activity on prostate 104.84±0.44 µg/ml and cervical 359.91±0.45 µg/ml cancer cell lines than *R. officinalis* ($P<0.05$). Crude methanol extract of *R. officinalis* showed greater activity on colorectal (301.99±0.53 µg/ml) (Table 4.3) cancer cell lines than *O. gratissimum* extract ($P<0.05$). There was atleast one extract that showed significant difference in mean IC_{50} values of both plants on prostate, colorectal and cervical cancer cell lines ($P<0.05$). There was no significant difference between means of methanol and ethylacetate extracts of *R. officinalis* on prostate cancer cell lines ($P>0.05$). The results showed that the antiproliferative activity was directly proportional to the polarity of solvent used in extraction as the most polar showed higher activity compared to the least polar.

Table 4.3. Antiproliferative activity of crude organic extracts of *O. gratissimum* and *R. officinalis* on prostate, colorectal and cervical cancer cell lines.

***O. gratissimum* (IC_{50} µg/ml)**				***R. officinalis* (IC_{50} µg/ml)**		
Cell lines	**Prostate**	**Colorectal**	**Cervical**	**Prostate**	**Colorectal**	**Cervical**
Crude Methanol	104.84 ± 0.44^{D}	586.68 ± 0.93^{C}	359.91 ± 0.45^{D}	147.38 ± 0.53^{B}	301.99 ± 0.53^{D}	432.47 ± 0.41^{D}
Crude Ethyl acetate	158.21 ± 0.38^{C}	626.33 ± 0.50^{B}	598.48 ± 0.47^{C}	182.48 ± 0.50^{B}	460.08 ± 0.14^{C}	522.80 ± 1.06^{C}
Crude Dichloromethane	967.21 ± 0.19^{B}	1094.41 ± 0.47^{A}	1761.50 ± 0.65^{B}	1459.10 ± 0.86^{A}	928.57 ± 0.49^{B}	931.63 ± 1.19^{B}
Crude Hexane	1259.56 ± 0.49^{A}	-	2874.81 ± 0.17^{A}	-	1104.04 ± 0.06^{A}	1001.10 ± 0.41^{A}
Doxorubicin drug	4.36 ± 0.22^{E}	6.39 ± 0.47^{D}	3.64 ± 0.33^{E}	4.36 ± 0.22^{C}	6.39 ± 0.47^{E}	3.64 ± 0.33^{E}

The results are expressed as mean ± standard deviation, n=3. Different letters in the same column represent significant difference between means at 95% confidence level in ANOVA.

4.3.2. Antioxidant activity-antiproliferative activity relationships

Correlations between antioxidant activity of crude extracts and antiproliferative activity on the cancer cell lines (prostate, colorectal and cervical) were evaluated using Pearson's Correlation Coefficient (r). The results are shown in table (4.4). From table 4.4, it was found that the antioxidant activity was positively correlated with the antiproliferative activity of crude extracts in cervical (0.987) and prostate (0.934) cancer cell lines of *O. gratissimum* and colorectal (0.938) and cervical (0.901) cancer cell lines of *R. officinalis* respectively. Therefore, the higher the antioxidant activity, the higher the antiproliferative activity on these cancer cell lines and vice versa. There was a negative correlation in prostate cancer cell line in *R. officinalis* (-0.048) and colorectal cancer cell lines in *O. gratissimum* (-0.584) (Table 4.4). The correlation was statistically significant with antioxidant activity in only cervical cancer cell lines presented to *O. gratissimum* extracts ($P<0.05$) while the correlation was not statistically significant with antioxidant activity in the prostate and colorectal cancer cell lines of *R. officinalis* and *O. gratissimum* and cervical cancer cell line of *R. officinalis* ($P>0.05$). Therefore, *O. gratissimum* extracts are more effective in prostate cancer conditions while *R. officinalis* extracts are more effective in colorectal cancer conditions.

Table 4.4. Pearson's Correlation coefficient (r) between the antioxidant activity and antiproliferative activity of crude extracts of *O. gratissimum* and *R. officinalis* on the cancer cell lines.

Cancer cell lines (r, p)					
O. gratissimum			*R. officinalis*		
Prostate	Colorectal	Cervical	Prostate	Colorectal	Cervical
0.934, 0.07	-0.584, 0.42	0.987, 0.01	-0.048, 0.95	0.938, 0.06	0.901, 0.1

The figures in the table are presented as Pearson's correlation (r), p-value).

4.4 Characterization of phenolic compounds of *O. gratissimum* and *R. officinalis* leaves extracts

4.4.1. FT-IR Analysis of Methanol fractions of *O. gratissimum* and *R. officinalis.*

The intense absorption at 3350 cm^{-1} (Figure 4.3) represents stretching of phenolic groups present in the extract. The band at 2900 represents stretching of hydroxyl groups like alcohols and water. The absorption at 2800 cm^{-1} represents a C-H group stretching which is SP_3 Hybridized (R_3C-H). Absorption at 1700 cm^{-1} is due to stretching of C=O carbonyl groups. The bend at 1550 cm^{-1} represents C=C bonds which is typical of aromatic compounds such as a benzene ring. Absorption at 1450cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. Absorption at 1350 cm^{-1} is due to Symmetric in-plane bending of $–CH_3$. The stretch at 1250 cm^{-1} is due to Nitro groups ($-NO_2$). The absorption at 1000 cm^{-1} represents C-O stretching vibrations. The weak band at 1000 cm^{-1} represents C-H bending. The weak band at 850 cm^{-1} is due to the terminal $C=CH_2$. These assignments are based on previous work on phenolic compounds in plants (Ashokkumar & Ramaswamy, 2014; Meenakshi, Umayaparvathi, Arumugam, & Balasubramanian, 2012; Singh, 2016).

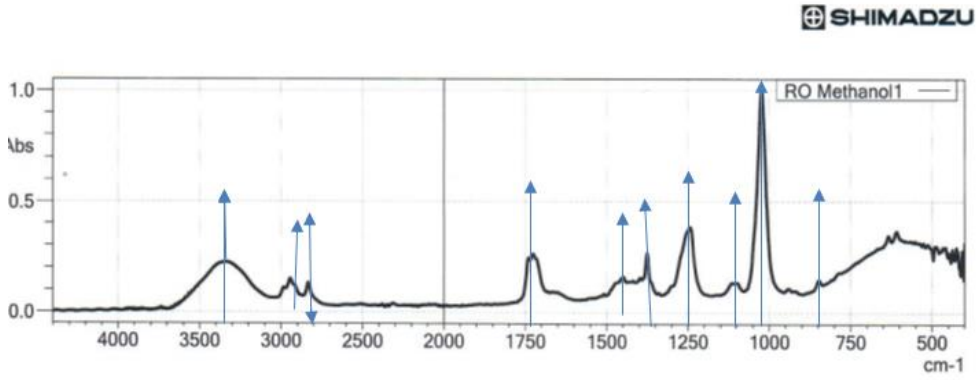


Figure 4.3 FT-IR spectrum showing Absorbance (Abs) of methanol fraction of *Rosmarinus officinalis* against wave number (cm^{-1})

The intense absorption at 3400 cm^{-1} (Figure 4.4) represents stretching of phenolic groups present in the extract. The band at 2950 represents stretching of hydroxyl groups like alcohols and water. The absorption at 2850 cm^{-1} represents a C-H group stretching which is SP^3 Hybridized (R_3C-H). Absorption at 1700 cm^{-1} represents stretching of C=O carbonyl groups. The bend at 1450 cm^{-1} represents C=C bonds which is typical of aromatic compounds such as a benzene ring. Absorption at 1400cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. Absorption at 1250 cm^{-1} represents Symmetric in-plane bending of $–CH_3$. The stretch at 1000 cm^{-1} is due to Nitro groups ($-NO_2$). The absorption at 1100 cm^{-1} represents C-O stretching vibrations. The weak band at 850 cm^{-1} is due to C-H bending. The weak band at 650 cm^{-1} represents terminal $C=CH_2$. These assignments are based on previous work on phenolic compounds in plants (Singh, 2016).

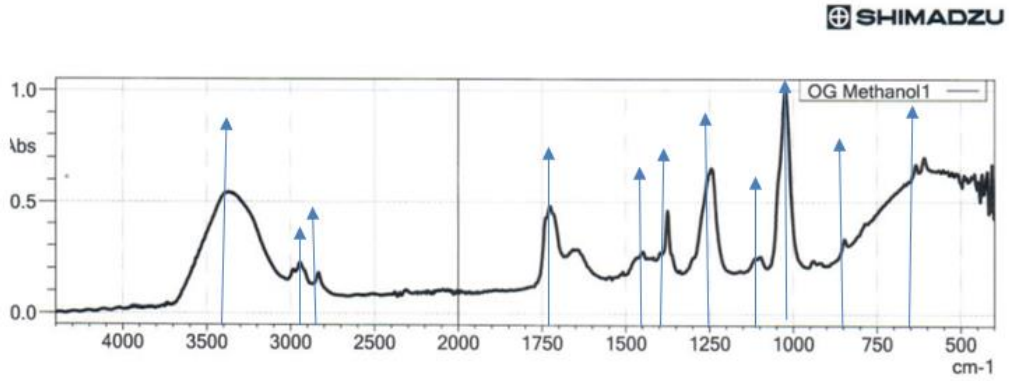


Figure 4.4 FT-IR spectrum showing Absorbance (Abs) of methanol fraction of *Ocimum gratissimum* against wave number (cm^{-1})

4.4.2 Qualitative characterization of phenolic compounds present in methanolic fractions of *O. gratissimum* and *R. officinalis* leaves crude extract by LCMS/MS after SPE cleanup.

The analysis of the extract showed presence of 34 compounds in *R. officinalis* methanol extract (Table 4.5). *R. officinalis* extract showed presence of Procyanidin (**6**), Latifoliamide

(**18**), Hesperidin (**28**) and Emetine (**32**) as phenolic compounds that were not in the *O. gratissimum* extract (Figure 4.5). The analysis revealed presence of 33 compounds characterized in *O. gratissimum* methanol extract (Table 4.5). *O. gratissimum* extract revealed presence of Tannic acid (**35**), Carboxystrictosinedine (**36**), Isoferullic acid (**37**), Ferrullic acid (**38**) and Psychotrin (**39**) as phenolic compounds that were not in *R. officinalis* extract (Figure 4.6). The characterization of observed compounds is as follows;

Table 4.5. Compounds identified from *R. officinalis* and *O. gratissimum* methanol extracts.

R. officinalis	Retention time (minutes)	*O. gratissimum*	Retention time (minutes)
Quinic acid (**1**)	2.06	Quinic acid (**1**)	2.06
Gallic acid (**2**)	2.265	Gallic acid (**2**)	2.265
Anustoline (**3**)	2.967	Anustoline (**3**)	2.967
Caffeic acid (**4**)	3.082	Caffeic acid (**4**)	3.082
Rutin (**5**)	3.128	Rutin (**5**)	3.128
Procyanidin (**6**)	8.306	Hydroxyphlorentin (**7**)	8.466
Hydroxyphlorentin (**7**)	8.466	Catechin (**8**)	8.48
Catechin (**8**)	8.48	Nepetrin (**9**)	8.90
Nepetrin (**9**)	8.90	Rosmarinic acid (**10**)	9.79
Rosmarinic acid (**10**)	9.79	Tannic acid (**35**)	11.415
Cirsimaritin (**11**)	13.81	Carboxylstrictosidine (**36**)	11.433
Rosmanol (**12**)	13.92	Isoferullic acid (**37**)	11.61
Genkwanin (**13**)	15.14	Ferrullic acid (**38**)	11.795
Cephalin (**14**)	15.57	Cirsimaritin (**11**)	13.81
Asiatic acid (**15**)	16.04	Rosmanol (**12**)	13.92
Quercetin (1**6**)	17.405	Genkwanin (**13**)	15.14
Isoquercetin (**17**)	18.558	Cephalin (**14**)	15.57
Latifoliamide(**18**)	18.669	Asiatic acid (**15**)	16.04
Diadzin (**19**)	18.826	Quercetin (**16**)	17.405
Benthamic acid (**20**)	21.91	Isoquercetin (**17**)	18.558
Augustic acid(**21**)	22.35	Diadzin (**19**)	18.826
Ellargic acid (**22**)	25.425	Psychotron (**39**)	21.117
Courmarin (**23**)	29.116	Benthamic acid (**20**)	21.91
Phlorizin (**24**)	29.148	Ellargic acid (**22**)	25.425
Hyperin (**25**)	29.161	Courmarin (**23**)	29.116
Catechin (**26**)	29.165	Phlorizin (**24**)	29.148
Naringin (**27**)	29.175	Hyperin (**25**)	29.161

R. officinalis	Retention time (minutes)	*O. gratissimum*	Retention time (minutes)
Hesperidin (**28**)	29.179	Naringin (**27**)	29.175
Gentisic acid (2**9**)	29.234	Gentisic acid (**29**)	29.234
Cathequin (**30**)	29.363	Cathequin (**30**)	29.363
Chlorogenic acid (**31**)	29.384	Chlorogenic acid (**31**)	29.384
Emetine (**32**)	29.405	Atropine (**33**)	29.719
Atropine (**33**)	29.719	Ursolic acid (**34**)	30.25
Ursolic acid (**34**)	30.25		

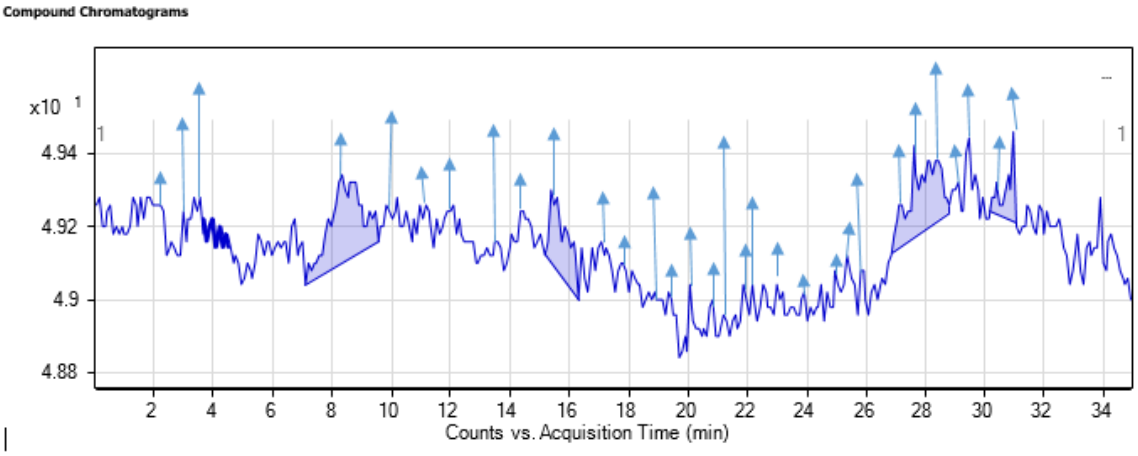


Figure 4.5 Liquid chromatography chromatogram showing abundance against retention time of compounds found in *R. officinalis* methanol extract.

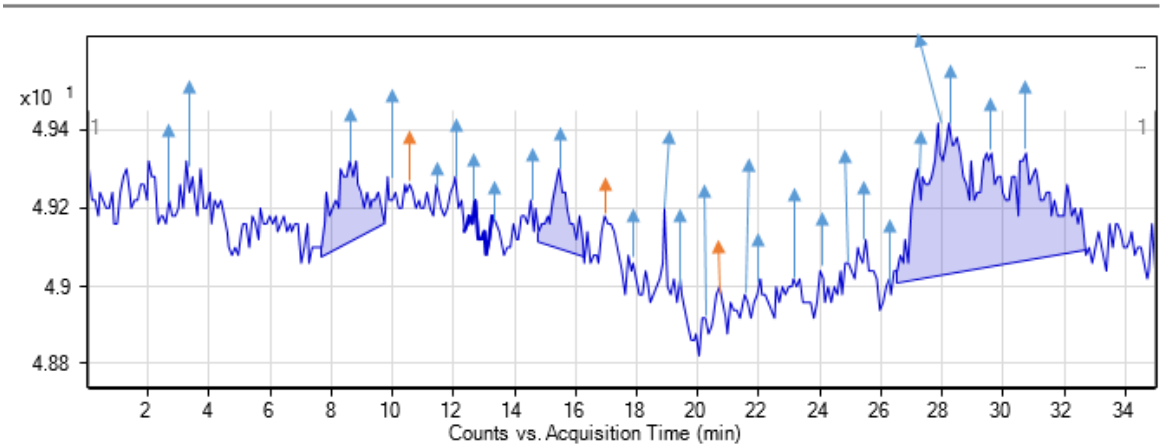


Figure 4.6 Liquid chromatography chromatogram showing abundance against retention time of compounds found in *O. gratissimum* methanol extract.

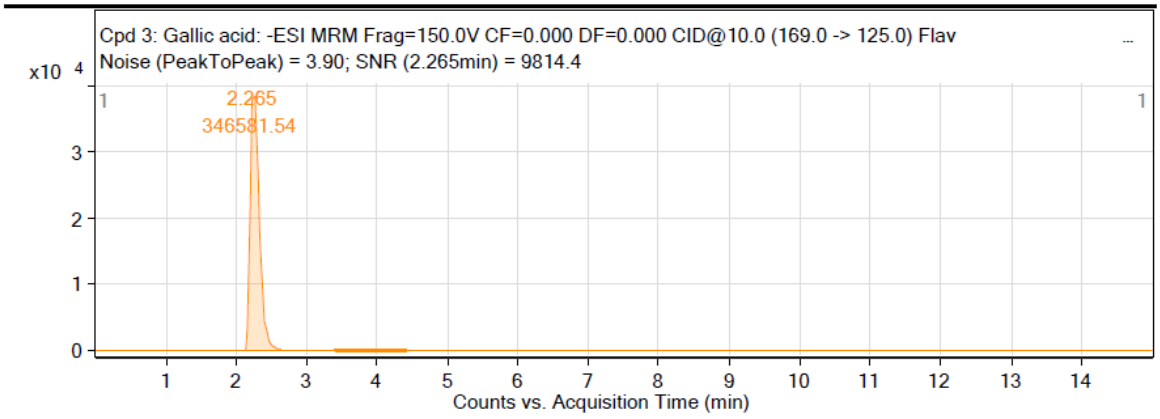
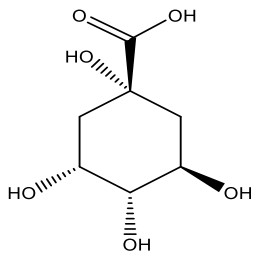


Figure 4.7 Liquid Chromatography chromatogram showing abundance against retention time (min) of Gallic acid (2) standard.

4.4.2.1 Quinic acid (1)

Peak 1 of retention time 2.06, whose precursor ion m/z 191.1 (Figure 4.8) was identified as Quinic acid (1), $C_7H_{12}O_6$ (Quatrin et al., 2019). It produced 2 fragment ions at m/z 127[M-COOH-H_2O-H] and 93[M-OH-COOH-O_2] whose neutral loss was 64.1 and 98.1 respectively (Baskaran, Pullencheri, & Somasundaram, 2016).



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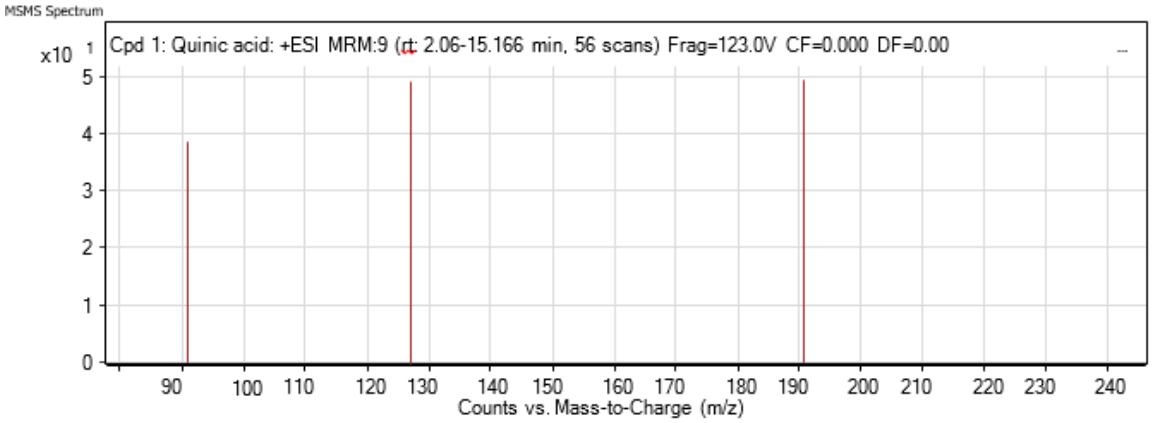
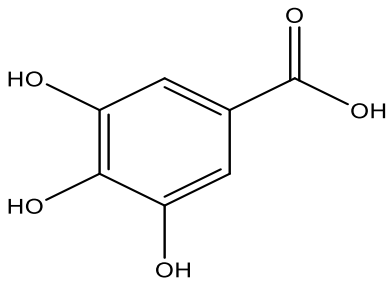


Figure 4.8 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Quinic acid (1) compound in the sample.

4.4.2.2 Gallic acid (2)

Peak 2 of retention time 2.265 and precursor ion m/z 169 showed two fragment ions at m/z 125 and 79 (Figure 4.9) and was identified as Gallic acid (2) $C_7H_6O_5$. The fragment ion at m/z 125 was due to the neutral loss of (44) a carbondioxide molecule, [M-CO_2]. The fragment ion at m/z 79 was due to the neutral loss of (90) two carboxyl molecules, [M-2COOH] (Quatrin et al., 2019). The retention time and fragment ion was compared to gallic acid standard which gave a similar retention time and fragment ions as in the sample.



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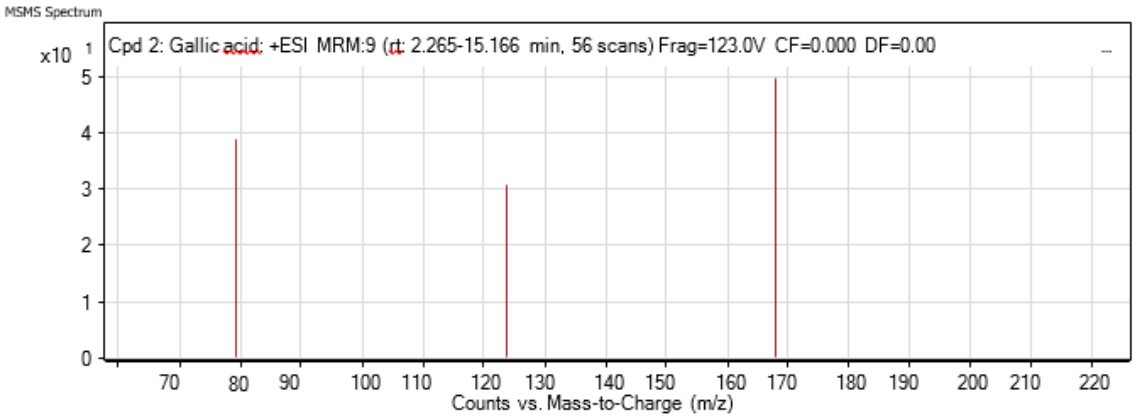
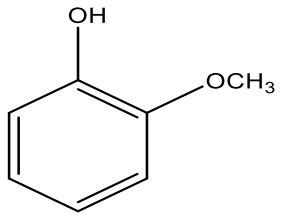


Figure 4.9 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Gallic acid (2) in the sample.

4.4.2.3 Anustoline (3)

Peak 3 of retention time 2.967 whose m/z 124.1 showed one fragment ion m/z 117.1 (Figure 4.10) causing a neutral loss of 7 which is due to loss of 7 hydrogen ions [M-H-$(H)_7$]. This was identified as Anustoline (3), $C_7H_8O_2$ (Kivilompolo, Obůrka, & Hyötyläinen, 2007).



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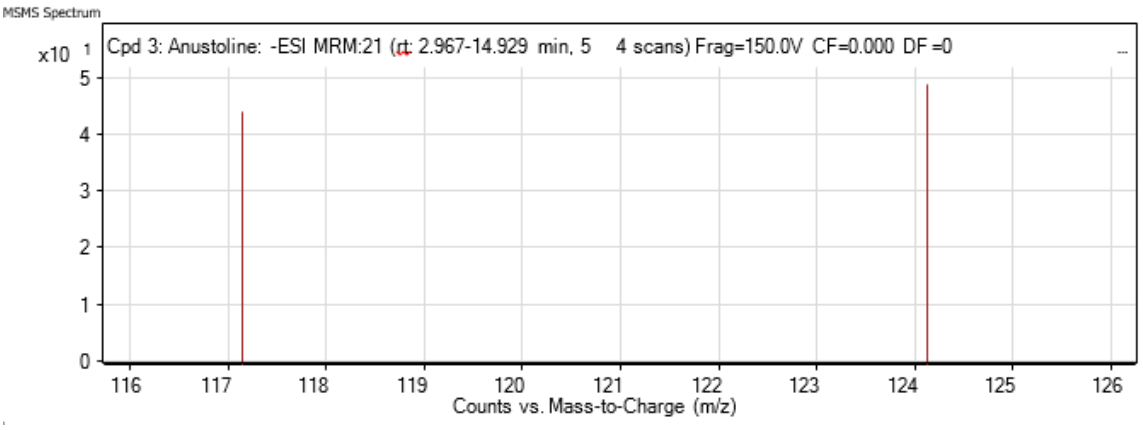
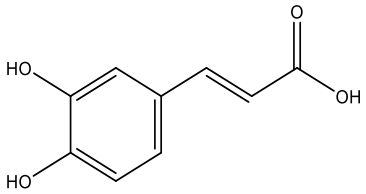


Figure 4.10 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Anustoline (3) compound in the sample

4.4.2.4 Caffeic acid (4)

Peak 4 whose retention time was 3.082 with m/z 179 (figure 4.11) was identified as Caffeic acid (4), $C_9H_8O_4$. It showed fragments at m/z 135 and 134 which is due to neutral loss of Carbondioxide [M-CO_2] (44) and a carboxyl [M-COOH] (45) respectively.



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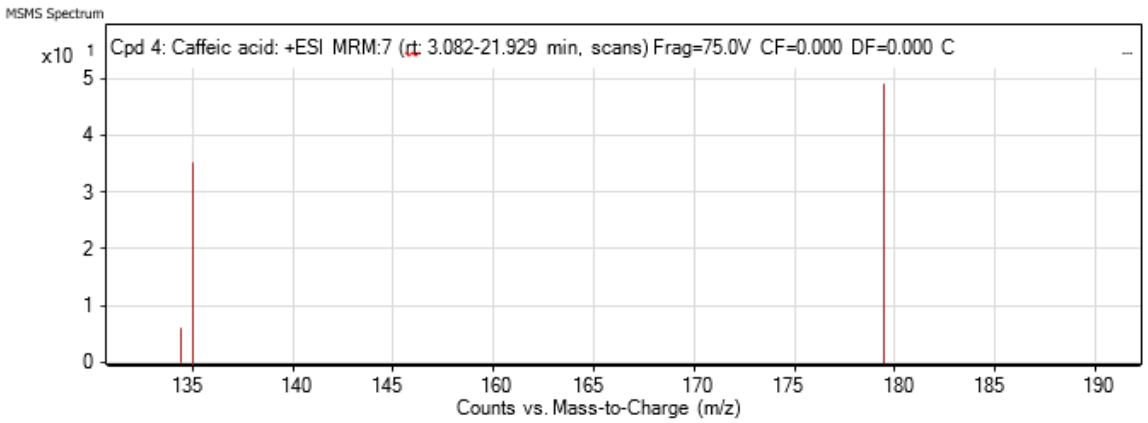
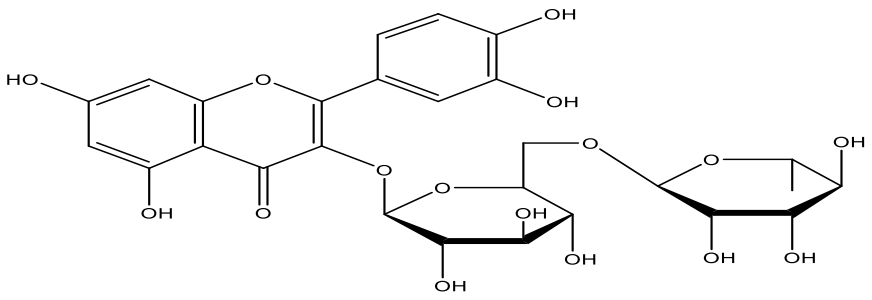


Figure 4.11 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Caffeic acid (14) compound in the sample.

4.4.2.5 Rutin (5)

Compound 5 of retention time 3.128 and m/z 609.5 was identified as Rutin (5), $C_{27}H_{30}O_{16}$. It showed two fragments at m/z 86.1 and 58.2 (Figure 4.12) which was due to the neutral loss of hydroxyl, carbondioxide and Homoplantaginin [M-OH-CO_2-$C_{22}H_{22}O_{11}$] (523.4) and apigenin-malonyl-glucoside and oxygen atom [M-O-$C_{24}H_{23}O_{14}$] (551.3) respectively (Xu et al., 2017). The liquid chromatography chromatograms and MS spectrum was compared to those of Rutin standard which gave a similar retention time and fragment ions as in the sample.



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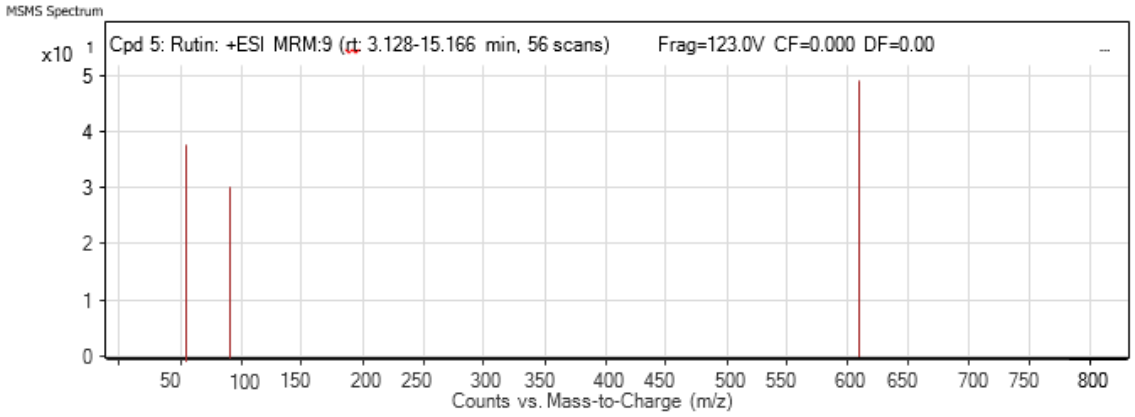
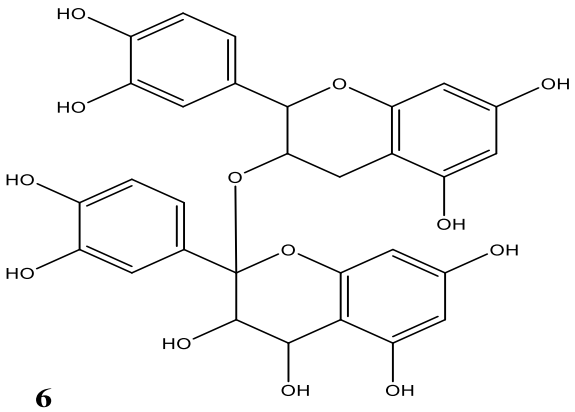


Figure 4.12 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Rutin (5) compound in the sample.

4.4.2.6 Procyanidin (6)

Peak 6 of retention time 8.306 and m/z 593.5 was identified as Procyanidin (6), $C_{30}H_{26}O_{13}$ (Proestos, Sereli, & Komaitis, 2015). It produced one fragment ion at 56.1 (Figure 4.13) which was due to the neutral loss of hydrogen molecule and apigenin-malonyl-glucoside molecules [M-H_2-$C_{24}H_{23}O_{14}$] (537.4) (Yan, Hu, Wang, Hong, & Ji, 2014).



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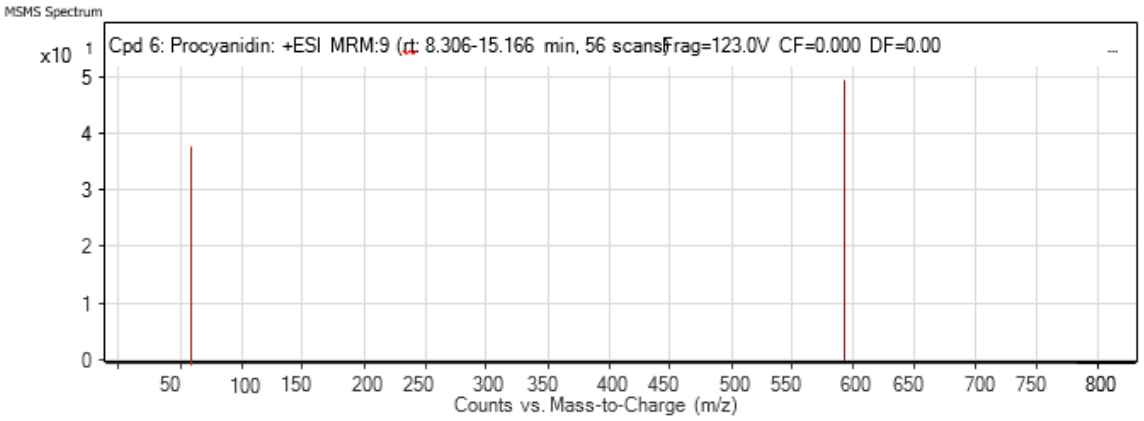
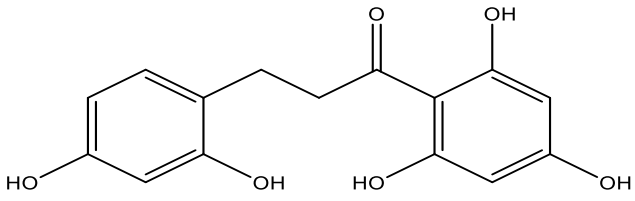


Figure 4.13 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Procyanidin (6) compound in the sample.

4.4.2.7 Hydroxyphloretin (7)

Peak 7 of retention time 8.466 whose precursor ion m/z 290.2 and produced two fragment ions at m/z 182.1 and 82 (Figure 4.14) was identified as Hydroxyphloretin (7), $C_{15}H_{15}O_6$. Neutral loss of 108.1 was due to loss of a methyl phenol molecule [M-C_7H_8O]. Neutral loss of 208.2 was due to loss of formyl radical (HCO·), a carboxyl group and $C_4H_6O_5$ [M-HCO-COOH-$C_4H_6O_5$]**.**



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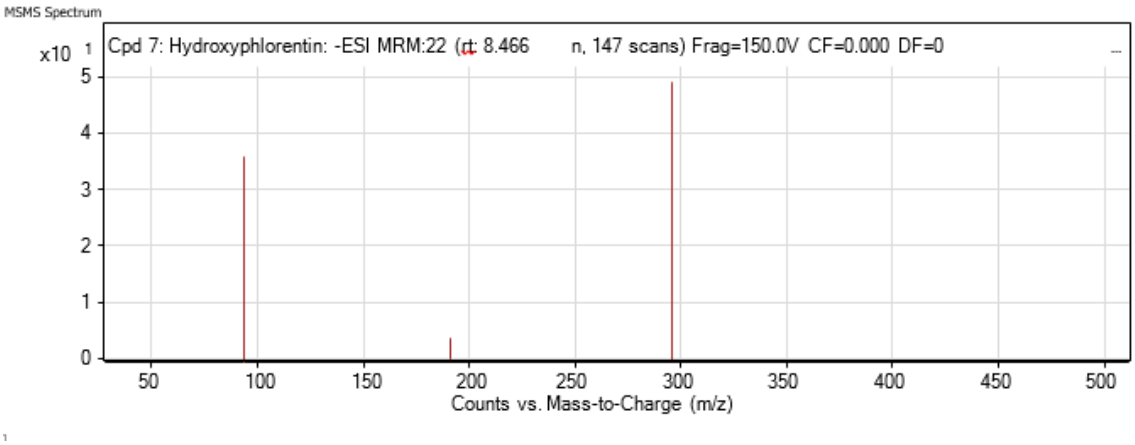
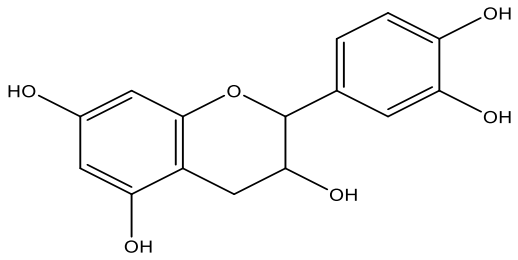


Figure 4.14 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Hydroxyphlorentin (7) compound in the sample.

4.4.2.8 Catechin (8)

Peak 8 of retention time 8.48 whose precursor ion m/z 289.2 and produced one fragment ion at m/z 55.2 (Figure 4.15) was identified as Catechin (8), $C_{15}H_{14}O_6$ (Quatrin, Pauletto, Maurer, Minuzzi, Nichelle, Carvalho, Maróstica, et al., 2019). The neutral loss of 234 was due to loss of a carbon monoxide and a tetra methyl butyl phenol molecule [M-CO-$C_{14}H_{22}O$] (Maity et al., 2013).



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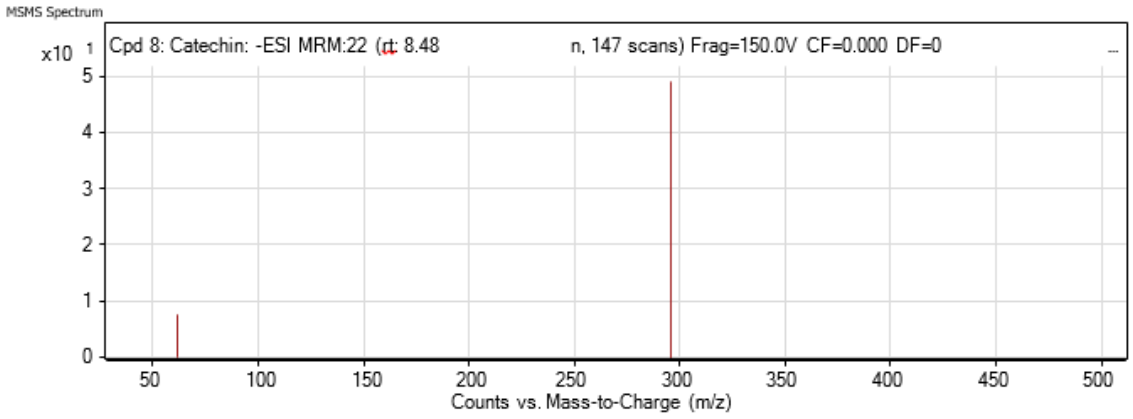
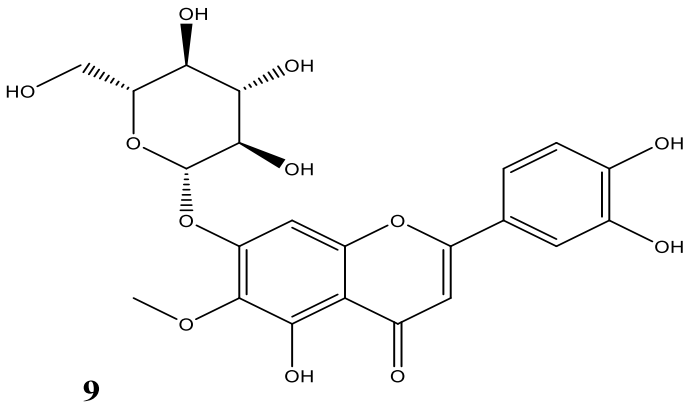


Figure 4.15 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Catechin (8) compound in the sample.

4.4.2.9 Nepetrin (9)

Peak 9 of retention time 8.90 with precursor ion m/z 477.1 was identified as Nepetrin (9), $C_{22}H_{22}O_{12}$. It gave one fragment ion at m/z 315.1 (Figure 4.16) which was due to the neutral loss of Methyl cinnamate (162) [M-$C_{10}H_{10}O_2$](Lee et al., 2018).



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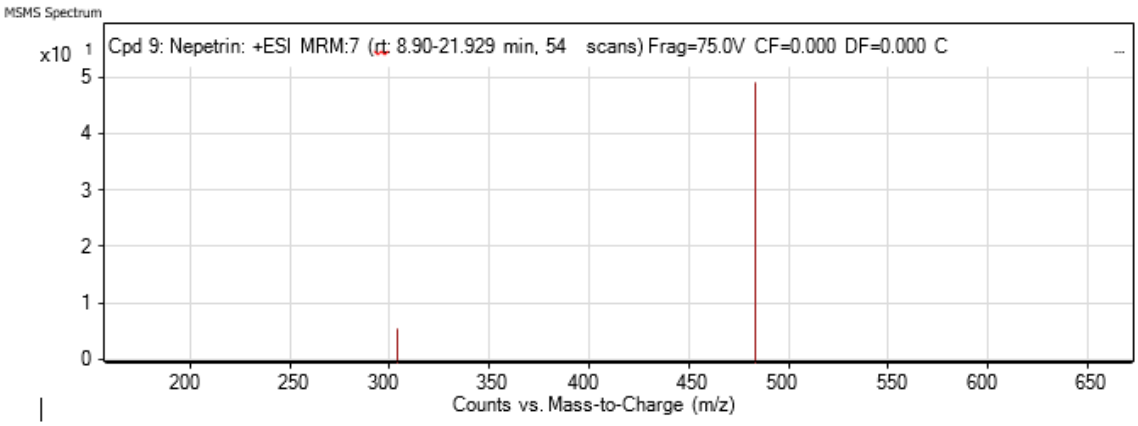
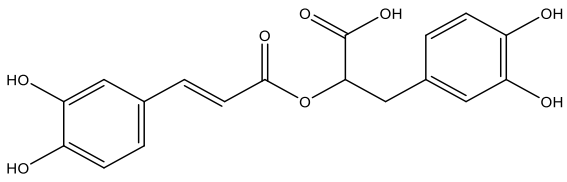


Figure 4.16 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Nepetrin (9) compound in the sample.

4.4.2.10. Rosmarinic acid (10)

Peak 10 of retention time 9.79 whose precursor ion of m/z 359.1 was identified as Rosmarinic acid (10), $C_{18}H_{16}O_8$ (Amoah & Biavatti, 2018). The precursor ion gave 2 fragmentation ions at 123.0 and 161.0 (Figure 4.17) whose neutral loss was due to (236.1) loss of Ethyl glucuronide and CH_2 molecules [M-$C_8H_{14}O_7$-CH_2] and (198.1) loss of water and Caffeic acid molecules [M-H_2O-$C_9H_8O_4$] respectively (Damasius, Kaskokiene, & Marušk, 2014).



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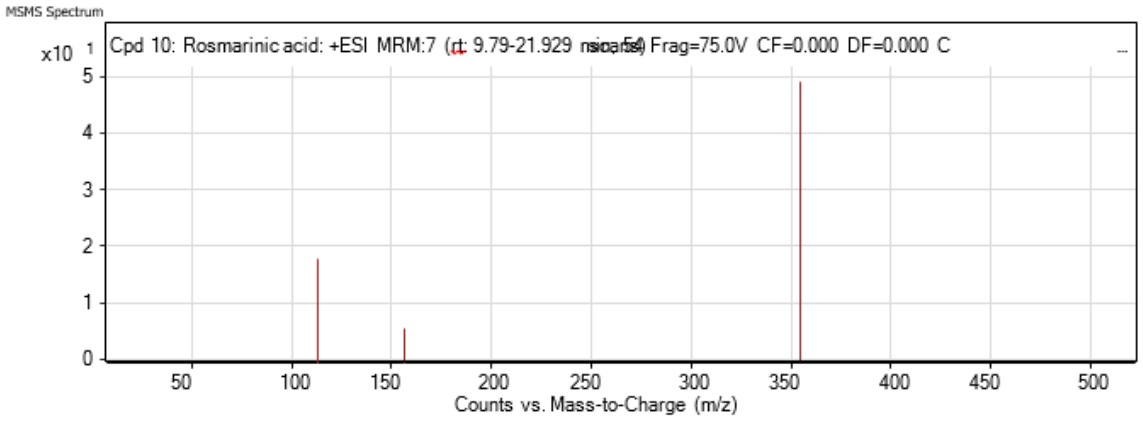
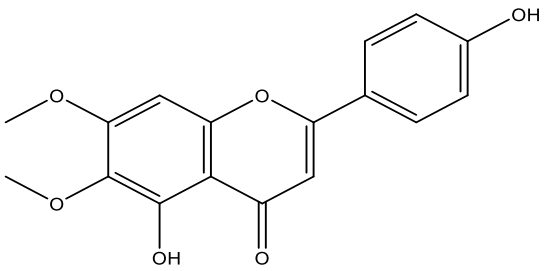


Figure 4.17 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Rosmarinic acid (10) compound in the sample.

4.4.2.11 Cirsimaritin (11)

Peak 11 of retention time 13.81 and precursor ion m/z 313.1 showed two fragment ions at m/z 283.0 and 298.1 (Figure 4.18) and this was identified as Cirsimaritin (11), $C_{17}H_{14}O_6$. The fragment ions were due to the neutral loss of (30.1) two methyl groups [M-CH_3-CH_3] and (15) one methyl group [M-CH_3] respectively (Baskaran et al., 2016).



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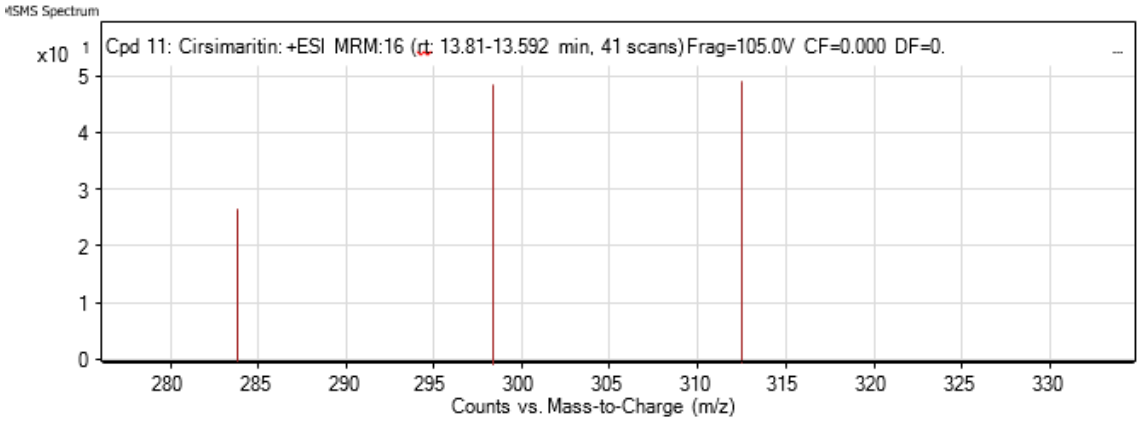
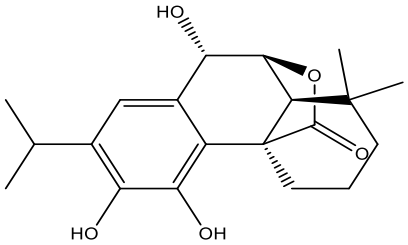


Figure 4.18 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Cirsimaritin (11) compound in the sample.

4.4.2.12 Rosmanol (12)

Peak 12 of retention time 13.92 and precursor ion m/z 345.2 showed two fragment ions at m/z at 283.2 and 301.2 (figure 4.19) was identified as Rosmanol (12), $C_{20}H_{26}O_5$ (Borrás-Linares et al., 2014). The fragment ions were due to the neutral loss of (62) one water molecule and one carbondioxide molecule [M-H_20-CO_2] and (44) one carbondioxide molecule [M-CO_2] respectively.



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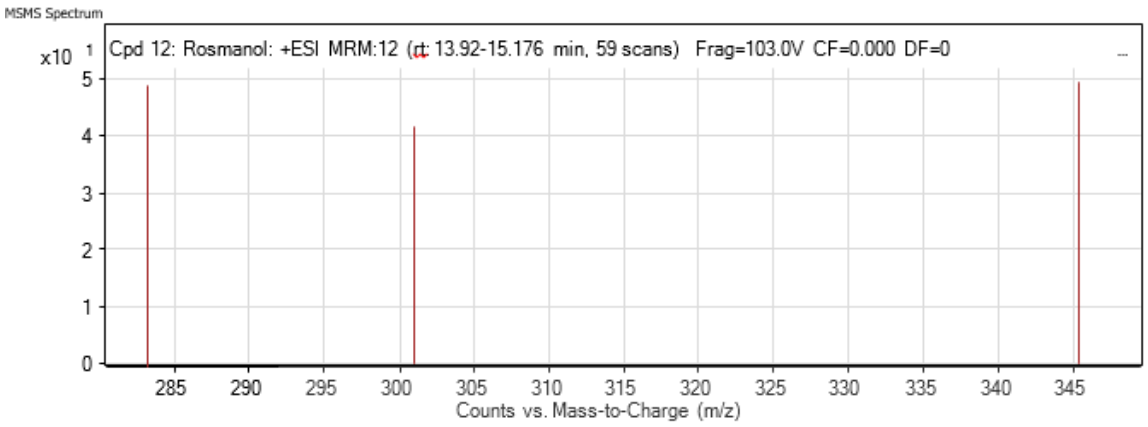
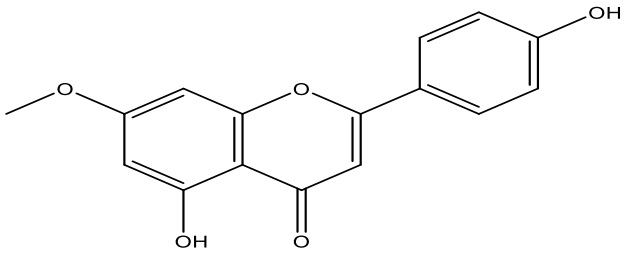


Figure 4.19 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Rosmanol (12) compound in the sample.

4.4.2.13 Genkwanin (13)

Peak 13 of retention time 15.14 and precursor ion m/z 283.1 showed one fragment ion at m/z 268.1 (Figure 4.20) was identified as Genkwanin (13), $C_{16}H_{12}O_5$ (Borrás-Linares et al., 2014). The fragment ion was due to the neutral loss of (15), a methyl group [M-CH_3].



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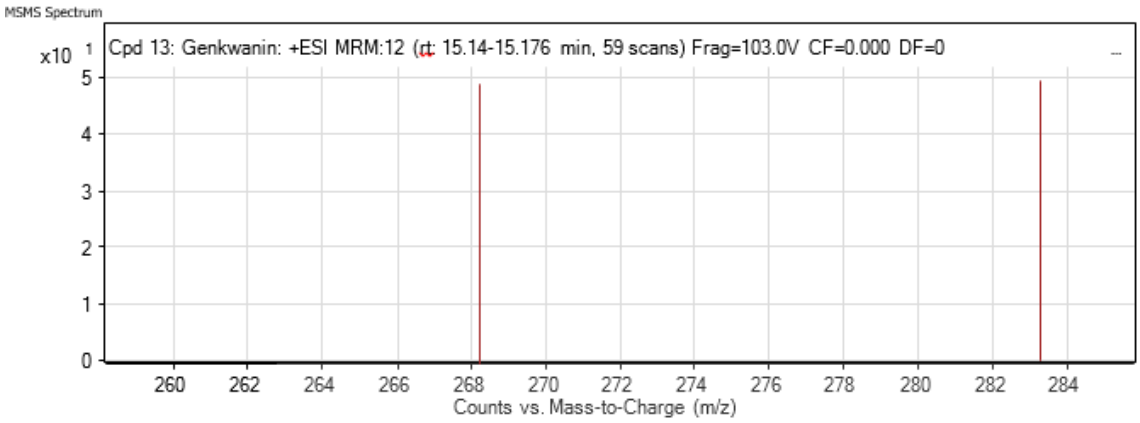
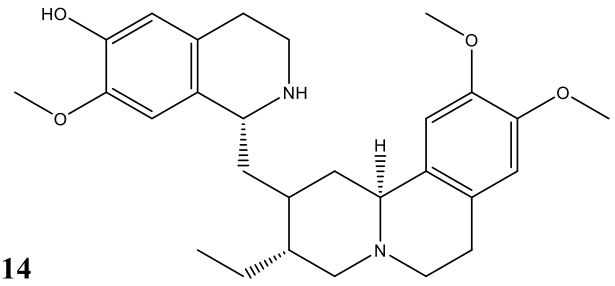


Figure 4.20 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Genkwanin (13) compound in the sample.

4.4.2.14 Cephalin (14)

Peak 14 of retention time 15.57 and precursor ion m/z 637.1 showed two fragment ions at m/z 65.1 and 91.0 (Figure 4.21) was identified as Cephalin (14), $C_{31}H_{62}N_2O_{11}$ structure. The fragment ion at 65.1 was due to the neutral loss of (572), hydrogen ion, 2 water molecules and apigenin-malonyl-glucoside molecule [M–H-$(H_20)_2$-$(C_{24}H_{23}O_{14})$]. The fragment ion at m/z 91.0 was due to neutral loss of (546.1) a hydrogen ion, a peroxide molecule and a homoplantaginin [M-H-H_2O_2-$C_{22}H_{22}O_{11}$].



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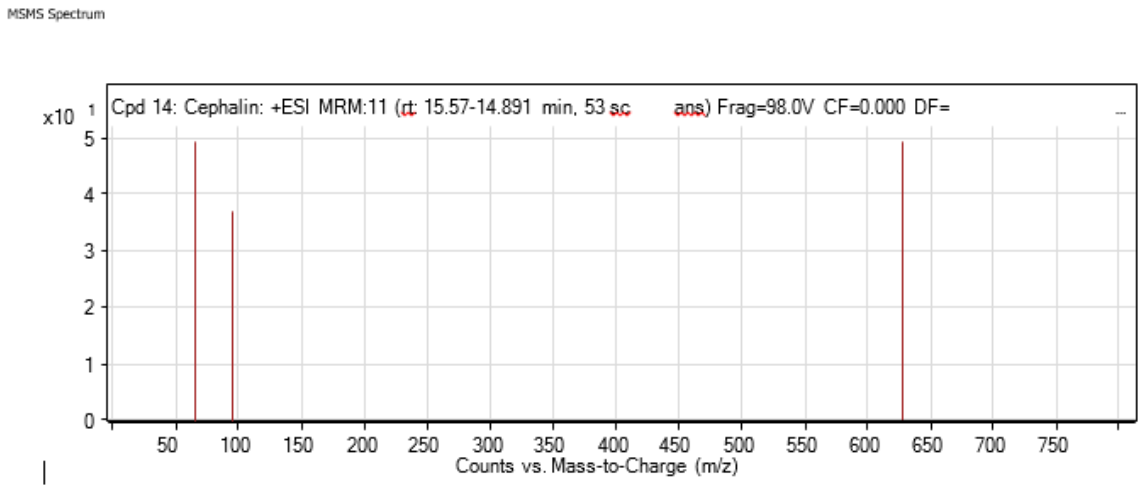
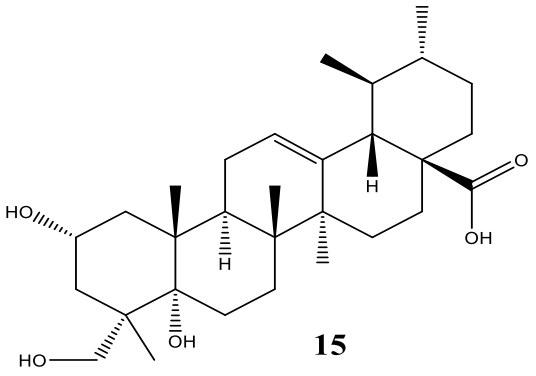


Figure 4.21 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Cephalin (14) compound in the sample.

4.4.2.15 Asiatic acid (15)

Peak 15 of retention time 16.04 and precursor ion m/z 487.3 showed no fragment ion but was identified as Asiatic acid (25), $C_{30}H_{48}O_5$ (Borrás-Linares et al., 2014).



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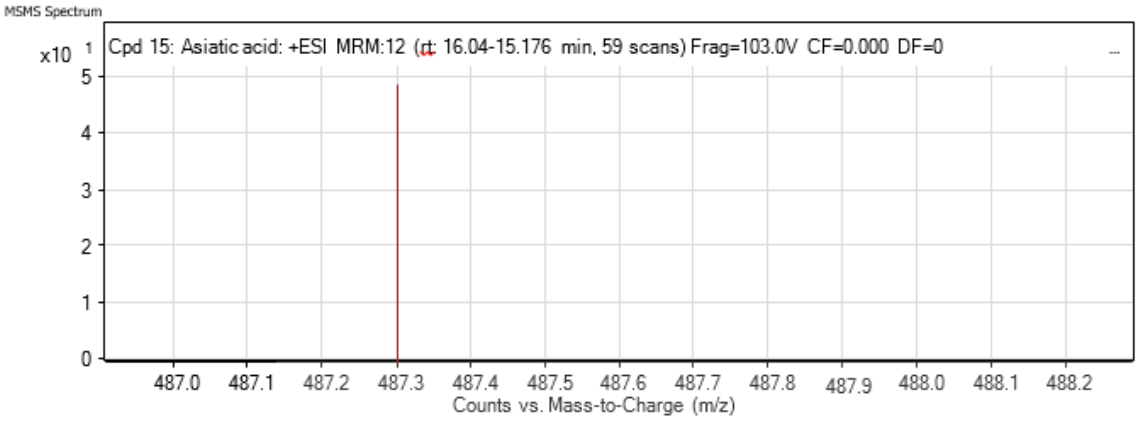
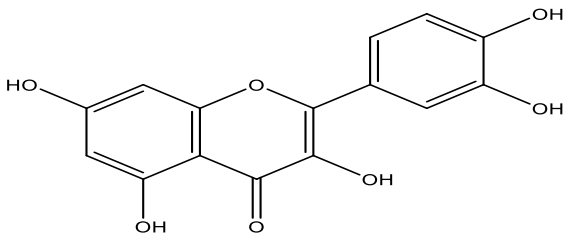


Figure 4.22 Mass spectrum showing % relative abundance against mass to charge ratio (m/z) of Asiatic acid (15) compound in the sample.

4.4.2.16 Quercetin (16)

Peak 16 of retention time 17.405 and precursor ion m/z 301.2 showed two fragment ions at m/z 168 and 77 (Figure 4.23) was identified as Quercetin (16), $C_{15}H_{10}O_7$ (Plessi, Bertelli, & Miglietta, 2016; Ren et al., 2019). The fragment ions at m/z 168 was due to the neutral loss of (133.2) a carboxyl group and 2 carbondioxide molecules [M-COOH-2CO_2]. The fragment ion at 77 was due to the neutral loss of (224.2), four hydrogen ions and nonyl phenol [M-4H-$C_{15}H_{24}O$].



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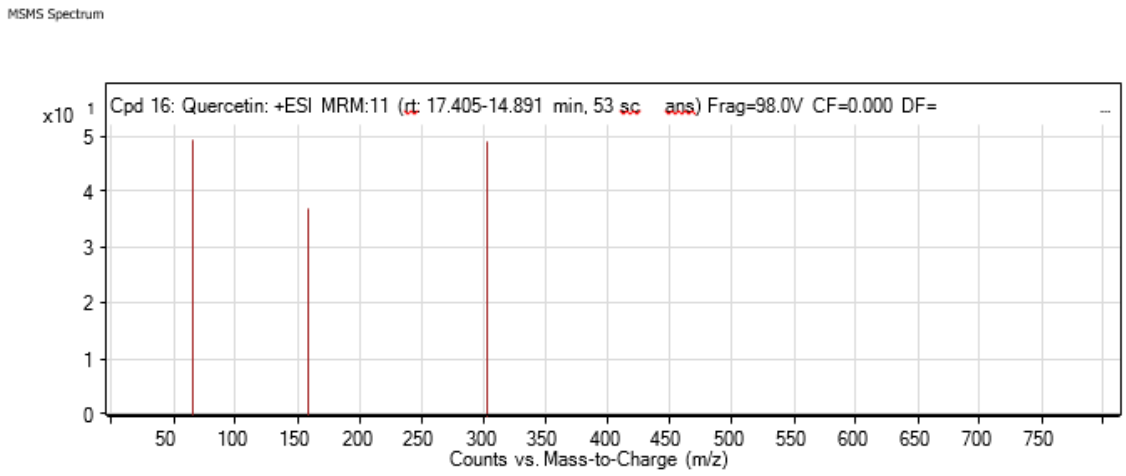
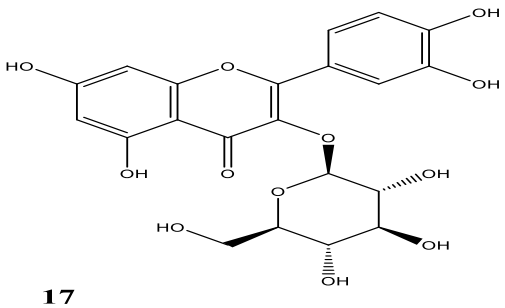


Figure 4.23 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Quercetin (16) compound in the sample.

4.4.2.17 Isoquercetin (17)

Peak 17 of retention time 18.558 and precursor ion m/z 463.1 showed one fragment ion at m/z 163.1 (Figure 4.24) was identified as Isoquercetin (17), $C_{21}H_{20}O_{12}$. The fragment ion at m/z 163.1 was due to the neutral loss of (300) an oxygen molecule and trimethyl pentadecanone [M-O_2-$C_{18}H_{36}O$].



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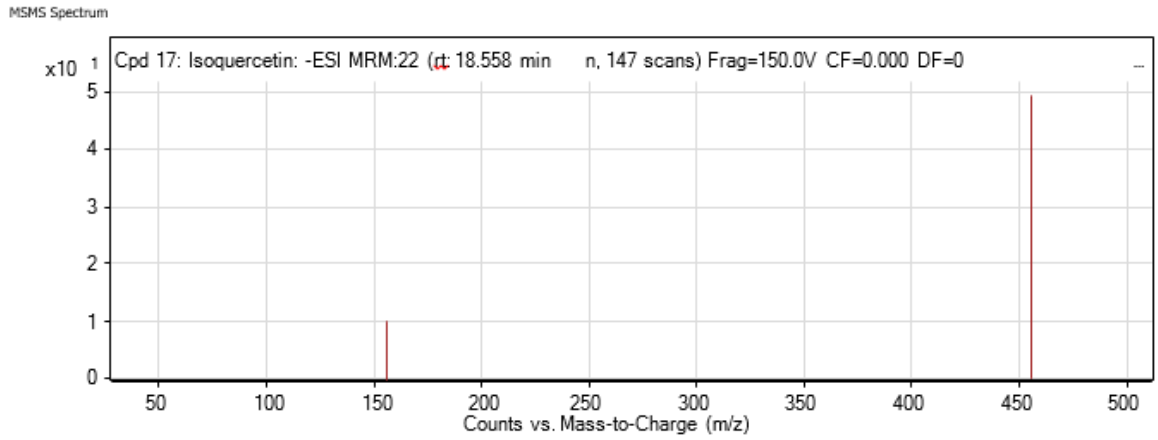
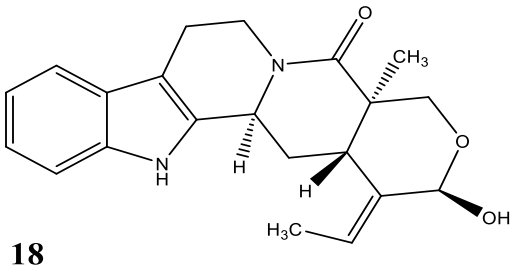


Figure 4.24 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Isoquercetin (17) compound in the sample.

4.4.2.18 Latifoliamide (18)

Peak 18 of retention time 18.669 and precursor ion m/z 351.1 showed one fragment ion at m/z 130.1 (Figure 4.25) was identified as Latifoliamide (18), $C_{21}H_{24}N_2O_3$. The fragment ion at m/z 130.1 was due to the neutral loss of (221), [M-CO-CO_2-$C_4H_6O_5$-CH_3].



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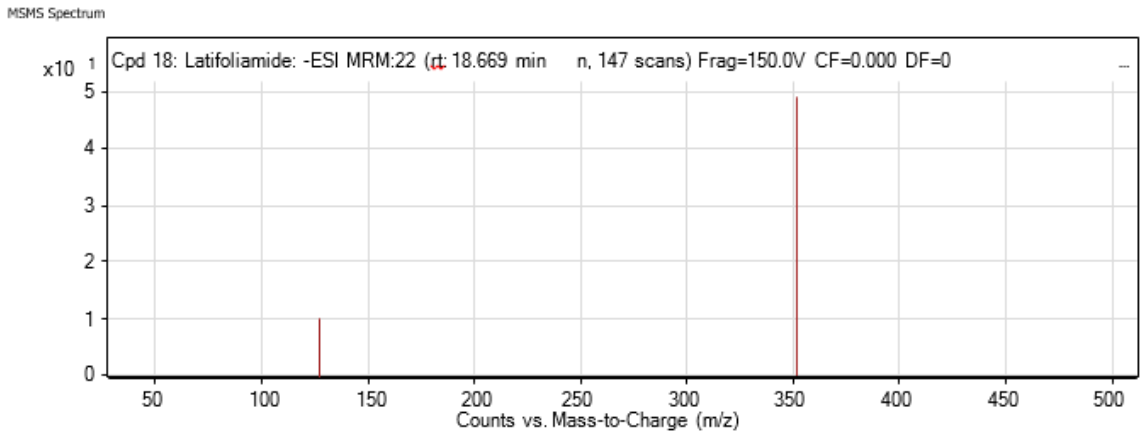
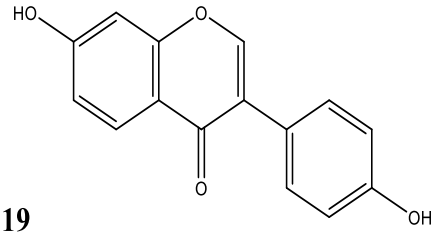


Figure 4.25 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Latifoliamide (18) compound in the sample.

4.4.2.19 Diadzin (19)

Peak 19 of retention time 18.826 and precursor ion m/z 253.2 showed two fragment ions at m/z 91.1 and 86.2 (Figure 4.26) was identified as Diadzin (19), $C_{15}H_{10}O_4$. The fragment ions at m/z 91.1 was due to the neutral loss of (162.1), [M-$C_{10}H_{10}O_2$]. The fragment ion at 86.2 was due to the neutral loss of (167), a carboxylic group and benzoic acid molecule [M-COOH-C_6H_5COOH] (Plessi et al., 2016).



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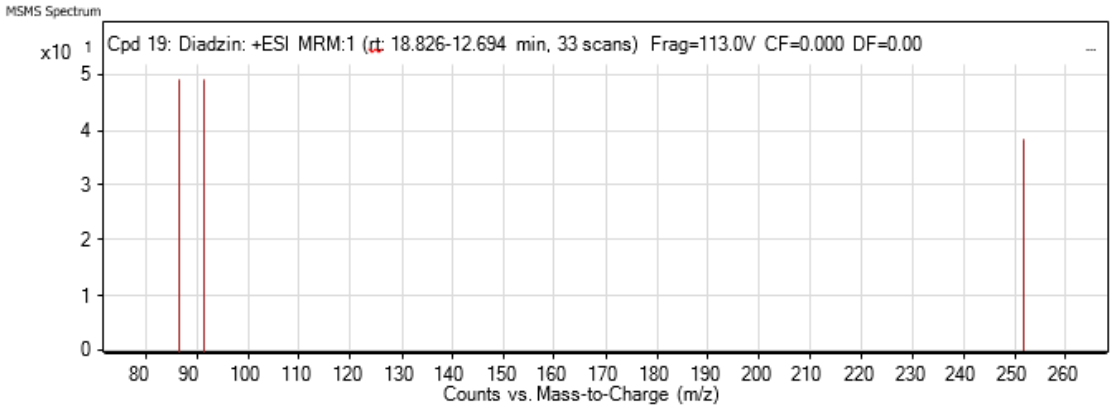
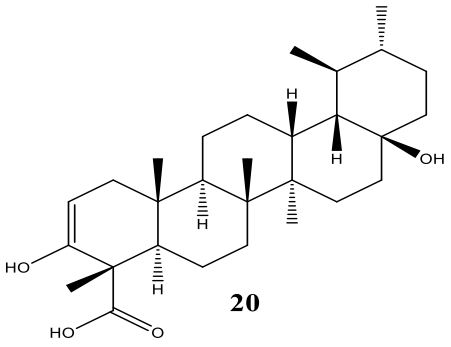


Figure 4.26 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Diadzin (19) compound in the sample.

4.4.2.20 Benthamic acid (20)

Peak 20 of retention time 21.91 and precursor ion m/z 471.3 (Figure 4.27) showed no fragment ions but was identified as Benthamic acid (20), $C_{30}H_{48}O_4$ (Borrás-Linares et al., 2014).



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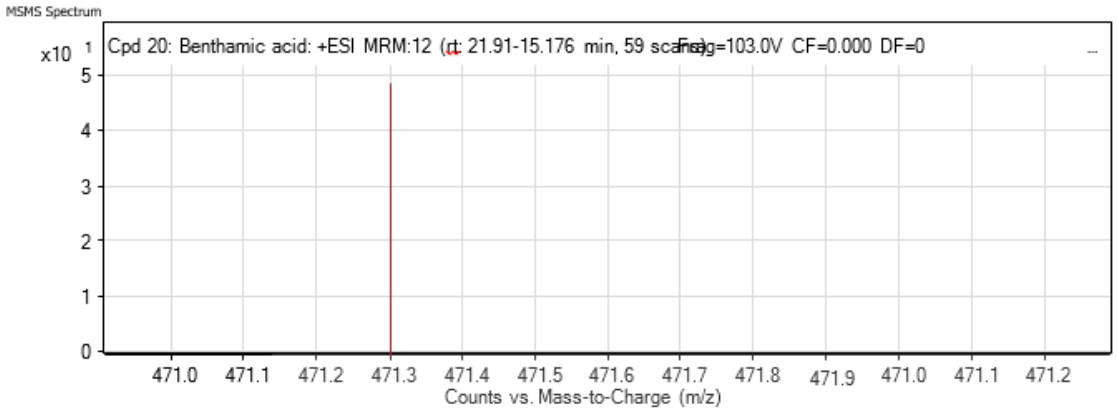
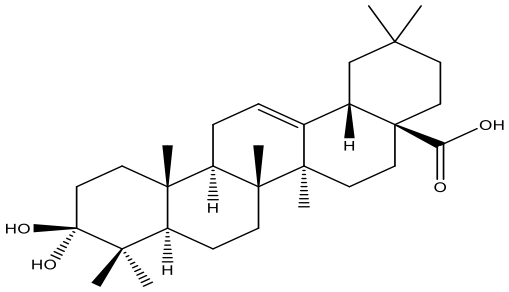


Figure 4.27 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Benthamic acid (20) compound in the sample.

4.4.2.21 Augustic acid (21)

Peak 21 of retention time 22.35 and precursor ion m/z 471.3 (Figure 4.28) did not show any fragment ion but was identified as Augustic acid (21) $C_{30}H_{48}O_4$ (Borrás-Linares et al., 2014).



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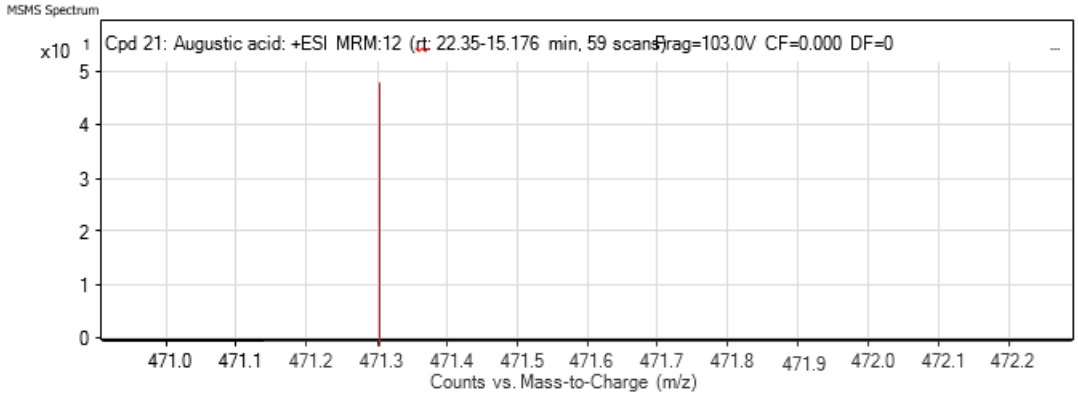
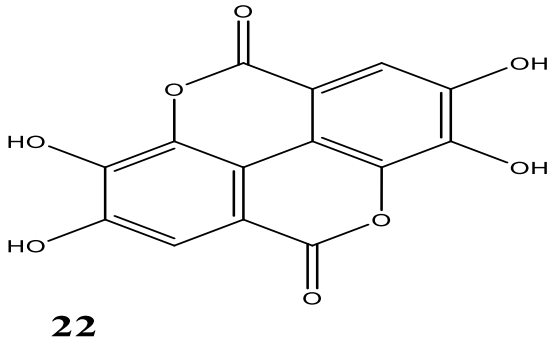


Figure 4.28 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Augustic acid (21) compound in the sample.

4.4.2.22 Ellargic acid (22)

Peak 22 of retention time 25.425 and precursor ion m/z 301.1 showed one fragment ion at m/z 83 (Figure 4.29) and was identified as Ellargic acid (22) $C_{14}H_6O_8$ (Quatrin et al., 2019). The fragment ions at m/z 83 was due to the neutral loss of (218.1), a water molecule and hydroxyphenyl methyl phenol [M-H_2O-$C_{13}H_{12}O_2$].



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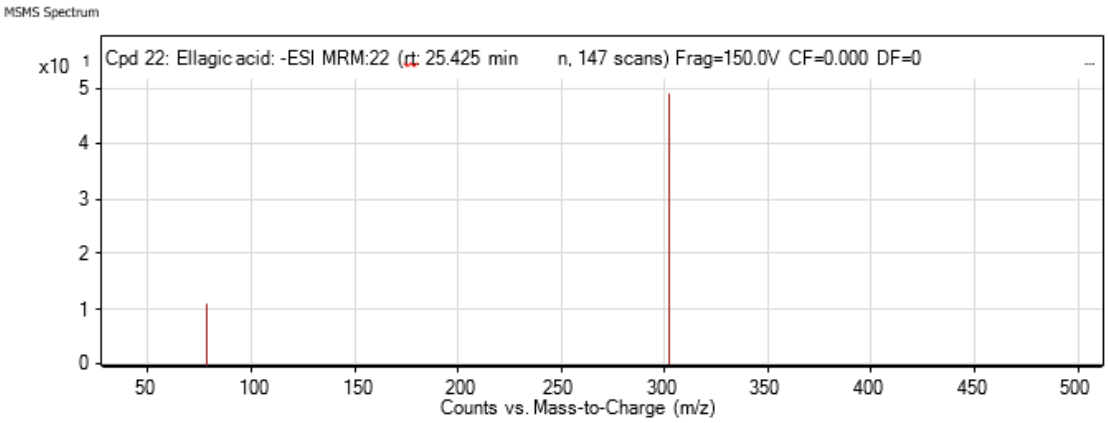
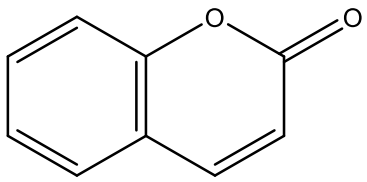


Figure 4.29 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Ellargic acid (22) compound in the sample.

4.4.2.23 Courmarin (23)

Peak 23 of retention time 29.116 and precursor ion m/z 145 showed two fragment ions at m/z 103.1 and 91.1 (Figure 4.30) and was identified as Courmarin (23) $C_9H_6O_2$. The fragment ion at m/z 103.1 was due to the neutral loss of (41.9), [M-HCO-CH]. The fragment ion at m/z 91.1 was due to the neutral loss of (53.9) 3 water molecules, [M-3(H_2O)] (Proestos et al., 2015).



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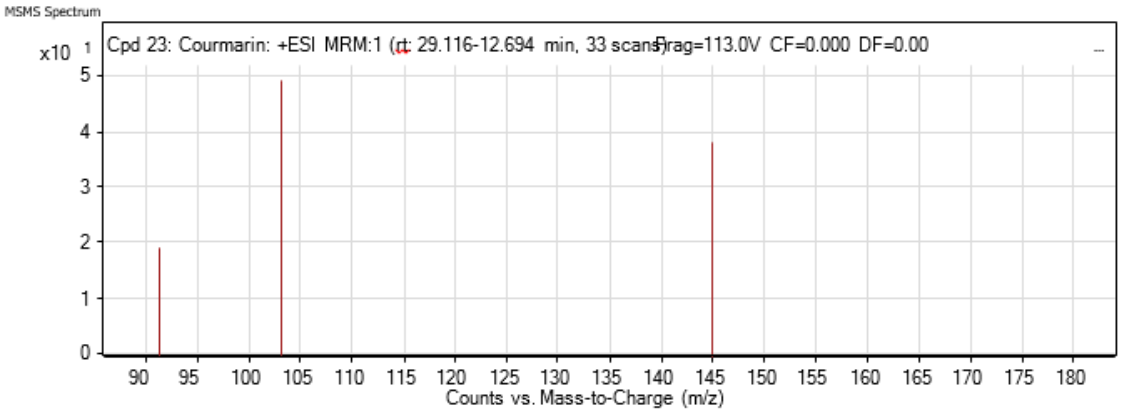
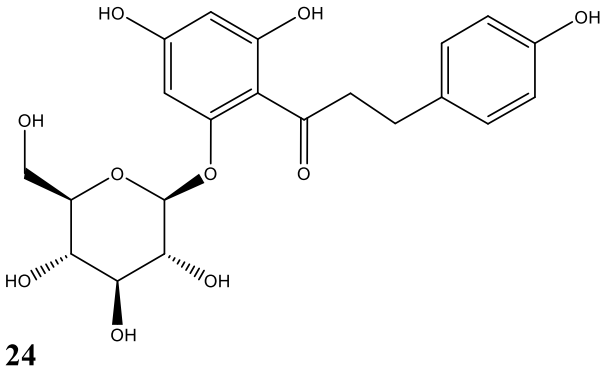


Figure 4.30 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Coumarin (23) compound in the sample.

4.4.2.24 Phlorizin (24)

Peak 24 of retention time 29.148 and precursor ion m/z 435.2 showed one fragment ion at m/z 196 (Figure 4.31) and was identified as Phlorizin (24) $C_{21}H_{24}O_{10}$. The fragment ion at

m/z 196 was due to the neutral loss of (239.2), 2 hydrogen molecules, a methyl group and ethyl glucuronide [M-2H-CH_3-$C_8H_{14}O_7$] (Quatrin et al., 2019).



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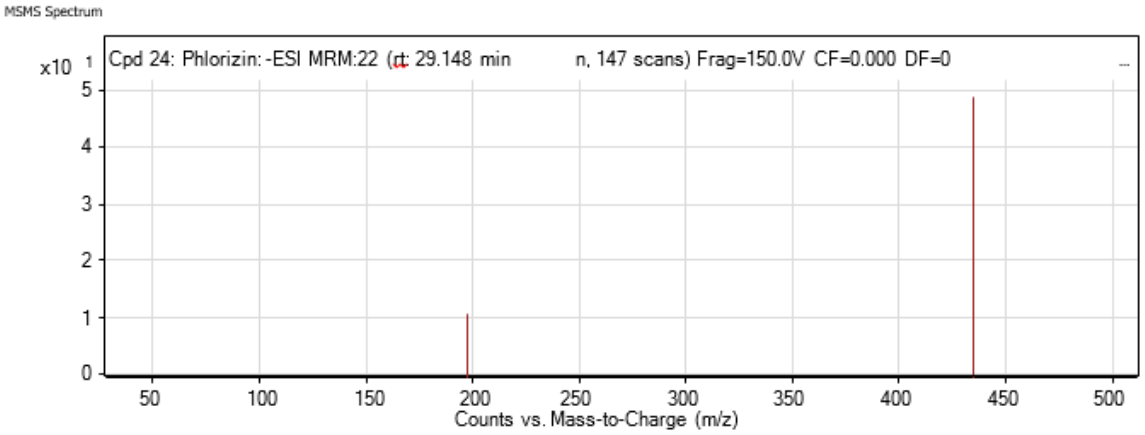
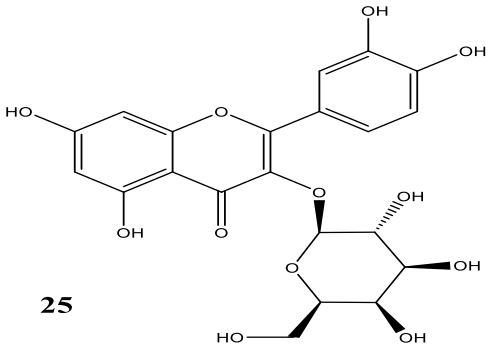


Figure 4.31 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Phloridizin (24) compound in the sample.

4.4.2.25 Hyperin (25)

Peak 25 of retention time 29.161 and precursor ion m/z 463.2 showed two fragment ions at m/z 215.1 and 171.1 (Figure 4.32) and was identified as Hyperin (25) $C_{21}H_{20}O_{12}$. The fragment ion at m/z 215.1 was due to the neutral loss of (248.1), [M-2(CH)-$C_8H_{14}O_7$]. The fragment ion at m/z 171.1 was due to the neutral loss of (292.1), [M-CO_2-2(CH)-$C_8H_{14}O_7$] (Quatrin et al., 2019; Rajauria, 2018).



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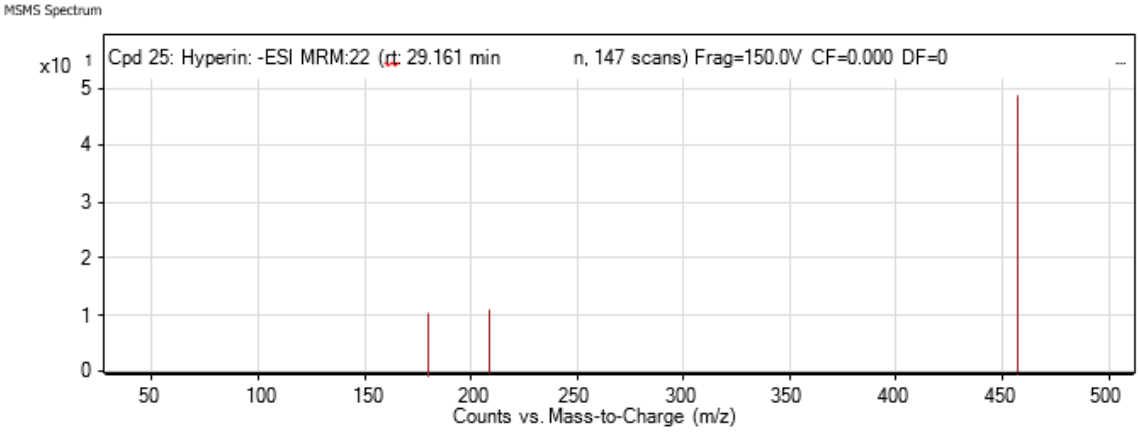
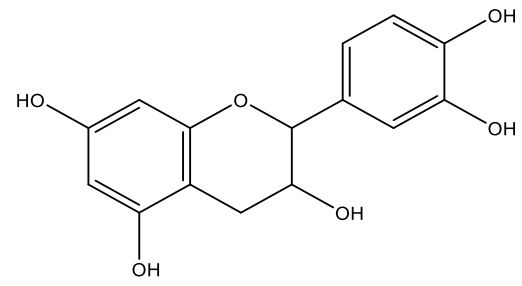


Figure 4.32 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Hyperin (25) compound in the sample.

4.4.2.26 Catechin (26)

Peak 26 of retention time 29.165 and precursor ion m/z 289.2 showed one fragment ion at m/z 176.1 (Figure 4.33) and was identified as Catechin (26) $C_{15}H_{14}O_6$. The fragment ion at m/z 176.1 was due to the neutral loss of (113.1), [M-3H-$C_6H_6O_2$] (Quatrin et al., 2019; Rajauria, 2018).



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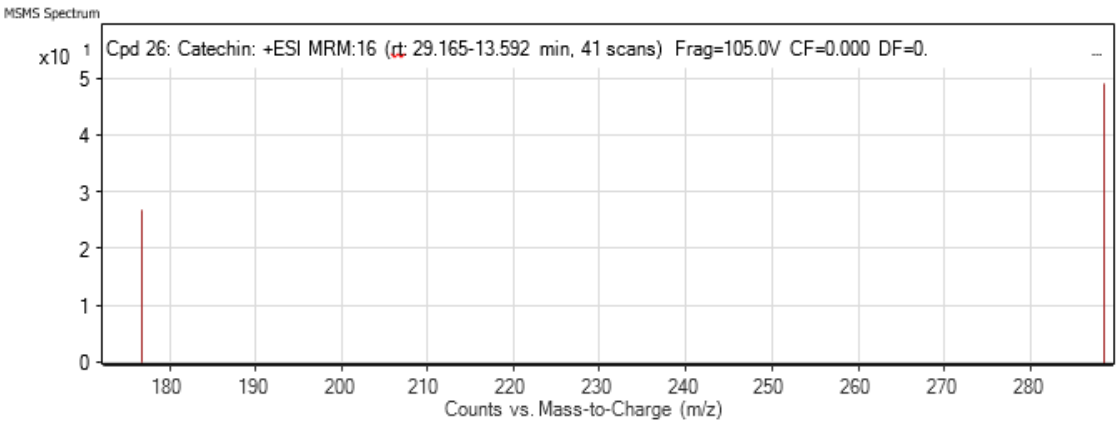
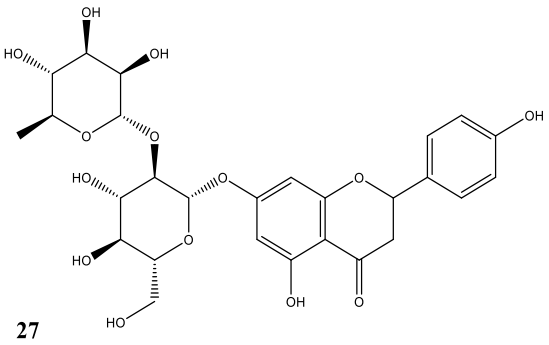


Figure 4.33 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Catechin (26) compound in the sample.

4.4.2.27 Naringin (27)

Peak 27 of retention time 29.175 and precursor ion m/z 579.1 showed one fragment ion at m/z 84.1 (Figure 4.34) and was identified as Naringin (27) $C_{27}H_{32}O_{14}$. The fragment ion at m/z 84.1 was due to the neutral loss of (495), a water molecule, methyl group and homoplantaginin [M-H_2O-CH_3-$C_{22}H_{22}O_{11}$] (Borrás-Linares et al., 2014).



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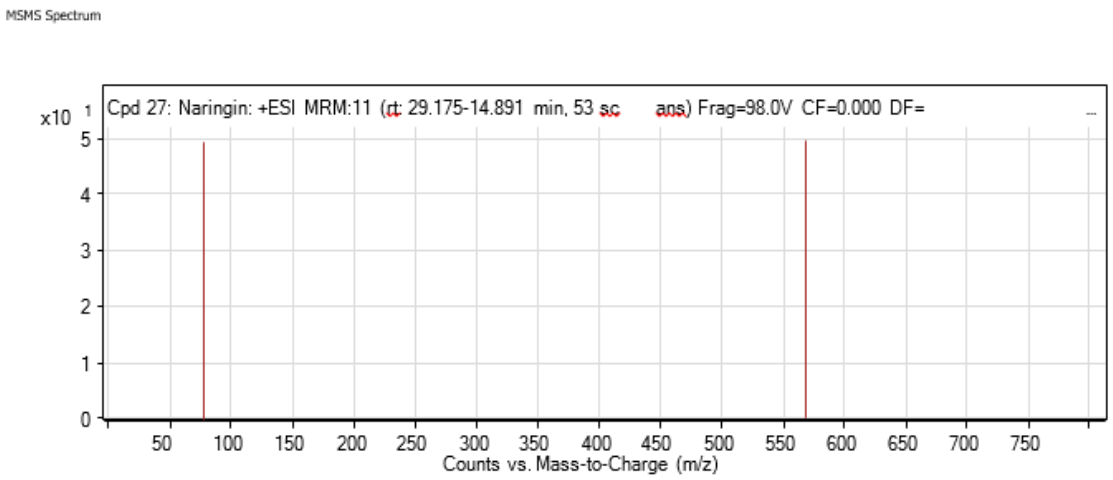
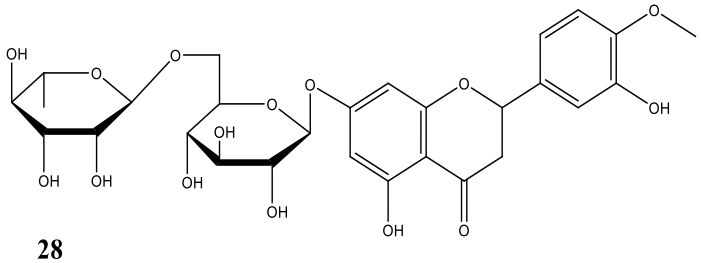


Figure 4.34 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Naringin (27) compound in the sample.

4.4.2.28 Hesperidin (28)

Peak 28 of retention time 29.179 and precursor ion m/z 609.1 showed one fragment ion at m/z 84.1 (Figure 4.35) and was identified as Hesperidin (28) $C_{28}H_{34}O_{15}$. The fragment ion at m/z 84.1 was due to the neutral loss of (525), water molecule, carboxyl group and Homoplantaginin [M-H_2O-COOH-$C_{22}H_{22}O_{11}$] (Borrás-Linares et al., 2014).



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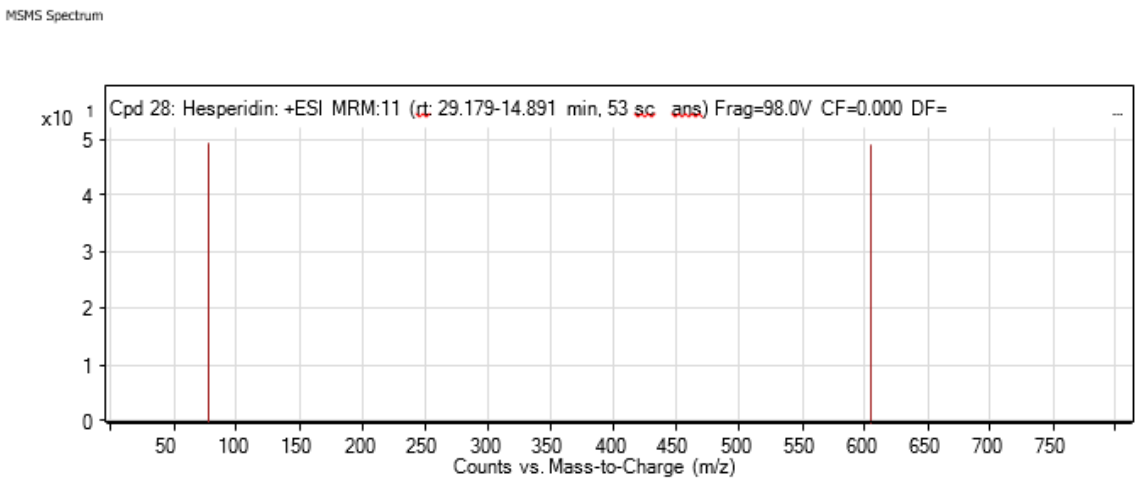
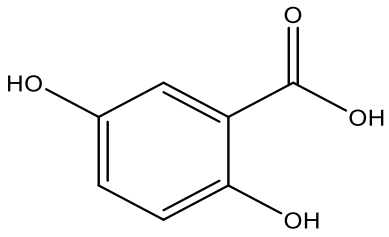


Figure 4.35 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Hesperidin (28) compound in the sample.

4.4.2.29 Gentisic acid (29)

Peak 29 of retention time 29.234 and precursor ion m/z 153 showed two fragment ions at m/z 108 and 109 (Figure 4.36) and was identified as Gentisic acid (29) $C_7H_6O_4$. The fragment ion at m/z 109 was due to the neutral loss of (44) a carbondioxide molecule, [M-CO_2]. The fragment ion at m/z 108 was due to the neutral loss of (45) a carboxyl group, [M-CO_2H] (Kivilompolo et al., 2007).



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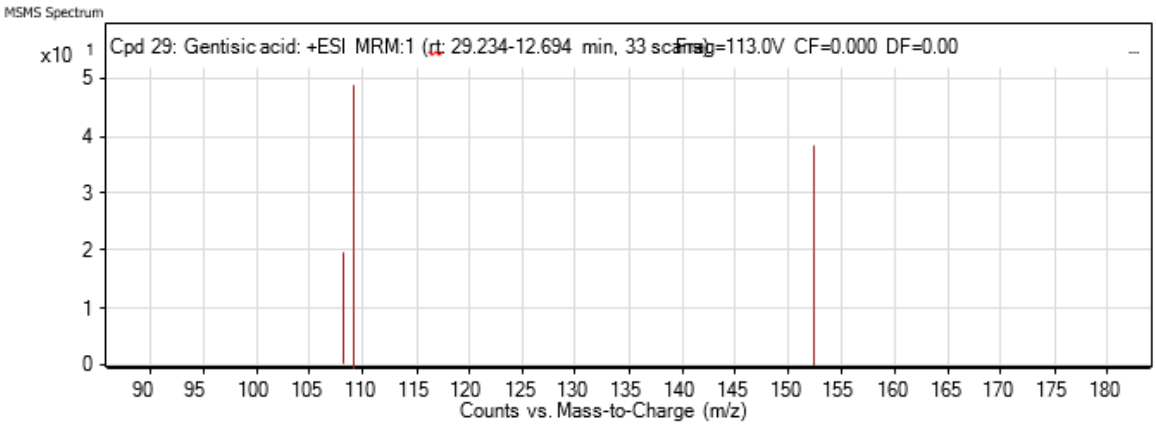
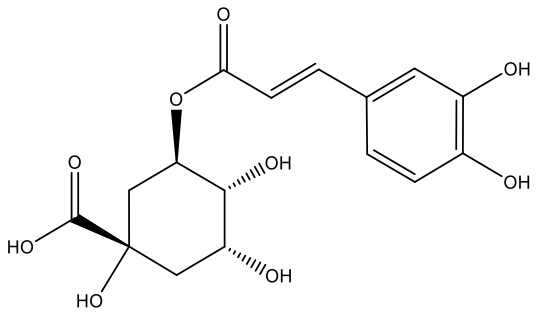


Figure 4.36 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Gentisic acid (29) compound in the sample.

4.4.2.30 Chlorogenic acid (31)

Peak 31 of retention time 29.384 and precursor ion m/z 353.1 showed one fragment ion at m/z 98.1 (Figure 4.37) and was identified as Chlorogenic acid (31) $C_{16}H_{18}O_9$. The fragment ion at m/z 109 was due to the neutral loss of (244.1) 2 benzoic acid molecules, [M-2(C_6H_5COOH)].



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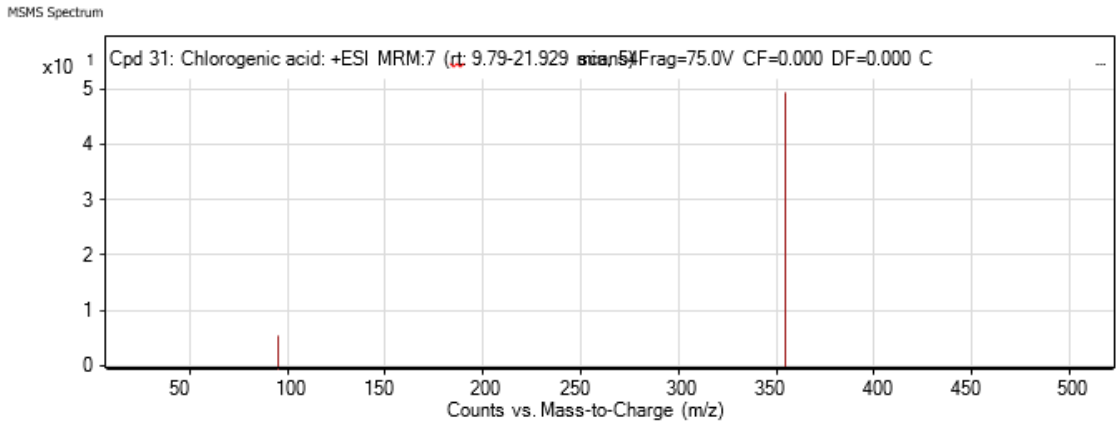
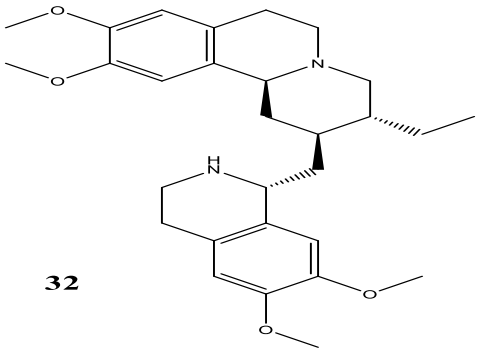


Figure 4.37 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Chlorogenic acid (31) compound in the sample.

4.4.2.31 Emetine (32)

Peak 32 of retention time 29.405 and precursor ion m/z 479.1 showed two fragment ions at m/z 119 and 91 (Figure 4.38) and was identified as Emetine (32) $C_{29}H_{40}N_2O_4$. The fragment ion at m/z 119 was due to the neutral loss of (360.1), [M-H-COH-$C_{24}H_{26}O$]. The fragment ion at m/z 91 was due to the neutral loss of (388.1), [M-CO_2H-CH-$C_{24}H_{26}O$] (Borrás-Linares et al., 2014; Rajauria, 2018).



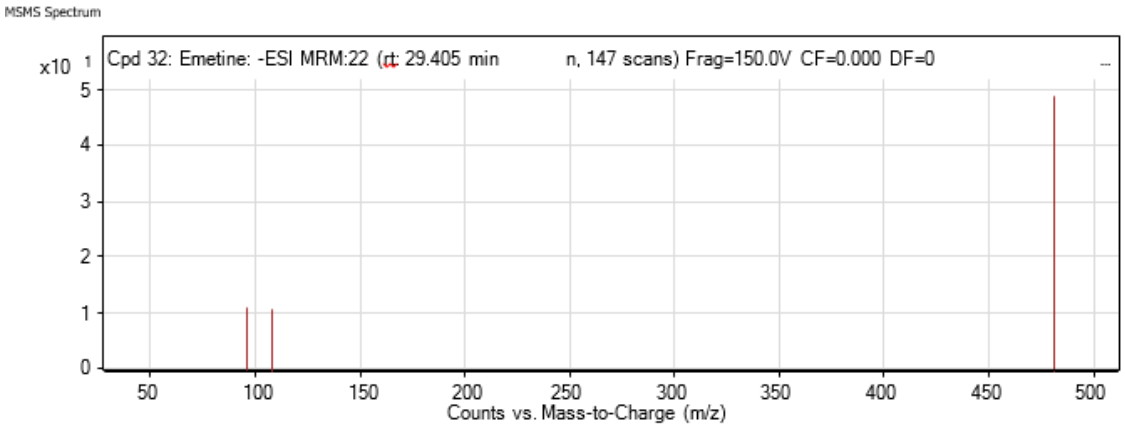
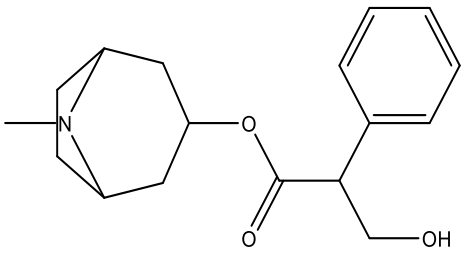


Figure 4.38 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Emetine (32) compound in the sample.

4.4.2.32 Atropine (33)

Peak 33 of retention time 29.719 and precursor ion m/z 288.2 showed two fragment ions at m/z 124.1 and 93.1 (Figure 4.39) and was identified as Atropine (33) $C_{17}H_{23}NO_3$. The fragment ion at m/z 124.1 was due to the neutral loss of 164.1, [M-2H-$C_{10}H_{10}O_2$]. The fragment at 93.1 was due to neutral loss of 195.1, [M-$C_9H_8O_4$-CH_3] (Borrás-Linares et al., 2014; Rajauria, 2018).



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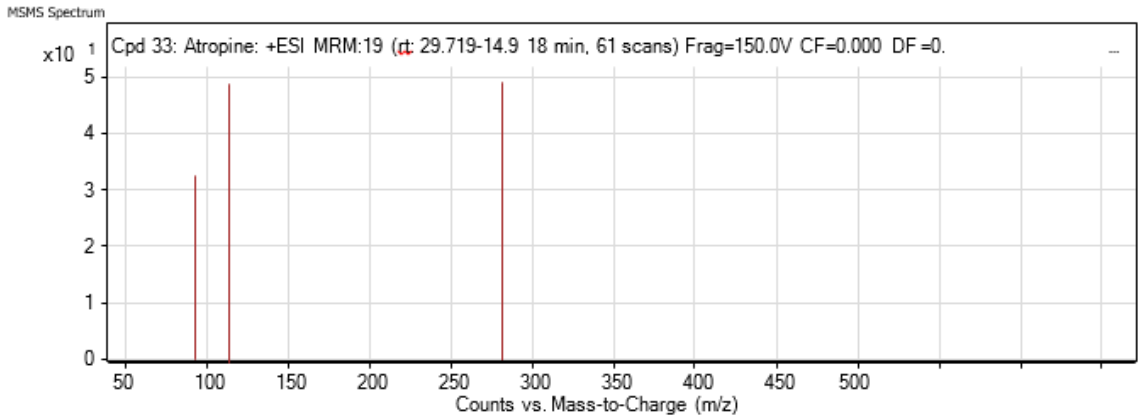
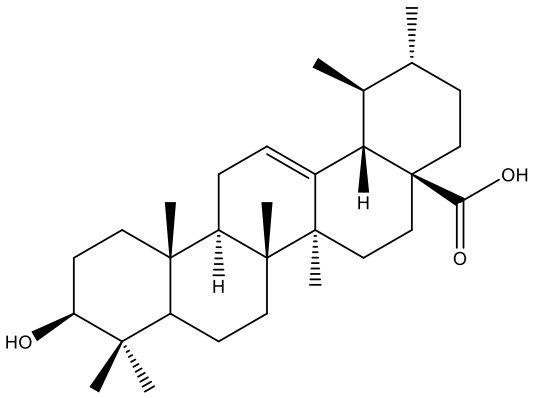


Figure 4.39 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Atropine (33) compound in the sample.

4.4.2.33 Ursolic acid (34)

Peak 34 of retention time 30.25 and precursor ion m/z 455.4 (Figure 4.40) did not show any fragment ions but was identified as Ursolic acid (34) $C_{30}H_{48}O_3$ (Borrás-Linares et al., 2014).



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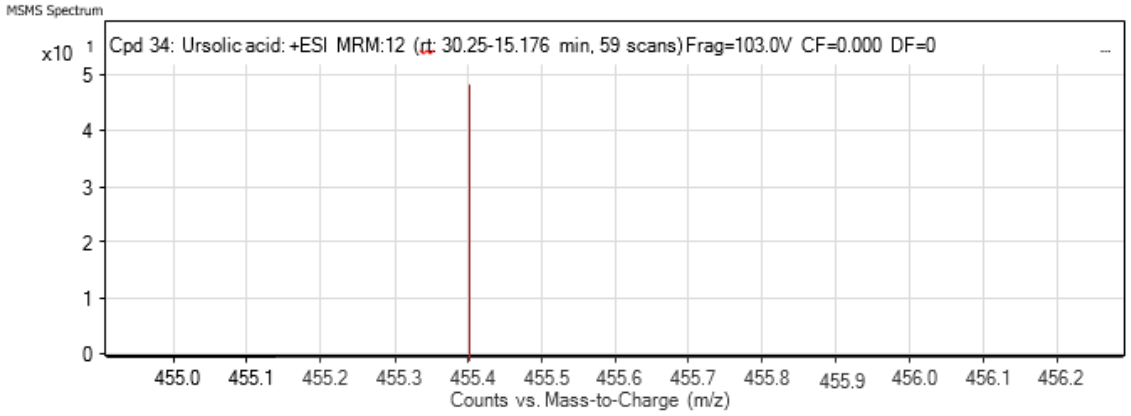
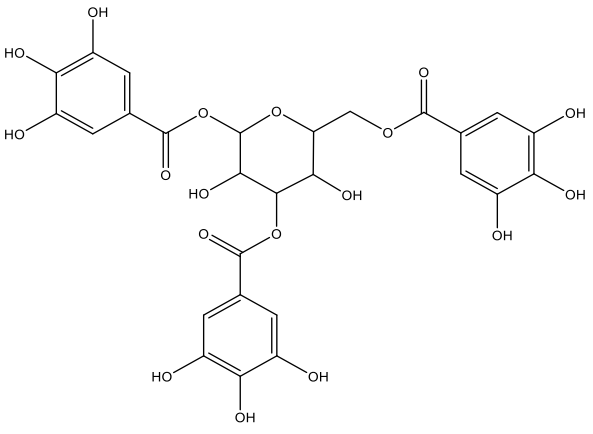


Figure 4.40 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Ursolic acid (34) compound in the sample.

4.4.2.34 Tannic acid (35)

Peak 35 of retention time 11.415 and precursor ion m/z 1699.1 showed one fragment ion at m/z 138 (Figure 4.41) and was identified as Tannic acid (35) $C_{76}H_{52}O_{46}$. The fragment ion at m/z 138 was due to the neutral loss of (1561.1), [M-2H-H_2O_2-3($C_{22}H_{22}O_{11}$)-C_6H_6O-COOH] (Rajauria, 2018).



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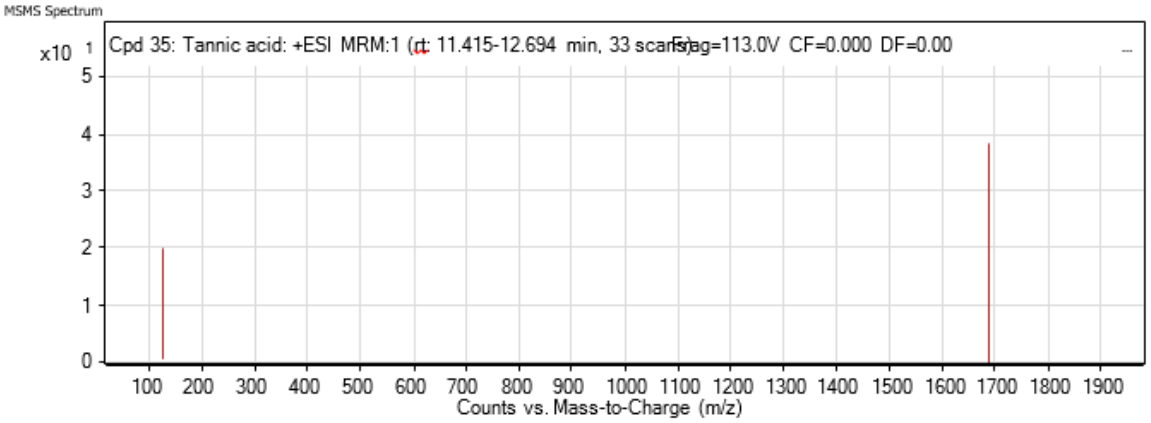
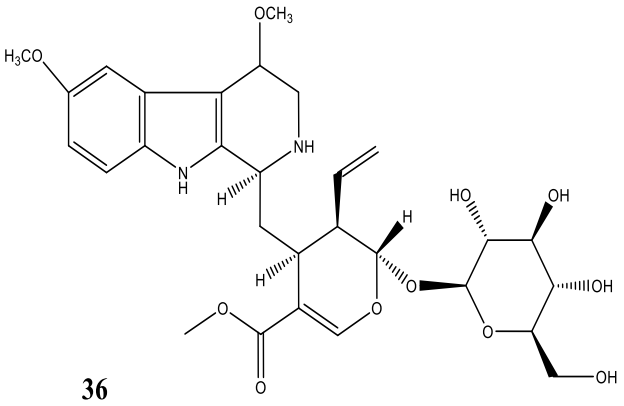


Figure 4.41 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Tannic acid (35) compound in the sample.

4.4.2.35 Carboxylstrictosidine (36)

Peak 36 of retention time 11.433 and precursor ion m/z 573.1 showed one fragment ion at m/z 91.1 (Figure 4.42) and was identified as Carboxylstrictosidine (36) $C_{28}H_{34}N_2O_{11}$. The fragment ion at m/z 91.1 was due to the neutral loss of (482) a carbondioxide molecule and a methyl group, [M-2H-H_2O-$C_{22}H_{22}O_{11}$].



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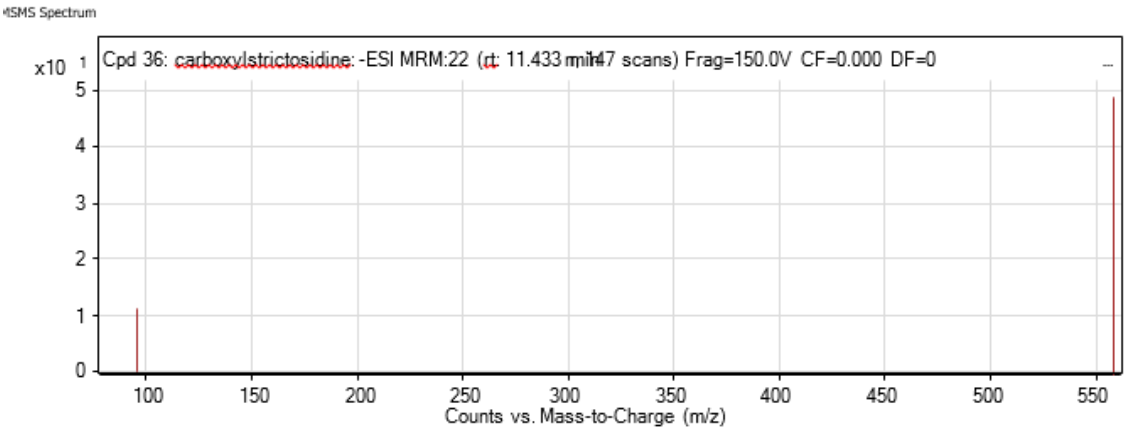
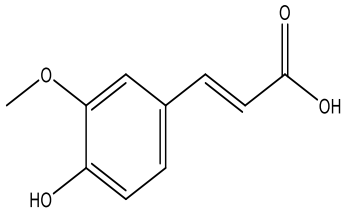


Figure 4.42 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Carboxystrictosinedine (36) compound in the sample.

4.4.2.36 Isoferullic acid (37)

Peak 37 of retention time 11.61 and precursor ion m/z 193.1 showed one fragment ion at m/z 105.1 (Figure 4.43) and was identified as Isoferullic acid (37) $C_{10}H_{10}O_4$. The fragment ion at m/z 91.1 was due to the neutral loss of (88), [M-CO_2-CO_2].



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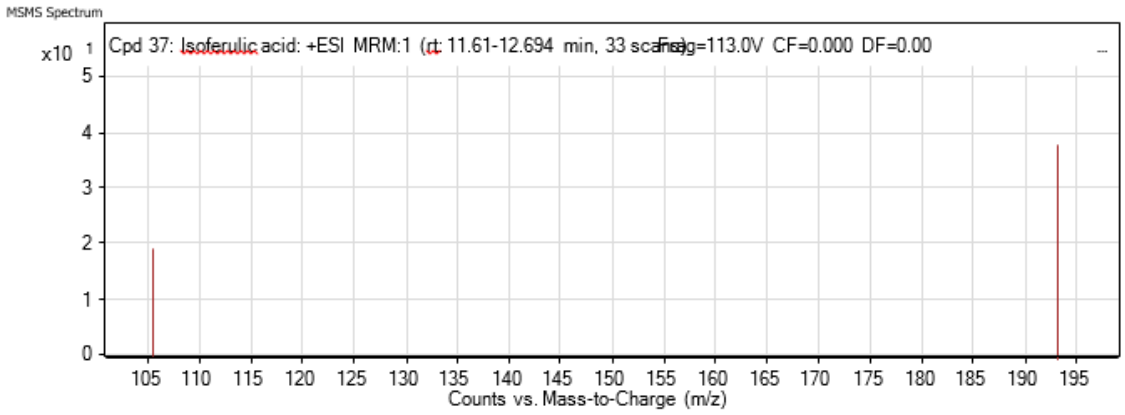
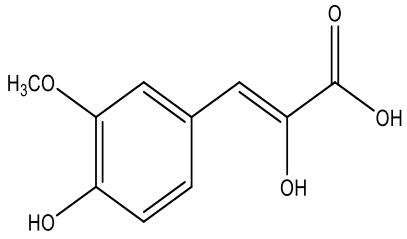


Figure 4.43 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Isoferrulic acid (37) compound in the sample.

4.4.2.36 Ferrullic acid (38)

Peak 38 of retention time 11.795 and precursor ion m/z 193.1 showed one fragment ion at m/z 163.1 (Figure 4.44) and was identified as Ferrullic acid (38) $C_{10}H_{10}O_4$. The fragment ion at m/z 163.1 was due to the neutral loss of (30), [M-OCH_2] (Proestos et al., 2015).



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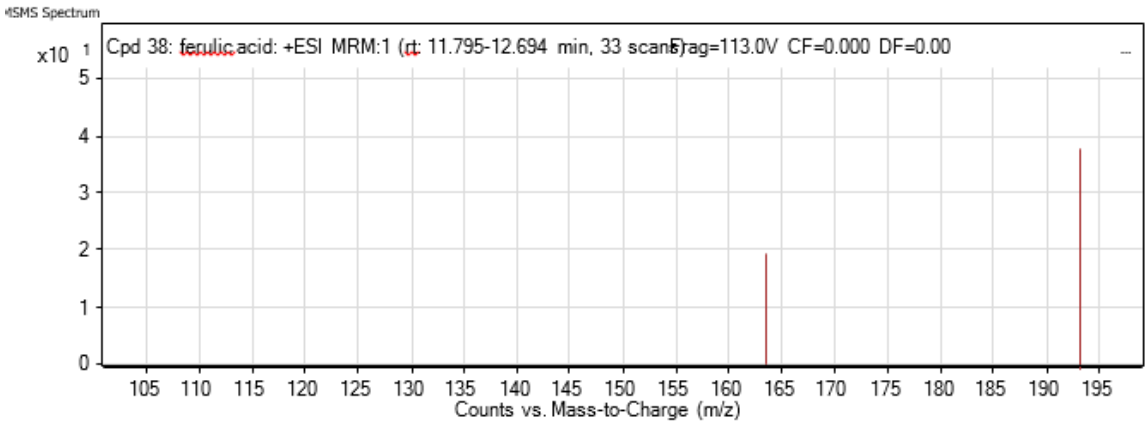
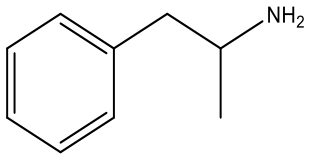


Figure 4.44 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Ferrulic acid (38) compound in the sample.

4.4.2.38 Psychotron (39)

Peak 39 of retention time 21.117 and precursor ion m/z 119.1 showed one fragment ion at m/z 91.0 (Figure 4.45) and was identified as Psychotron (39) $C_8H_{12}N$. The fragment ion at m/z 91.0 was due to the neutral loss of (28.1), [M-CH-CH_3] (Proestos et al., 2015).



39

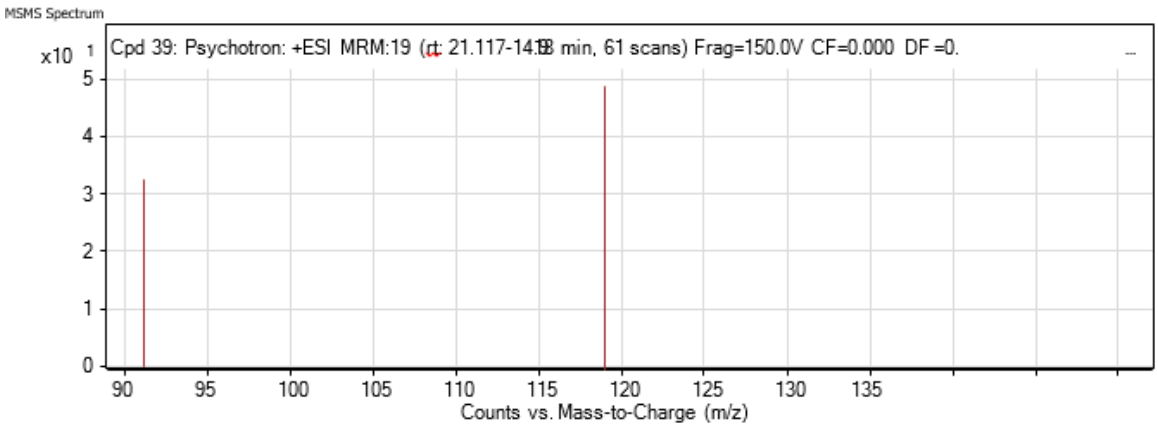


Figure 4.45 Mass spectrum showing %relative abundance against mass to charge ratio (m/z) of Psychotron (39) compound in the sample.

4.5 *In vitro* antiproliferative activity of phenolic crude isolates of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves against selected cancer cell lines.

Ethyl acetate fractions showed the highest activity on all cancer cell lines tested *R. officinalis* had IC_{50} of 8.54±0.47, 196.02±0.03, 181.47±0.5 for prostate, colorectal and cervical cancer cell lines respectively while *O. gratissimum* had IC_{50} of 6.39±0.26, 261.31±0.27, 119.34±0.38 µg/ml for prostate, colorectal and cervical cancer cell lines respectively (table 4.6). The hexane fractions showed the lowest activity for *R. officinalis* there was no activity against prostate cancer cell line and IC_{50} of 972.26±0.44 and 902.69±0.6 µg/ml for colorectal and cervical cancer cell lines respectively. *O. gratissimum* 1019.26±0.28 µg/ml for prostate cancer cell line while there was no activity against colorectal and cervical cancer cell lines (Table 4.6). The phenolic fractions were observed to be highly effective on cancer cells than the corresponding crude extracts while the SPE isolates have been observed to be less toxic compared to the crude extracts. This is evident

by the increase in their inhibitory concentrations required to reduce 50% of the cell number (IC_{50}).

The mean IC_{50} values of the control, doxorubicin on all cancer cell lines (4.36±0.22, 6.39±0.47, 3.64±0.33 µg/ml for prostate, colorectal and cervical cancer cell lines respectively) is statistically different from the means of all phenolic isolates of the two plants ($P<0.05$). There is statistically significant difference in the mean IC_{50} values between the six (methanol, ethylacetate, dichloromethane and hexane fractions plus SPE isolates of methanol and ethylacetate) groups of *R. officinalis* and *O. gratissimum* ($P<0.05$), using one-way ANOVA.

Table 4.6 Minimum inhibitory concentration (IC_{50}) values required to kill 50% of the cancer cells by the plants' fractions.

	R. officinalis (IC_{50} µg/ml)			*O. gratissimum* (IC_{50} µg/ml)		
	Prostate (DU145)	Colorectal (CT26)	Cervical (HeLa229)	Prostate (DU145)	Colorectal (CT26)	Cervical (HeLa229)
Methanol fraction	28.28±0.49^{C}	272.32±0.56^{D}	385.43±0.52^{C}	16.16±0.14^{E}	357.39±0.34^{D}	201.21±0.23^{D}
Ethylacetate fraction	8.54±0.47^{D}	196.02±0.03^{E}	181.47±0.5^{D}	6.39±0.26^{F}	261.31±0.27^{E}	119.34±0.38^{E}
Dichloromethane fraction	812.49±0.50^{A}	773.41±0.35^{C}	569.30±0.58^{B}	626.13±0.25^{B}	922.21±0.66^{A}	833.73±1.11^{A}
Hexane fraction	-	972.26±0.44^{A}	902.69±0.6^{A}	1019.26±0.28^{A}	-	-
Methanol solid phase extract	488.90±1.01^{B}	521.29±0.50^{B}	578.74±0.65^{B}	571.00±0.01^{C}	666.49±0.52^{B}	602.20±0.34^{B}
Ethylacetate solid phase extract	429.30±0.26^{B}	512.02±0.04^{B}	550.75±0.53^{B}	510.35±0.33^{D}	572.54±0.46^{C}	535.88±0.82^{C}
Doxorubicin drug	4.36±0.22^{E}	6.39±0.47^{F}	3.64±0.33^{E}	4.36±0.22^{G}	6.39±0.47^{F}	3.64±0.33^{F}

The results are expressed as mean inhibitory concentrations ± standard deviation. Different letters in the same column represent significant difference between means at 95% confidence level in ANOVA.

4.6. *In vitro* cytotoxicity activity of phenolic crude isolates of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves against normal vero cell lines and their selectivity indices

4.6.1 Cytotoxicity activity of the extracts

There is a significant difference between doxorubicin drug and all the plant extracts in mean CC_{50} values ($P<0.05$). Doxorubicin drug was potentially very toxic to normal vero cells compared to all extracts as shown in the table (4.7). The cytotoxic concentrations (µg/ml) of different extracts of *R. officinalis* and *O. gratissimum* that killed 50% of normal vero cells (CC_{50}) are shown in table 4.7 below;

Table 4.7. CC_{50} values of extracts on normal Vero cells.

Extract	*R. officinalis* (µg/ml)	*O. gratissimum* (µg/ml)
Crude methanol	468.55 ± 0.51^{E}	610.85 ± 0.64^{C}
Crude ethyl acetate	599.27 ± 0.24^{D}	642.0431 ± 0.07^{C}
Crude dichloromethane	1253.00 ± 0.62^{C}	1210.00 ± 1.74^{B}
Crude hexane	-	-
Ethyl acetate fraction	401.09 ± 0.08^{F}	495.60 ± 0.35^{D}
Methanol fraction	378.38 ± 0.55^{G}	355.04 ± 0.04^{E}
Dichloromethane fraction	1644.64 ± 0.58^{B}	1694.18 ± 0.10^{A}
Hexane fraction	-	-
Methanol Solid phase extract	1897.12 ± 0.11^{A}	1860.46 ± 0.40^{A}
Ethyl acetate Solid phase extract	1841.27 ± 0.47^{A}	1749.68 ± 0.60^{A}
Doxorubicin drug	6.36 ± 0.45^{H}	6.36 ± 0.45^{F}

The results are expressed as mean cytotoxicity concentrations ± standard deviation. Different letters in the same column represent significant difference between means at 95% confidence level in ANOVA.

From the CC_{50} values shown in the table 4.7 above, the CC_{50} values ranged from 355.04 ± 0.04 to 1897.12 ± 0.11 µg/ml. Methanol fraction of *O. gratissimum* showed the least CC_{50} value of 355.04 ± 0.04 µg/ml which is potentially harmful while the SPE extract of methanol showed the highest CC_{50} value of 1897.12 ± 0.11 µg/ml which is potentially non-toxic (IC_{50}<10µg/ml is potentially very toxic; IC_{50} 10-100 µg/ml is potentially toxic; IC_{50} 100-1000µg/ml is potentially harmful and IC_{50}>1000µg/ml is potentially nontoxic (Hussain et al., 2010)). The results obtained show that all the extracts under investigation were less toxic to normal vero cells, compared to the positive control which was doxorubicin drug (6.36 ± 0.45 µg/ml) which is potentially very toxic (IC_{50}<10µg/ml is potentially very toxic; IC_{50} 10-100 µg/ml is potentially toxic; IC_{50} 100-1000µg/ml is potentially harmful and IC_{50}>1000µg/ml is potentially nontoxic (Hussain et al., 2010).

4.6.2. Selectivity indices of all extracts and doxorubicin drug

The extracts of SPE phenolic isolates of both plants had the highest selectivity index since they showed selectivity on all cells, followed by the ethyl acetate and methanolic fractions and then the crude extracts than the positive control, doxorubicin drug ($P<0.05$) as shown in the table (4.8) below. It is also shown in the table that doxorubicin drug is not selective on normal vero cells (with selectivity indices of 1.459, 0.995, 1.747 for prostate, colorectal and cervical cancer cell lines respectively) (Table 4.8) which is shown by its selectivity indices being lower than 3 which is recommended (Njuguna et al., 2018). There was no significant difference in selectivity indices of ethylacetate fractions of *R. officinalis* and *O. gratissimum* on prostate cancer cells ($P>0.05$). Significant difference was observed in crude methanol extracts of *R. officinalis* and *O. gratissimum* on prostate cancer cell lines

($P<0.05$). No significant difference was observed in selectivity indices of ethyl acetate and methanolic crude extracts of both plants in prostate cancer cell lines ($P>0.05$).

Table 4.8 CC_{50}, IC_{50} and selectivity index of the extracts and reference drug. The results are expressed as mean inhibitory / cytotoxicity concentrations ± standard deviation.

Extract	*Rosmarinus officinalis*				*Ocimum gratissimum*			
	Vero	Prostate (DU145)	Colorectal (CT26)	Cervical (HeLa229)	Vero	Prostate (DU145)	Colorectal (CT26)	Cervical (HeLa229)
Methanolic extract	468.55 ± 0.51	147.38 ± 0.53 (3.18)	302.32 ± 0.53 (1.55)	432.47 ± 0.41 (1.08)	610.85 ± 0.64	104.84 ± 0.44 (5.82)	359.91 ± 0.93 (1.70)	586.68 ± 0.45 (1.04)
Ethyl acetate extract	599.27 ± 0.24	182.48 ± 0.50 (3.28)	460.08 ± 0.14 (1.30)	522.80 ± 1.06 (1.15)	642.04 ± 0.07	158.21 ± 0.38 (4.06)	598.48 ± 0.50 (1.07)	626.33 ± 0.47 (1.03)
Dichloromethane Extract	1253.00 ± 0.62	1459.10 ± 0.86 (0.86)	928.57 ± 0.49 (1.08)	931.63 ± 1.19 (1.07)	1210.00 ± 1.74	967.21 ± 0.19 (1.03)	1094.41 ± 0.47 (1.11)	1761.50.00 ± 0.65 (0.69)
Hexane extract	-	-	1104.04 ± 0.06	1001.10 ± 0.41	-	1259.56 ± 0.49	-	2874.81 ± 0.17
Methanolic fraction	401.09 ± 0.08	28.28 ± 0.49 (14.18)	272.32 ± 0.56 (1.47)	385.43 ± 0.52 (1.04)	495.60 ± 0.35	16.16 ± 0.14 (30.68)	357.39 ± 0.34 (1.39)	201.21 ± 0.23 (2.46)
Ethyl acetate fraction	378.38 ± 0.55	8.54 ± 0.47 (44.31)	196.02 ± 0.03 (1.93)	181.47 ± 0.50 (2.09)	355.04 ± 0.04	6.39 ± 0.26 (55.60)	261.31 ± 0.27 (1.36)	119.34 ± 0.38 (2.98)
Dichloromethane fraction	1644.64 ± 0.58	812.49 ± 0.50 (2.02)	773.41 ± 0.35 (2.13)	569.30 ± 0.58 (2.89)	1694.18 ± 0.10	626.13 ± 0.25 (2.71)	922.21 ± 0.66 (1.84)	833.73 ± 1.11 (2.03)
Hexane fraction	-	-	972.26 ± 0.44	902.69 ± 0.60	-	1019.26 ± 0.28	-	-
Methanol solid phase extract	1897.12 ± 0.11	488.90 ± 1.01 (3.88)	521.29 ± 0.50 (3.64)	578.74 ± 0.65 (3.28)	1860.46 ± 0.40	571.00 ± 0.01 (3.26)	666.49 ± 0.52 (2.79)	602.20 ± 0.34 (3.09)
Ethylacetate Solid phase extract	1841.27 ± 0.47	429.29 ± 0.26 (4.29)	512.02 ± 0.04 (3.60)	550.75 ± 0.53 (3.34)	1749.68 ± 0.60	510.35 ± 0.33 (3.43)	572.54 ± 0.46 (3.06)	535.88 ± 0.82 (3.27)
Doxorubicin	6.36 ± 0.45	4.36 ± 0.22 (1.459)	6.39 ± 0.47 (0.995)	3.64 ± 0.33 (1.747)				

CHAPTER FIVE: DISCUSSION OF RESULTS

5.1.1 Percentage yield of the crude leaf extracts

From the results shown in Table 4.1, methanol gave the highest yield (54.14% for *Rosmarinus officinalis* and 27.66% for *Ocimum gratissimum*) while *n*-hexane showed the least (21.35% and 3.19% respectively). This could be due to the differences in polarity as methanol being the most polar of all gave the highest yield. This is because it extracted many compounds from the leaves, the least yield was observed with *n*-hexane extracts because it was the least polar of all solvents used. Differences in solvent polarities used for extraction is known to play a key role in increasing the solubility of phytochemical compounds (Felhi et al., 2017; Naima et al., 2015). Further, differences in the structure of phytochemical compounds also determine their solubility in solvents of different polarities (Felhi et al., 2016). Indeed, the four solvents used had different polarities arranged as hexane < dichloromethane < ethyl acetate < methanol (Felhi et al., 2016). This change is related not only to the differences in the polarity of extracts of the components but also to the solvents used, which also plays a vital role in increasing the solubility of phytochemicals. Therefore, the results of the current study confirmed the effect of different solvent polarities on the yield of plant extracts and confirms the richness of *Rosmarinus officinalis* than *Ocimum gratissimum* leaves in polar phytochemicals. Extraction yield can also differ due to difference in concentration of solvent used, methods of extraction, time and temperatures of extraction. However, these conditions were all optimized when it came to this study because these were all constant for all extractions and only the different solvents were used and thus only solvent polarity playing key in this study.

The results obtained in this study are consistent with those of (Aziz et al., 2018) who assessed the effects of solvent polarity on the phytochemical yields from *O. gratissimum* L leaf extracts. The same observation was made by Bomma et al. (2018) who compared different extraction methods in different *Ocimum* species (*O. gratissimum, O. basilicum, , O. x africanum, O. americanum, O. campechianum* and *O. tenuiflorum*). Maceration method was among the methods under study which used different extraction solvents (methanol, acetone, n-butanol) and concluded that methanol is a suitable solvent for extracting plant bioactive compounds.

5.1.2 Total phenolic content and antioxidant activity of the crude organic extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves

The antioxidant activity (IC_{50}-5.39 ± 0.09) (Table 4.2) of methanolic extract of *R. officinalis* as well as its total phenolic content (TPC) (476.80 ± 0.40 µg/ml GAE) (Figure 4.2) were the highest ($p < 0.05$) compared to n-hexane, dichloromethane and ethylacetate crude extracts of *O. gratissimum* and *R. officinalis*. This is because most phenolic compounds responsible for antioxidant activity have polar functional groups which are easily dissolved in polar solvents like methanol (Widyawati, Dwi, Budianta, & Kusuma, 2014). Similar results of antioxidant activity were reported by (Kontogianni et al., 2013) after carrying out methanolic extraction of rosemary leaves by Soxhlet apparatus. The antioxidant activity (IC_{50}-5.79 ± 0.09) (Table 4.2) of methanolic extract of *O. gratissimum* as well as its total phenolic content (TPC) (401.07 ± 6.47 µg/ml GAE) (Figure 4.2) were lower than the results observed in *R. officinalis*. Similar results of antioxidant activity were reported by Ekunwe et al. (2013) for *O. gratissimum* leaf extracts after fractionating ethanol extracts into different solvents (chloroform, ethylacetate and n-butanol). Similar results

were also obtained by Mariod, Ibrahim, Ismail, and Ismail (2013) after fractionating *O. gratissimum* ethanolic leaves extract with different solvents (chloroform, ethylacetate and n-butanol). The antioxidant activity of plant phenolic compounds is attributed to their redox properties, which allows them to act as reducing agents, hydrogen donators (Equation 8), singlet oxygen quenchers and metal chelators (Cook & Samman, 1996). The DPPH test measures the hydrogen atom or electron donating capacity of extracts to the stable radical DPPH formed in solution (Tepe, Daferera, Sokmen, Sokmen, & Polissiou, 2005). Thus, methanol extracts which had the highest total phenolic contents also had the highest antioxidant activity. Therefore, *R. officinalis* leaves had a better antioxidant potential than *O. gratissimum* leaves due to higher TPC.

$$C_6H_{12}O + \cdot OH \rightarrow H_2O + C_6H_{11}O\cdot \qquad \text{(Equation 8)}$$

A hydroxyl radical seizing a hydrogen atom from a phenol molecule resulting in a water molecule and a phenol radical).

5.1.3 Characterization of phenolic compounds of *O. gratissimum* and *R. officinalis* leaves extracts

The methanol extracts of the plants were further fractioned and characterized using FT-IR and LC/MS spectroscopy. The FTIR spectra of both plant leaves showed absorption at 3400, 2900, 2800, 1700, 1550, 1350, 1250, 1100, 1000 and 900 cm^{-1}. Assignments based on previous studies on phenolic compounds in plants (Ashokkumar & Ramaswamy, 2014 ; Meenakshi et al., 2012; Singh, 2016) confirmed the presence of phenolic compounds in the extracts. The various functional groups observed in the extracts of the plant leaves reflected their biochemical profile which could be responsible for their various medicinal properties, including antiproliferative activity.

Further, LCMS/MS analysis of the methanolic extracts after solid phase extraction revealed the presence of 34 compounds in *R. officinalis* leaf extracts. Procyanidin, Latifoliamide, Hesperidin and Emetine were the phenolic compounds that were not identified in *O. gratissimum* extract. 8 compounds were reported for the first time in *R. officinalis* and these include Procyanidin, hydroxyplorentin, cephalin, Isoquercetin, Latifoliamide, Diadzin, hyperin and emetine. On the other hand, 33 compounds were characterized in *O. gratissimum* extract. The extract of *O. gratissimum* had Tannic acid, Carboxystrictosinedine, Isoferullic acid, Ferrullic acid and Psychotrin which were not identified in *R. officinalis* extract. 9 compounds were reported for the first time in *O. gratissimum* and these include Procyanidin, Carboxystrictosinedine, Isoferullic acid, Psychotrin, hydroxyplorentin, cephalin, Isoquercetin, Diadzin and hyperin. The standards used for quality control in the study (gallic acid and rutin) showed similar LC chromatograms and MS spectra with their corresponding compounds in the samples.

Venuprasad et al. (2014) reported the presence of polyphenols, flavonoids and fatty acids in ethanolic leaf fraction of *O. gratissimum* analyzed by LC-ESI–MS/MS. Oleanolic acid, Methyl acetate, Plamitic acid, 2-alpha, 3 beta-Dihydroxyolean-12-en-28-oic acid, Basilimoside, Apigenin-7,4,'-dimethyl ether, Hymenoxin, Salvigenin, Nevadensin, Xanthomicrol, Nepetoidin A, Apigenin, Luteolin, Methyl eugenol, Sinapic acid and Rosmarinic acid were reported. Similarly, the presence of (poly)phenolic compounds in *R. officinalis* were reported (Mena et al., 2016). The investigation in his study utilized ultra-high-performance liquid chromatography-electrospray ionization-mass spectrometry (UHPLC-ESI-MS^n) which afforded the identification and quantification of 57 compounds, 14 of which were reported in the plant extract for the first time. The *R. officinalis* extract

contained 24 flavonoids (mainly flavones), 5 phenolic acids, 24 diterpenoids (carnosic acid, carnosol, and rosmanol derivatives), 1 triterpenoid (betulinic acid), and 3 lignans (medioresinol derivatives). Carnosic acid was reported as the dominant phenolic compound in the extracts (Mena et al., 2016). The compounds identified in his study were Medioresinol, *p*-Coumaric acid, Luteolin-rutinoside, Luteolin-hexoside, Isorhamnetin-3-O-hexoside, 4-hydroxybenzoic acid, Apigenin-7-O-glucoside, Homoplantaginin (Hispidulin 7-glucoside) among others which have been previously identified in *R. officinalis* (Hossain, Rai, Brunton, Martin-Diana, & Barry-Ryan, 2010; Kontogianni et al., 2013; Pérez-Fons, Garzón, & Micol, 2010; Romo-Vaquero et al., 2012; Segura-carretero & Fernández-gutiérrez, 2011). Five phenolic acids were identified in the rosemary extract, and these included a hydroxybenzoic acid, two hydroxycinnamic acids and two rosmarinic acid derivatives, substantiating previous observations in this species (Hossain et al., 2010; Pérez-Fons et al., 2010).

5.1.4 *In vitro* anti-proliferative activity of the crude organic extracts of *Ocimum gratissimum* and *Rosmarinus officinalis* leaves against selected cancer cell lines

Various phytochemicals such as glucosinolates, phenolics, carotenoids, terpenoids, and alkaloids from plants have been reported to be key actors in cancer therapy (Graham, Quinn, Fabricant, & Farnsworth, 2000; Kaur et al., 2011; Omara et al., 2020 a). In this study, *in vitro* anti-proliferative activity of crude organic extracts of *O. gratissimum* and *R. officinalis* leaves against human prostate (DU145), colorectal (CT26) and cervical (HeLa 229) cancer cells as well as the normal Vero cells were investigated. It was found that both *O. gratissimum* and *R. officinalis* extracts had anticancer activity on the tested cancer cells. All the plants extracts were highly selective on the normal Vero cells (Table 4.6). Methanol

crude extracts showed high cytotoxicity ($p < 0.05$) on the cancer cell lines, yet it also proved to be selective on the normal Vero cells. Methanol solvents extracts can be used for anticancer studies as previously reported by Emeka et al. (2015). This is supported by the results of the antioxidant activity which showed that methanol extracts showed the highest antioxidant activity. Indeed, the antioxidant and antitumor activities of plant extracts have been always reported to be positively related with each other (Kathiriya, Das, Kumar, & Mathai, 2010; Li, Chan, Guo, & Yu, 2007). This is highly because these extracts contain polyphenols which have a hydroxyl molecule which acts as a reducing agents against oxidants that would result into cancers as indicated earlier in chapter two section (2.5.9) and thus showing great antioxidant potential. In a preceding study (S. I. N. Ekunwe et al., 2010), partially purified *O. gratissimum* fractions (1.61 mg/mL) were reported to be effective in inhibiting the proliferation of prostate adenocarcinoma (PC-3) cells. The fractions exhibited antiproliferative activity against PC-3 cells in a concentration dependent manner.

The IC_{50}<10µg/ml is potentially very toxic; IC_{50} 10-100 µg/ml is potentially toxic; IC_{50} 100-1000µg/ml is potentially harmful and IC_{50}>1000µg/ml is potentially nontoxic (Hussain et al., 2010). From the results of anti-proliferative study, doxorubicin drug and ethylacetate fractions of both *O. gratissimum* and *R. officinalis* were potentially very toxic and hexane crude extracts were potentially nontoxic to all cancer cell lines (Table 4.3). The selectivity index (SI) is the ability of an extract to inhibit the growth of cancer cells more than it does to the normal cells. This was calculated from the ratio of CC_{50} values on normal Vero cells to the IC_{50} values on cancer cells ($SI = CC_{50}/IC_{50}$) for each of the extracts. An extract with the $SI > 3$ is considered to be highly selective and has the potential to be used

in the management cancer (Njuguna et al., 2018). From the results obtained, doxorubicin drug was not selective to normal vero cells as it showed selectivity indices less than 3 while SPE isolates of ethylacetate and methanol of both plants showed better selectivity indices as they showed values greater than 3.

As expected, the fractions showed better activity than the crude extracts of the plants with the ethyl acetate fraction being the most active amongst all (Table 4.6). This could be attributed to much greater activity of the polyphenols than in crude extracts where the polyphenols could have faced interferences from other components in the extracts (S. I. N. Ekunwe et al., 2010). It was observed that the isolates obtained through solid phase extraction had lower toxicity levels than the crude extracts and fractions. This could be due to synergistic effects in the crude extracts (Borrás-Linares et al., 2015). Among the fractions, ethyl acetate fraction showed better antiproliferative activity on the cells than the methanol fraction. This could be due to the fact that ethyl acetate solvent was passed through the column before methanol and it had extracted most of the active compounds from the plant extracts. This was still carried on to the solid phase extracted isolates where it was observed that ethyl acetate isolates showed higher activity ($p < 0.05$). The results of anticancer activity of *Rosmarinus officinalis* extracts on the prostate, cervical and colorectal cells are in agreement with previous studies (Akshay et al., 2019; Hussain et al., 2010) whereas that of *Ocimum gratissimum* also agreed with those of previous authors (Aiello et al., 2019; Ekunwe et al., 2010; Torres et al., 2018). *Ocimum gratissimum* showed better activity on prostate cancer (DU145) and cervical cancer (HeLa229) cell lines than *R. officinalis* while *R. officinalis* extracts showed greater activity on colorectal (CT26) cancer cell line.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSION

The results of this study showed that *O. gratissimum* and *R. officinalis* has phenolic compounds and antiproliferative activity against human prostate (DU145), colorectal (CT26) and cervical (HeLa 229) cancer cells. Methanol was the best solvent of extraction as shown by the high antioxidant activity of its extracts. Therefore, results show selective action and potential use of these plants to generate lead compounds for use in developing drugs against prostate, colorectal and cervical cancers. This therefore supports the isolation and use of polyphenols of these plants in pharmaceuticals to combat cancer since some of the compounds that have been characterized in the plants (such as rosmarinic acid, quercetin, gallic acid, rutin) have been reported to have anticancer activity.

6.2 RECOMMENDATION

The study recommends further studies on isolation and use of these plants extracts for pharmacological use against the cancer cells that have been studied.

Studies of antiproliferative activity of these plants in vivo models is recommended.

Isolation of the pure compounds as well as investigation of the mechanism of cytotoxicity of compounds from the leaves of *O. gratissimum* and *R. officinalis* should be undertaken.

The study also recommends further studies on the chemical composition and antiproliferative activity of roots of these plants and this has not been investigated. Further, antiproliferative activity studies in pure compounds of *O. gratissimum* and *R. officinalis*. The study also recommends investigation of polyphenols of *O. gratissimum* and *R. officinalis* on other cancer cell lines because some of the polyphenols could be inactive on the cell lines investigated in this study yet active on the other cell lines that have not been studied.

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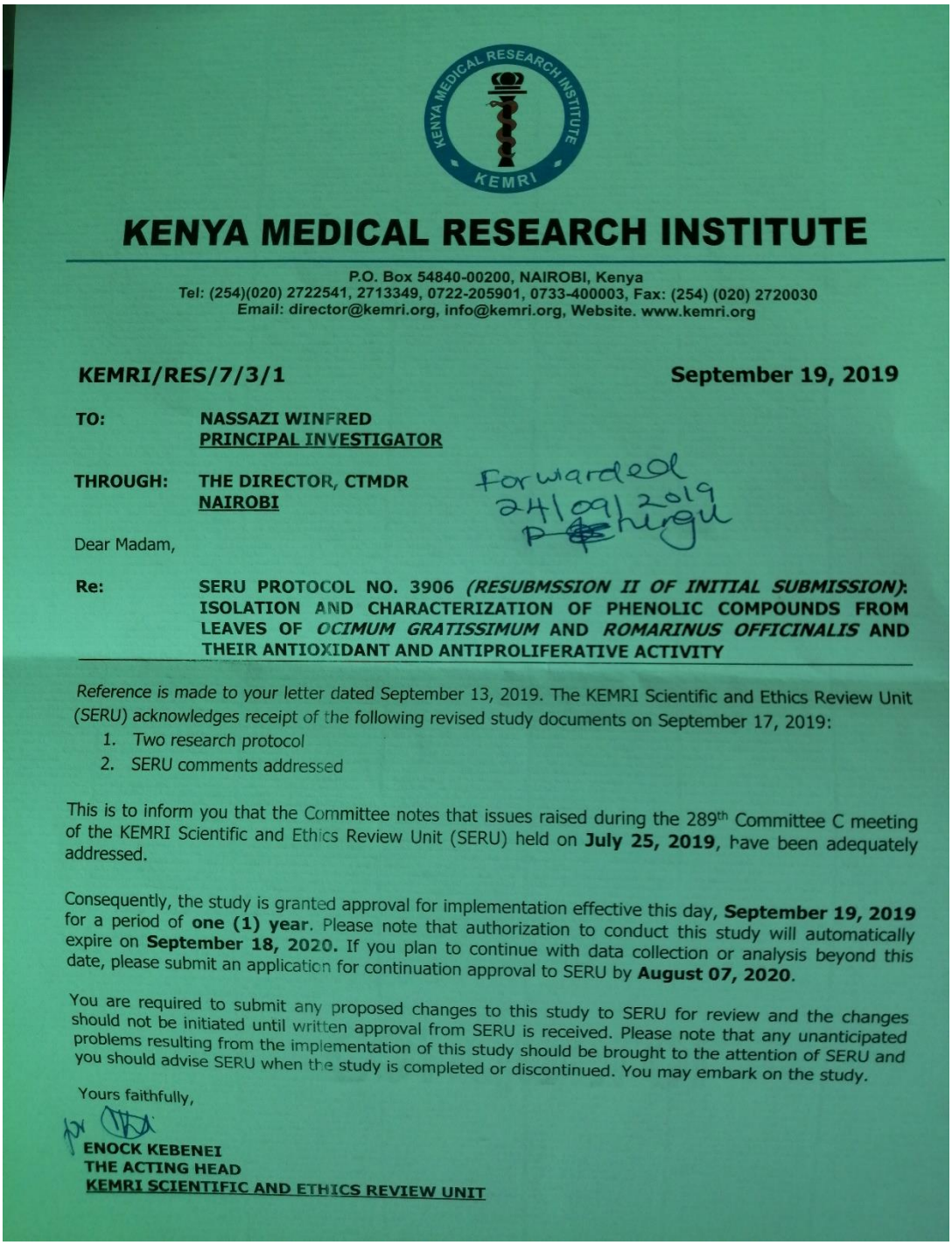
APPENDICES

APPENDIX I: STUDY APPROVAL FORM



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KEMRI/RES/7/3/1 **September 19, 2019**

TO: **NASSAZI WINFRED**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CTMDR**
NAIROBI

Forwarded
24/09/2019

Dear Madam,

Re: **SERU PROTOCOL NO. 3906 *(RESUBMSSION II OF INITIAL SUBMISSION)*: ISOLATION AND CHARACTERIZATION OF PHENOLIC COMPOUNDS FROM LEAVES OF *OCIMUM GRATISSIMUM* AND *ROMARINUS OFFICINALIS* AND THEIR ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY**

Reference is made to your letter dated September 13, 2019. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the following revised study documents on September 17, 2019:

1. *Two research protocol*
2. *SERU comments addressed*

This is to inform you that the Committee notes that issues raised during the 289th Committee C meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **July 25, 2019**, have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **September 19, 2019** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **September 18, 2020.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **August 07, 2020**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued. You may embark on the study.

Yours faithfully,

ENOCK KEBENEI
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT