ANALYSIS OF PHENOLICS FROM *CENTELLA ASIATICA* AND *VERNONIA AMYGDALINA* AND THEIR ROLE AS ANTIBACTERIAL AND ANTIOXIDANT COMPOUNDS

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ABSTRACT

Six species of Malaysian medicinal plants were selected on the basis of their known medicinal values. The plants chosen were *Aloe vera* - leaf and pulp, *Azadirachta indica* - leaf, *Carica papaya* - leaf, *Centella asiatica* - whole plant, *Hymenocallis speciosa* - leaf, tuber and root, *Vernonia amygdalina* - leaf and stem. Aqueous and ethanolic extract of the plant tissues were tested for their antimicrobial properties against five test microorganisms.

Of the six plants tested, the aqueous and ethanolic extract of *C. asiatica* (whole plant) exhibited highly significant antimicrobial activity against the bacterial strains while the ethanolic extract of *Vernonia amygdalina* (leaf) showed distinct inhibition only towards *B. cereus*. *C. asiatica* and *V. amygdalina* were selected as candidates for further evaluation of their medicinal properties.

Ammonium sulphate precipitation was conducted to determine if antimicrobial activity was due to peptides present in the plant. The antimicrobial activity for ethanolic plant extracts was observed in the supernatant with their pellet showing insignificant antimicrobial activity. The highest antimicrobial activity was observed in the ammonium sulphate supernatant of *C. asiatica* against *S. aureus* and *B. cereus*. Since phenolic compounds are responsible for their antimicrobial and antioxidant properties, they were investigated in *C. asiatica* and *V. amygdalina*.

Total phenolic content (TPC) of plant extracts were determined with Folin-Coicalteu assay. In *C. asiatica*, the TPC of the ethanolic extract was the highest 4.006 ± 0.032 mg GAE/g d.w followed by methanolic extracts 3.346 ± 0.029 mg GAE/g d.w and aqueous extracts 1.120 ± 0.063 mg GAE/g d.w. In *V. amygdalina*, the TPC was highest in methanolic extract 1.168 ± 0.101 mg GAE/g d.w, followed by aqueous extract and ethanolic extract had the lowest TPC.

The antioxidant properties of the extracts were determined by DPPH radical scavenging assay and FRAP. Using the DPPH assay, ethanolic extract of *C. asiatica* showed high radical scavenging activity whereas the lowest activity was observed in the aqueous extracts. All three extracts of *V. amygdalina* showed weak radical scavenging activity. Using the FRAP assay, reducing capability of *C. asiatica* and was observed as methanol > ethanol > aqueous > ascorbic acid and the reducing capability of *V. amygdalina* extracts was observed as methanol > aqueous > ethanol > ascorbic acid. Protective effect of the plant extracts were tested against hydrogen peroxide induced haemolysis *in vitro*. The ethanolic and methanolic extracts of *C. asiatica* and *V. amygdalina* showed strong protective effect against hydrogen peroxide.

Biologically active phenolic compounds present were elucidated with reverse-phase high performance liquid chromatography (RP-HPLC), liquid chromatography mass spectroscopy (LC-MS) and fourier transform infrared spectroscopy (FT-IR). RP-HPLC of *C. asiatica* methanolic extract presented two major compounds: chloramphenicol and benzoic acid. The identification of these compounds was confirmed by FT-IR analysis.

The phenolic compounds in *V. amygdalina* were not well separated hence LC-MS was done. Analysis by LC-MS identified thirty eight phenolic compounds in *C. asiatica* and forty in *V. amygdalina* including gallic acid derivatives, hydroxybenzoic acid, hydroxycinnamic acid, flavonoids, purine alkaloids and phenolic terpenes and lignins. Amongst them, the presence of phloridzin which is known for its antidiabetic properties was detected in the methanolic extract of *V. amygdalina*.

ABSTRAK

Enam spesies tumbuhan perubatan Malaysia telah dipilih berdasarkan nilai-nilai perubatan mereka yang diketahui. Tumbuh-tumbuhan yang dipilih adalah *Aloe vera* - daun dan pulpa, *Azadirachta indica* - daun, *Carica papaya* - daun, *Centella asiatica* – seluruh tumbuhan, *Hymenocallis speciosa* - daun, ubi dan akar, *Vernonia amygdalina* - daun dan batang. Ekstrak berair dan etanol tisu tumbuhan telah diuji untuk aktiviti antimikrobial mereka terhadap lima mikroorganisma ujian.

Daripada enam tumbuhan yang diuji, ekstrak berair dan etanol *C. asiatica* (seluruh tumbuhan) menunjukkan aktiviti antimikrobial yang amat ketara terhadap semua mikroorganisma yang diuji manakala ekstrak etanol daripada *V. amygdalina* (daun) menunjukkan aktiviti yang ketara hanya terhadap *B. cereus*. *C. asiatica* dan *V. amygdalina* telah dipilih untuk membuat penyilidikan lanjut terhadap ciri-ciri perubatan mereka.

Presipitasi ammonium sulfat telah dilakukan untuk menentukan samada aktiviti antimikrobial adalah berdasarkan peptida yang hadir dalam tumbuhan. Didapati bahawa aktiviti antimikrob adalah dalam supernatan dengan pelet menunjukkan aktiviti yang tidak jelas bagi kedua-dua ekstrak etanol tumbuhan yang dikaji. Diperhatikan bahawa aktiviti antimikrobial adalah tertinggi bagi supernatan *C. asiatica* terhadap *S. aureus* dan *B. cereus*. Tatkala sebatian fenol mempengaruhi kegiatan antimikrob serta antioksida, ia telah dikaji dalam *C. asiatica* dan *V.amygdalina*.

Kandungan fenol keseluruhan dalam ekstrak tumbuhan telah ditentukan dengan ujian Folin-Coicalteu. Kandungan fenol keseluruhan dalam *C. asiatica* adalah tertinggi dalam ekstrak etanol iaitu sebanyak 4.006 ± 0.032 mg GAE/g d.w diikuti oleh ekstrak methanol sebanyak 3.346 ± 0.029 mg GAE/g d.w dan ekstrak berair 1.120 ± 0.063 mg GAE/g d.w. Kandungan fenol dalam *V. amygdalina* pula adalah tertinggi bagi ekstrak

methanol iaitu sebanyak 1.168 ± 0.101 mg GAE /g d.w, diikuti oleh ekstrak berair dan ekstrak etanol mempunyai kandungan fenol yang terendah.

Ciri-ciri antioksida ektrak tumbuhan telah ditentukan dengan ujian DPPH serta FRAP. Ekstrak etanol *C. asiatica* menunjukkan aktiviti memerangkap radikal yang tinggi manakala aktiviti terendah telah diperhatikan dalam ekstrak berair. Ketiga-tiga ekstrak *V. amygdalina* menunjukkan aktiviti memerangkap radikal yang lemah. Dengan menggunakan ujian FRAP, aktiviti pengurangan bagi ekstrak *C. asiatica* adalah diperhatikan seperti berikut methanol > etanol > berair > asid askorbik. Manakala aktiviti pengurangan bagi ekstrak *V. amygdalina* adalah diperhatikan seperti berikut methanol > berair > etanol > asid askorbik. Keupayaan antihemolisis ekstrak tumbuhan yang dikaji telah ditentukan dengan menggunakan ujian hemolisis in vitro dengan eritrosit arnab. Ekstrak methanol *C. asiatica* telah menunjukkan keupayaan melindungi tertinggi terhadap hidrogen peroksida, ini diikuti dengan ekstrak etanol, manakala ekstrak berair menunjukkan kesan yang tidak tetap. Bagi *Vernonia amygdalina* pula, ekstrak etanol serta methanol telah menunjukkan keupayaan melindungi yang tinggi terhadap hidrogen peroksida manakala ekstrak berairnya didapati mempunyai keupayaan melindungi yang terendah.

Sebatian fenol yang hadir dalam ekstrak tumbuhan telah ditentukan dengan reverse-phase high performance liquid chromatography (RP-HPLC), liquid chromatography mass spectroscopy (LC-MS) serta fourier transform infrared spectroscopy (FT-IR). RP-HPLC ekstrak metanol *C. asiatica* telah membentangkan dua komponen penting iaitu kloramfenikol serta asid benzoik. mengenalpasti sebatian fenol yang hadir. Pengenalpastian dua kompoun fenolik ini telah disahkan oleh analisis FT-IR. Komponen fenol tidak dapat ditentukan bagi *V. amygdalina*, maka LCMS telah dijalankan.

Analisis dengan LC-MS pula telah mengenalpasti kehadiran tiga puluh lapan sebatian fenolik serta empat puluh dalam *V. amygdalina* termasuk derivatif gallic, asid hydroksibenzoic, asid hydroksicinamik, flavonoid, alkaloid purin, terpen fenolik dan lignin. Phloridzin yang telah dikesan dalam ekstrak methanol *V. amygdalina*. memiliki ciri-ciri antidiabetik.

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LIST OF SYMBOLS AND ABBREVIATIONS

AU	-	absorbance unit
α	-	alpha
ATCC	-	American Type Culture Collection
AMPs	-	Antimicrobial peptides
ANOVA	-	Analysis of variance
bp	-	base pair
β	-	beta
BHA	-	butylated hydroxyanisole
BHT	-	butylated hydroxytoluene
C_3	-	carbon 3
C_6	-	carbon 6
cm	-	centimetres
R^2	-	coefficient of determinant
r	-	correlation coefficient
p	-	correlation coefficient
°C	-	degree Celsius
DNA	-	deoxyribonucleic acid
DPPH	-	2,2-diphenxl-1-picrylhydrazyl
$FeCl_3.6H_2O$	-	ferric (III) chloride
FRAP	-	Ferric Reducing Antioxidant Power
$FeSO_4.7H_2O$	-	ferrous sulphate
FT-IR	-	Fourier Transform Infrared Spectroscopy
g/*l*	-	gram per litre
HSV-1	-	Herpes Simplex Virus-1
$FeCl_3$	-	iron (III) chloride haxahydrate
IPB	-	isotonic phosphate buffer
HCl	-	hydrochloric acid
H_2O_2	-	hydrogen peroxide
OH·	-	hydroxyl
HIV	-	Human Immunodeficiency Virus
ITS2	-	Internal transcriber spacer 2

kDa	-	kiloDalton
LC-MS	-	Liquid Chromatography-Mass Spectroscopy
m	-	metres
µ	-	micro
ml	-	millilitre
mg/ml	-	milligram per millilitre
mM	-	millimolar
mg GAE/g d.w.	-	milligram gallic acid equivalent per gram dry weight
mmol AAE/g d.w.	-	milimole ascorbic acid equivalent per gram dried weight
mmol Fe^{2+} /g d.w.	-	milimole ferrous sulphate per gram dried weight
mmol TE/g d.w.	-	milimole Trolox equivalent per gram dried weight
MS	-	mutans streptococci
nm	-	nanometre
O_2^-	-	oxide anion
PCR	-	Polymerase chain reaction
Tween 80	-	Polyoxyethylene sorbitan mono-oleate
TBE-EDTA	-	Tris-Borate-EDTA
TFA	-	trifluoroacetic acid
RP-HPLC	-	Reverse Phase-High Performance Liquid Chromatography
TMV	-	Tobacco Mosaic Virus
TPC	-	Total phenolic content
ROS	-	reactive oxygen species
RNA	-	ribonucleic acid
SD	-	standard deviation
TPTZ	-	2,4,6-tri(2-pyridyl)-S-triazine
UTI	-	urinary tract infection
VZV	-	Varicella Zoster Virus
v/v	-	volume to volume
v/w	-	volume to weight

1.0 INTRODUCTION

Currently, the emergence of bacterial resistance towards antibiotics has become a growing problem. Fleming (1929) discovered the first antibiotic, penicillin which was a mould that was able to lyse *Staphylococcus aureus* cells. Pharmacological industries have produced various new antibiotics ever since, but microorganisms have slowly developed resistance to these drugs because bacteria have the genetic ability to transmit and acquire resistance to drugs (Cohen, 1992).

Plants have been used to maintain human health because they possess chemical compounds that are able to prevent and cure diseases (Nascimento *et al.*, 2000). Furthermore, plant products are a better alternative compared to antibiotics and other synthetic drugs which display negative side effects such as sensitization reactions, and disruption of the metabolic processes in the body via interaction with the body system (Jamil *et al.*, 2007). Hence antimicrobial agents from plants are a more reliable and effective source to fight these microorganisms without the development of resistance.

Novel antimicrobial agents obtained from traditional medicinal plants can be used to treat diseases and inhibit microbial infections (Hemaiswarya *et al.*, 2008; Jain, 1994). These natural products exhibit selective toxicity towards pathogenic bacteria and are generally harmless to host cells (Craig, 1998). The morphological parts of different medicinal plants such as the leaves, seeds, stem, roots, fruits, sap, or tuber are prepared as extracts, infusions, decoctions, powders and are utilized to treat various diseases in humans, plants and animals (Nostro *et al.*, 2000).

In various human cultures around the world, more than 35,000 plant species have been exploited for medicinal purposes (Lewington, 1993). However, this number could be inaccurate because knowledge on the indigenous uses of plants are usually not

well documented and are passed on orally from one generation to another (Jantan, 2004).

Malaysia is covered by tropical rainforest which is home to more than 20,000 species of angiosperms and 600 species of ferns whereby 15% of angiosperms and 13% of fern species were reported to possess medicinal properties (Puteh, 2005). Some of these have been commonly used as folk medicine for hundreds of years (Zaidan *et al.*, 2005). Various compounds are synthesized by biologically and chemically diverse plants in the tropical rain forest and these compounds act as defence agents against microorganisms and diseases. Furthermore, knowledge of the medicinal and chemical properties of these plants products could lead to the design and synthesis of new drugs (Jantan, 2004).

Antimicrobial agents from plants target and destroy biochemical and morphological components of microorganisms not found in host cells (Samy & Gopalakrishnakone, 2010). Researchers are currently investigating natural sources such as plant extracts for antimicrobial agents including phenolic compounds. Antimicrobial agents in plants are plant secondary metabolites and are constantly present in active forms in all plants but in some plants they are activated by plant tissue damage or microbial infection (Osbourn, 1996). Major secondary metabolites in plants include alkaloids, flavones (flavonoids, flavonols, quinones), essential oils, lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids (Samy & Gopalakrishnakone, 2010).

Phenolic compounds are the largest group of secondary metabolites in plants ranging from simple structures with one aromatic ring to complex polymers such as tannins and lignins (Gurib-Fakim, 2006). These compounds serve as defence metabolites to prevent infection and provide oxidative stability in case of injuries (Ćetković *et al.*, 2007; Croteau *et al.*, 2000). Various studies have established that

simple phenolic acids are potent antimicrobial agents (Pereira *et al.*, 2007; Kishimoto *et al.*, 2005; Stoilova *et al.*, 2005; Winkelhausen *et al.*, 2005; Amić *et al.*, 2003).

According to a study conducted by Amić *et al.* (2003) phenolic compounds also possess antioxidant properties and act by scavenging free radicals due to the presence of conjugated ring structures or hydroxyl groups. Therefore it is important to closely study the antioxidant properties of the plant alongside with their antimicrobial properties.

Antioxidants are substances that inhibit free radical-induced oxidative stress in biological systems by neutralizing free radicals, quenching singlet and triplet oxygen, and decomposing peroxides (Thirunavukkarasu *et al.*, 2011; Anderson *et al.*, 2001b). Antioxidants protect the body against oxidative damage initiated by reactive oxygen species. Oxidative damage is thought to be linked to diseases such as cancer, diabetes, shock, arthritis, acceleration of the ageing process and tissue injuries (Shahidi, 1997).

Polyphenols found in medicinal plants are diverse compounds that possess significant antioxidant capacities (Anderson *et al.*, 2001b). It was deduced that flavonoids (Pietta, Simonetti, & Mauri, 1998) phenolic acids, and phenolic diterpenes (Shahidi *et al.*, 1992) are the main phenolic compounds that contribute to the antioxidant properties of plant extracts.

In this study, six medicinal plants of Malaysia that are common and easily accessible: *Aloe vera* (aloe), *Azadirachta indica* (neem), *Carica papaya* (papaya), *Centella asiatica* (pegaga), *Hymenocallis speciosa* (green-tinged spiderlily), and *Vernonia amygdalina* (bitter leaf) were screened for antimicrobial activity. Plants that showed significant antimicrobial activity *Centella asiatica* (*C. asiatica*) and *Vernonia amygdalina* (*V. amygdalina*) were selected and further studies were conducted to identify the compound(s) present in the plant responsible for the antimicrobial activities.

C. asiatica is a member of the Umbelliferae family. This plant is a perennial creeper, flourishing in moist areas, growing naturally in India, Sri Lanka, Madagascar, Africa, Australia, China, Indonesia and Malaysia (James & Dubery, 2009). It has been used in ayurvedic medicine for centuries for the treatment of wound healing, asthma, ulcers, leprosy, lupus, vein diseases, and in memory enhancement (Orhan *et al.*, 2013; Hashim *et al.*, 2011; James & Dubery, 2009). Extracts of this plant were found to be antibacterial and antifungal in properties and also displaced anticancer and antioxidant properties (Hashim *et al.*, 2011).

V. amygdalina is a perennial shrub of 2 to 5 metres in height. It grows throughout tropical Africa and is used to treat malaria, diabetes mellitus, gastrointestinal tract conditions and sexually transmitted diseases. Currently grown in Malaysia, the leaves are locally used for the treatment of hyperglycaemia in diabetes mellitus and hypertension (Atangwho *et al.*, 2013). *V. amygdalina* is a member of the Asteraceae family. Based on recent studies conducted, extracts of this plant were found to be antimicrobial, antifungal, anticancer and antidiabetic (Oga *et al.*, 2013; Ijeh & Ejike, 2011). Antioxidant activity and phenolic content were reported in leaf extracts using different extraction techniques (Atangwho *et al.*, 2013; Fasakin *et al.*, 2011; Salawu *et al.*, 2011; Anyasor *et al.*, 2010; Owolabi *et al.*, 2008) but bioactive phenolic compounds responsible for the antioxidant activity were not identified.

The purpose of the research was to identify compound(s) from plant source that are antimicrobial and to provide further information on the bioactive phenolic compounds responsible for the antioxidant and antimicrobial activity in both *C. asiatica* and *V. amygdalina*.

1.2 Objectives of the research

1. To screen six medicinal plants for antimicrobial activity.
2. To evaluate the antioxidant capacity of selected plants that show strong antimicrobial activity.
3. To identify the polyphenolic compounds present in the selected plants.
4. To evaluate correlations of polyphenolic compounds with the bioactivities.

2.0 LITERATURE REVIEW

2.1 Medicinal Plants as Antimicrobial Agents

Microorganisms are evolving and are becoming resistant towards commercially available antibiotics. Bacteria that are resistant towards antibiotics, antiseptics and disinfectants cause major health problems. Currently, bacterial resistance has resulted in nosocomial infections causing serious health problems in the hospitals. Bacterial resistance may be due to mobile genetic elements such as plasmids, transposons, naked DNA or bacteriophages (Levy & Marshall, 2004; Marchese & Schito, 2000).

Antibiotics were discovered in the nineteenth century, and administered to patients routinely. They have successfully solved public health hazards caused by bacterial infections, but certain antibiotics also cause delirious side effects. These side effects include adverse immune response, allergic reaction, hypersensitivity, nausea, depression of the bone marrow, thrombocytopenic purpura, agranulocytosis and other previously uncommon diseases (Busani *et al.*, 2012; Ghosh *et al.*, 2008; Ahmad *et al.*, 1998). Antibiotics react with the body system and disrupt important metabolic processes (Conlon *et al.*, 2003; Hancock, 1997a; Lehrer *et al.*, 1991). Apart from that, the indiscriminate use of antibiotics in the treatment of infectious disease and the design of antibiotics with limited chemical scaffolds and few advances since the 1980s, led to the development of multiple drug resistant bacteria (Talbot *et al.*, 2006; Shah, 2005; Service, 1995; Davies, 1994). A potential solution to this problem is by using alternative antimicrobial agents discovered from nature. The extraction of bioactive components including antimicrobial peptides and phenolic compounds from natural sources like medicinal plants to treat bacterial infections seem attractive (Conlon *et al.*, 2003; Hancock, 1997a; Lehrer *et al.*, 1991).

The knowledge of using plants to naturally alleviate human health has been around for decades. Around 70,000 plants have the potential to treat various ailments (Busani *et al.*, 2012; Jamil *et al.*, 2007; Nascimento *et al.*, 2000). With few exceptions, naturally occurring plant materials have little side effects on the human body when consumed at the right dosage and are more affordable compared to synthetic alternatives (Ciocan & Bara, 2007). In fact, 80% of the world population depend on plant based medicines (Azaizeh *et al.*, 2003).

Although synthetic drugs and antibiotics brought about a revolution in disease management, medicinal plants serve as a raw material for some important modern medicine and were used to cure deadly diseases even before synthetic drugs were discovered. The World Health Organization states that the best sources of a variety of drugs are from plants (Santos *et al.*, 1995). Medicinal plants serve as a cure for diseases for millions of people inhabiting remote places in the world, who are unable to gain access to synthetic drugs and depend on traditional healers (Bhattacharjee, 2000).

Plant drugs are placed in two categories (i) complex mixtures such as infusions, essential oils, tinctures or extracts and (ii) pure, active compounds from plant extracts. Pure compound from plant extracts with strong and specific activity and/or a small therapeutic index requires an accurate and reproducible dosage (Hamburger & Hostettmann, 1991). Plant extracts are selected as antimicrobial agent after thorough biological evaluation of the safety and efficacy of the extracts followed by the process of identifying active compounds, formulating dosage, efficacy and determining the pharmacokinetic profile of the new drug (Tanaka *et al.*, 2006).

2.2 Medicinal Plants of Malaysia

The natural vegetation of Malaysia is made up of tropical rainforest which is home to an estimated 15000 plant species (Lattif *et al.*, 1984). A study by Burkill (1966), recorded about 1300 plant species were used as traditional medicine in the Malaysian Peninsula. The tropical rainforest is rich with bioactive compounds which act as defence agent against pests, diseases and predators. Defensive compounds from the rainforest have greater diversity compared to those from temperate regions (Coley & Barone, 1996).

Plant extracts from local plants have been used in the treatment of various health ailments in Malaysia (Ong *et al.*, 2011; Siti *et al.*, 2009; Ong & Norzalina, 1999). Elliott and Brimacombe (1986) found that about 25% of the drugs in modern medicine originated from tropical rainforest plant. Various plants such as tongkat ali (*Eurycoma longifolia*), hempedu bumi (*Andrographidis paniculata*), ginseng (*Ginseng baloba*), tumeric (*Curcuma domestica*) and misai kucing (*Orthosiphon stamineus)* were examples used in traditional medicine.

In Malaysia, plant samples are chosen for scientific research based on a random selection or ethno botanical knowledge of the indigenous usage of plants by traditional medicine practitioners (Jantan, 2004). Collection of plants in the earlier years of medicinal plant research in Malaysia was mainly for phytochemical screening. The beginning of medicinal plant research was started as early as 1954 by Arthur, on the phytochemical screening of 205 plants in Sabah followed by the screening of 200 species in peninsular Malaysia for alkaloid by Douglas and Kiang (1957). Most phytochemical screenings were done to isolate bioactive alkaloids because most of the natural compounds are alkaloids.

2.3 Plant Derived Antibiotics

Antibiotics function through bacteriostatic or bactericidal actions by accessing the intracellular targets of bacteria. Gram-positive bacteria do not possess an outer membrane and are relatively susceptible to antimicrobial agents. Gram-negative bacteria consist of an outer membrane which is a permeability barrier and is the main determining factor of antimicrobial resistance in bacteria together with additional resistance mechanism such as efflux and β-lactamases that work together to promote antimicrobial resistance (Hancock, 1997b; Nikaido, 1994).

Plant derived antibiotics that overcome the outer membrane barrier are effective as antimicrobial agents. Plants have multiple methods of defence against pathogens where some defence mechanism are pre-formed while others are triggered after recognition of pathogen attack (Jones & Dangl, 2006). Two groups of plant antibiotics that are involved in plant defence mechanism are phytoalexins and phytoanticipins. It is important to note that the distinction between phytoalexins and phytoanticipins is not based on the chemical structure but how these compounds are produced in the plant (VanEtten *et al.*, 1994).

2.3.1 Phytoalexins

Phytoalexins (Figure 1-1) are plant antibiotics comprising of a variety of small molecules of 500 Da (Hemaiswarya *et al.*, 2008) and are synthesized *de novo* after the exposure of plant towards microbial pathogens (Grayer & Kokubun, 2001). These plant antibiotics consist of a diverse structural space such as terpenoids, glycosteroids, flavonoids and polyphenols. Most of these structures have weak antimicrobial activity compared to bacterial and fungal origin. However plant derived antibiotics have adopted a different pathway to fight bacteria despite the fact that these antibacterial compounds have low potency (Hemaiswarya *et al.*, 2008). Upon detection of pathogen in the plant, phytoalexin production requires transcriptional and/or translational activity. The mechanism triggered involves trafficking and secretion of antimicrobial compounds to the infected area (Bednarek & Osbourn, 2009).

An example of phytoalexin in plants is scopoletin, a hydroxycoumarin found in tobacco plants. This compound displays antimicrobial activity *in vitro* (Matros & Mock, 2004; Chong *et al.*, 2002; Valle *et al.*, 1997). It was shown that a decrease in scopoletin and scopolin (glucoside form of scopoletin) levels is linked with decreased resistance towards Tobacco Mosaic Virus (TMV) infections in tobacco plants (Chong *et al.*, 2002).

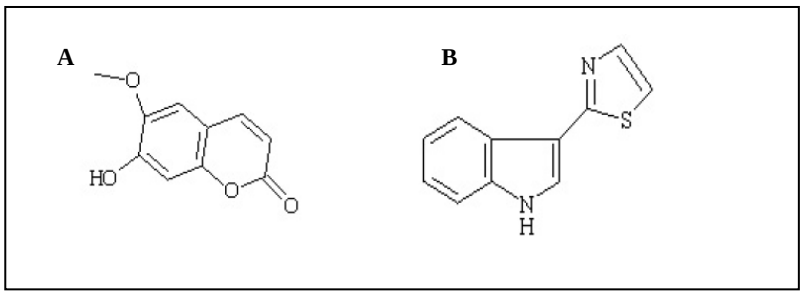

Figure 1- 1: Examples of plant phytoalexin structures
The chemical class of the compound is followed by species common name in parentheses followed by its scientific name. Scopoletin from (tobacco) *Nicotiana tabacum* (**A**) and camalexin from (thale cress) *Arabidopsis thaliana* (**B**).

2.3.2 Phytoanticipins

VanEtten *et al.* (1994) defined phytoanticipin (Figure 1-2) as a low molecular weight antimicrobial compounds present in the plants before pathogenic attack or are produced in the plants by pre-existing constituents after infection by pathogen. Some phytoanticipins are present on plant surfaces while others are sequestered as pre-existing compounds in vacuoles or organelles and are released after attack by pathogen through a hydrolysing enzyme (González-Lamothe *et al.*, 2009).

An example of a glycosylated phytoanticipin is saponin, found in many plant species. Saponin comprises of three major groups of compounds such as triterpenoid, steroid or steroidal glycoalkaloid (Osbourn, 1996). An example of a saponin is avenacin which was discovered in oat roots (Morrissey & Osbourn, 1999). Avenacin forms complexes with sterols in fungal membrane resulting in the loss in membrane integrity due to pore formation. Avenacin A-1, which is the major form of avenacin functions as a chemical barrier in the epidermal layer of oat root tip cells and in emerging lateral root (Osbourn *et al.*, 1994), acting as a chemical defence towards pathogen attack (Papadopoulou *et al.*, 1999).

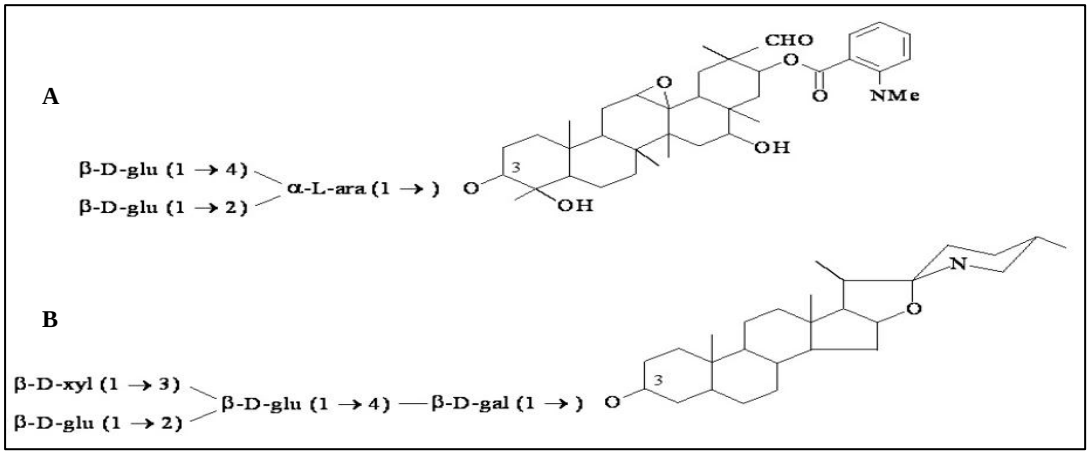

Figure 1- 2: Examples of plant antibiotic phytoalexin structures
The major oat root saponin avenacin A-1 (**A**) and the saponin α-tomatine from tomato (*Solanum lycopersicum*) (**B**).

2.4 Bioactive Components in Plants

There are about 100,000 bioactive compounds produced in plants also identified as the aromatic secondary metabolites. Secondary metabolites are mostly derived from isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways and differ from plant primary metabolites as they are not involved in intermediary metabolism of the plant. The numerous secondary metabolites present in plants are a result of plant evolution towards improved defence against microbes and predators giving plants their antimicrobial trait (Dixon, 2001). Most secondary metabolites are constitutive in healthy plants while others may exist as inactive precursors activated by tissue damage or pathogenic infections (Osbourn, 1996).

Medicinal properties of plants are due to the combinations of secondary metabolites such as alkaloids, steroids, tannins, and phenolic compounds that are synthesized and deposited in specific or in all parts of the plant. These medicinal properties are specific in a plant family, genus and species proving the fact that combinations of secondary metabolites are distinct between plant taxa (Parekh *et al.*, 2005; Balandrin *et al.*, 1985). Composition of secondary metabolites varies between (i) tissues such that the bark, heartwood, roots, branch basses and wound tissues have higher concentration, (ii) species and (iii) seasons (Gottlieb, 1990).

Medicinal plant extracts and phytochemical constituents present in the plant tissues with well-known antimicrobial properties play an important role in promoting human health and are non-toxic to the human body (Sheeba, 2010). Plant extracts work in synergy with synthetic antibiotics against drug resistant bacteria (Ncube *et al.*, 2008). The antimicrobial activity is due to the recognition of potential target sites in microorganisms by plant secondary metabolites which resembles endogenous

metabolites, ligands, hormones, signal transduction molecules or neurotransmitters (Parekh *et al.*, 2005).

Bioactive compounds also give plant their odour such as terpenoids whereas plant pigments are from quinones and tannins. In addition, these compound provide flavour, for example some herbs and spices used by humans to season their food (Cowan, 1999). Secondary metabolites are important as active compounds in medicinal preparation thus it is isolated and identified (Taylor *et al.*, 2001). However, isolating specific active compounds identified in the plant extracts are tedious because extracts contain mixtures of structurally related compounds with different degree of bioactivity and cytotoxicity (Jaki *et al.*, 2008).

2.4.1 Phenolic compounds

Phenolic compounds are compounds that possess at least one aromatic ring with a hydroxyl group or its substituent. This group of compounds was first discovered in lignin when it stimulated various physiological responses in plants and animals (Daniel, 2006). Phenolics are synthesized from cinnamic acid that is formed from phenylanine. The number of constitutive carbon atoms based on the basic phenolic skeleton distinguishes each phenols such as simple phenols, benzoic acid, phenylpropanoids and flavonoids (Michalak, 2006). They are classified based on the carbon atom numbers and biosynthetic pathway each group is derived (Daniel, 2006).

Most of the aromatic secondary metabolites synthesized in plants are phenolics and their oxygen substituted derivatives (Geissman, 1963). Subclasses in this group include phenols, phenolic acid, coumarins, flavones, flavonoids, flavonols, quinones and tannins. These compounds are important antimicrobial agents and serve as the plant defensive agent against pathogenic bacteria (Das *et al.*, 2010).

Phenolics are toxic to microorganisms due to the sites and number of hydroxyl groups on the phenol groups. Past research have revealed that highly oxidized phenols possesses stronger inhibitory actions towards a microorganism (Urs & Dunleavy, 1975). The microorganisms are inhibited by phenolic compound via enzyme inhibition through reaction with sulfydryl group or non-specific protein interactions (Mason & Wasserman, 1987).

2.4.1.1 Simple phenols and phenolic acids

Simple phenols and phenolic acids consist of a single substituted phenolic ring. Simple phenols comprises of phenolic alcohols, aldehydes, ketones and their glycosides. These compounds are colourless (as solids) and are easily oxidised upon exposure to air and alkaline conditions. Woody plants contain free phenols whereas metabolically active plants contain glycosylated phenols (Hopkinson, 1969). The number of hydroxyl groups and their site(s) determine their toxicity towards microorganisms (Scalbert, 1991; Urs & Dunleavy, 1975).

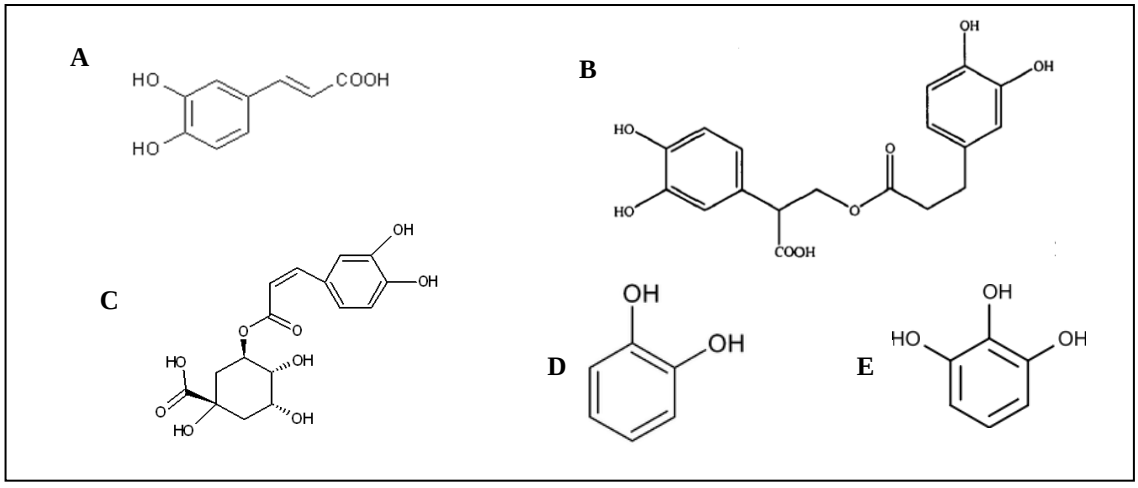

Figure 1- 3: Examples of simple phenols and phenolic acids
Figure displays the molecular structure of caffeic acid (**A**), rosmaric acid (**B**) chlorogenic **(C**), catechol (**D**) and pyrogallol (**E**).

Phenolic acids comprises of benzoic and cinnamic acids. Common benzoic acid includes p-hydroxy benzoic, vanillic and syringic acid found in lignin in angiosperms. Some phenolic acids common in plants are gentisic and protocatechuic acid (Daniel, 2006).

Purified aloe emodin that contains the polyphenols caffeic acid (Figure 1-3A), rosmaric (Figure 1-3B) and chlorogenic (Figure 1-3C) derivatives inhibits Herpes simplex virus-1 (HSV-1), Varicella zoster virus (VZV), pseudorabies and influenza virus (Sydiskis *et al.*, 1991). Analysis on the structure-activity of phenolic groups indicates that antimicrobial activity is related to the site(s) and number of hydroxyl groups (Li *et al.*, 2004). This is evident in catechol (Figure 1-3D) which has two hydroxyl groups and pyrogallol (Figure 1-3E) which has three hydroxyl groups, whereby both are toxic towards microorganisms. However increase in hydroxylation results in increase in inhibitory activity of microorganisms (Geissman, 1963). Some authors also discovered that highly oxidized phenolic compounds are better inhibitors (Scalbert, 1991; Urs & Dunleavy, 1975).

2.4.1.2 Coumarins

Coumarin (Figure 1-4A) comprises of phenolic substances with fused benzene and α-pyrone rings (O'Kennedy & Thornes, 1997). They are responsible for the characteristic odour of hay and possess antithrombotic (Thastrup *et al.*, 1985), anti-inflammatory (Piller, 1975), and vasodilatory (Namba *et al.*, 1988) properties. Coumarins were found to be toxic towards rodents, however based on recent studies, they were discovered to have “species-dependent metabolism” such that in humans toxic coumarin derivatives are excreted via the urine (Weinmann, 1997).

Coumarins such as warfarin (Figure 1-4B) have antiviral properties (Berkarda, 1993), and are used as an oral anticoagulant to inhibit HSV-1 that causes cold sores in humans (Eloff & McGaw, 2006). Based on a study conducted by O'Kennedy and Thornes (1997), it was discovered that coumarins inhibited *Candida albicans* in patients with vaginal candidiasis. Another important compound is calanoid (4-propyldipyranocoumarins) isolated from *Calophyllum lanigerum* var. *austrocoriaceum* and *C. inophyllum*, from the tropical rainforest trees of Sarawak, Malaysia (Currens *et al.*, 1996). *Callophylum* coumarins, classified as calonoids, inophyllums and cordatoloids, based on the C-4 substituent on the lactone ring, are natural reverse transcriptase inhibitors. Calanoid A has potential activity towards HIV-1 reverse transcriptase, hence it is a novel natural non-nucleotide reverse transcriptase inhibitor that may be useful when combined with other antiretroviral drugs (Cos *et al.*, 2004).

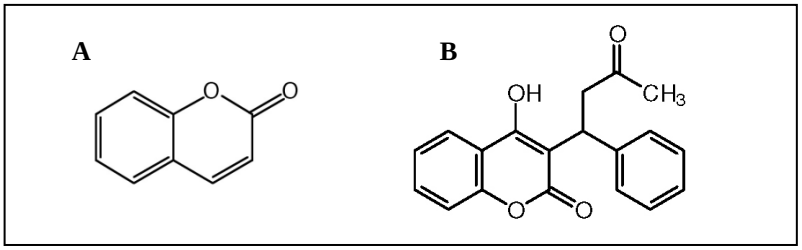

Figure 1- 4: Examples of coumarins
Figure displays the molecular structure of coumarin (**A**) and warfarin (**B**).

2.4.1.3 Quinones

Quinones (Figure 1-5A) are highly reactive compounds comprising of aromatic rings with two ketone substitutions. Quinones are coloured compounds responsible for the browning of cut or injured fruits and vegetables, colour of henna used as dye and an intermediate in the melanin synthesis pathway of skin (Schmidt, 1988; Fessenden & Fessenden, 1982). Oxidation and reduction reactions occur easily leading to the switch between diphenol (hydroquinone) and diketone (quinone), rendering the redox potential important in biological systems. This is observed through the role of ubiquinone (coenzyme Q) in mammalian electron transport system. Apart from that, vitamin K, a naphthoquinone possess antihaemorrhagic activity causing oxidation in body tissues (Cowan, 1999).

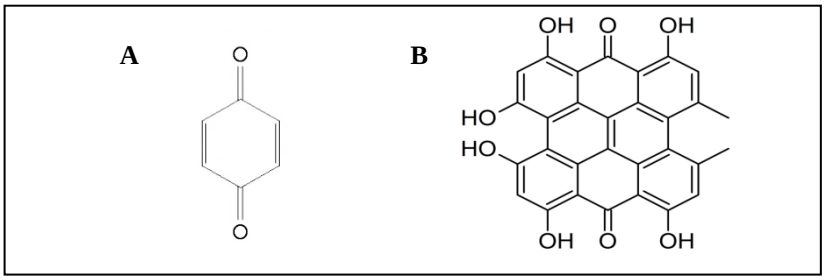

Figure 1- 5: Examples of quinone
Figure displays the molecular structure of quinone (**A**) and hypericin (**B**).

Quinones complex irreversibly with nucleophilic amino acids in proteins resulting in the loss of protein function (Stern *et al.*, 1996). This makes it a potential antimicrobial compound targeting surface-exposed adhesions, cell wall polypeptides and membrane bound enzymes in microorganisms. An anthraquinone isolated from a tree in Pakistan, *Cassia italica* was discovered to possess bacteriostatic activity against *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal activity against *Pseudomonas pseudomalliae* (Kazmi *et al.*, 1994). An antidepressant antharaquinone also known as hypericin (Figure 1-5B)

isolated from *Hypericum perforatum* (St. John's wort) was reported to possess antimicrobial activity (Duke, 1985).

2.4.1.4 Flavones, flavanol and flavanoids

Hydroxylated phenolics containing one carbonyl group form flavones (Figure 1-6A). Flavonol is formed by addition of a 3-hydroxyl group (Fessenden & Fessenden, 1982). Flavonoids are synthesized by plants in response to a microbial infection, and occur as a C_6 - C_3 unit linked to an aromatic ring (Dixon *et al.*, 1983). Flavonoids have a broad spectrum of antimicrobial activity due to their ability to form complexes with extracellular and soluble proteins and to interfere with bacterial cell walls (De Clercq, 2000). Lipophilic flavonoids disrupt microbial membranes (Tsuchiya *et al.*, 1996) and flavonoids lacking hydroxyl groups on their β-rings are active antimicrobials (Chaurasia & Vyas, 1977).

The most reduced form of C_3 in flavonoids are catechins (Figure 1-6B), commonly found in oolong green teas. Teas containing a mixture of catechin compounds have antimicrobial properties (Toda *et al.*, 1989) that inhibit *Vibrio cholerae* O1 (Borris, 1996), *Streptococcus mutans* (Tsuchiya *et al.*, 1996; Batista *et al.*, 1994; Sakanaka *et al.*, 1992; Sakanaka *et al.*, 1989), *Shigella* (Vijaya *et al.*, 1995), and other bacteria and microorganisms (Sakanaka *et al.*, 1992; Thomson & Schultes, 1978).

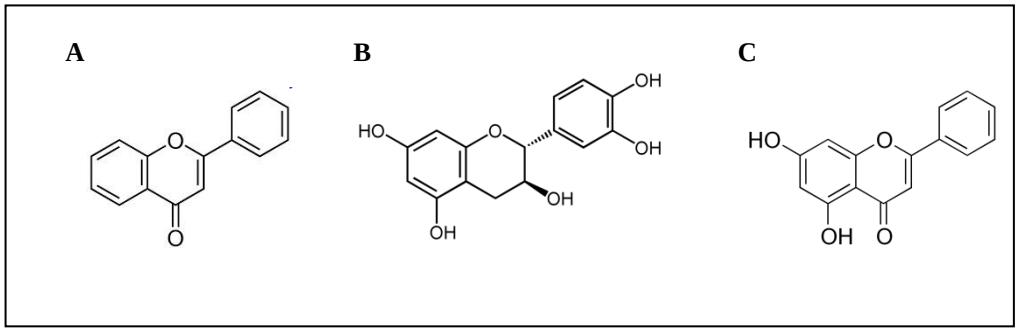

Figure 1- 6: Examples flavones, flavonoids and flavonols
Figure displays the molecular structure of flavone (**A**), catechin (**B**) and chrysin (**C**).

The various antimicrobial activities are (i) galangin, a 3,5,7-trihydrocyflavone from *Helichrysum aureonitens*, which inhibits HSV-1 and coxsackie B virus, (ii) isoquercitrin from *Waldstenia fragarioides* with anti-HSV activity, and (iii) swertifrancheside, glycyrrhizin and chrysin (Figure 1-6C) which inhibit HIV (Cos *et al.*, 2004; Jassim & Naji, 2003). Wogonin is a natural mono flavonoid, with rapid tissue distribution and prolonged rate of plasma elimination possessing anti-inflammatory, anticancer, neuroprotective, and antiviral activities (Tai *et al.*, 2005). This compound is advantageous in the development of antirabies and antiencephalitis therapies.

2.4.1.5 Tannins

Tannins (Figure 1-7A) are a group of polymeric phenolic substances of 500 to 3000 Da, found in the bark, wood, leaves, fruits, and roots of plants (Scalbert, 1991). They possess astringent property and are responsible in tanning leather and precipitating gelatine from solutions (Basri & Fan, 2005; Nizet *et al.*, 2001). Tannins are soluble in water, alcohol and acetone and form precipitates with proteins (Basri & Fan, 2005).

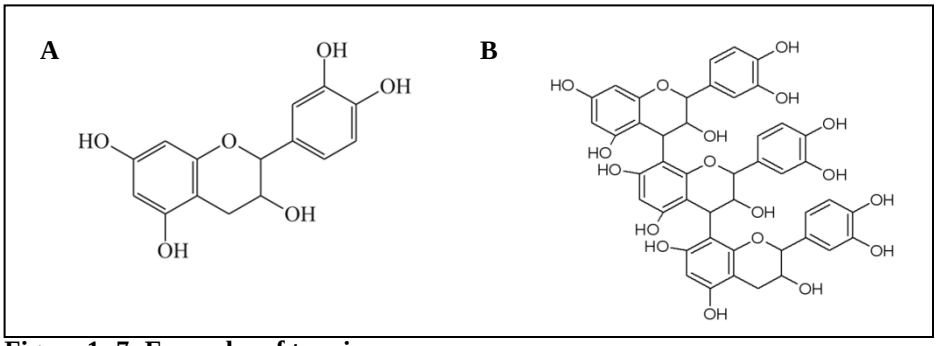

Figure 1- 7: Examples of tannins
Figure displays the molecular structure of tannin (**A**) and proanthocyanidins (**B**).

Tannins can be classified as hydrolysable or condensed forms. Hydrolyzable tannins exist as multiple esters with D-glucose conjugate on gallic acid subunit whereas condensed tannins (proanthocyanidins Figure 1-7B) are derived from flavonoid

monomers. Tannins are formed by condensation of flavan derivatives in woody tissues of plants or by polymerization of quinone units (Geissman, 1963).

Tannins were widely studied when tannin-containing beverages such as red wines and green teas were found to prevent and cure various illness when consumed over a period of time (Serafini *et al.*, 1994). Tannins can stimulate phagocytic cells and inhibit tumours and bacteria by forming complexes with microbial proteins via hydrogen bonding, hydrophobic effect or covalent bonding (Stern *et al.*, 1996; Haslam, 1996).

Tannins may displayed direct antimicrobial activity by inhibiting germ tubes of *Crinipellis perniciosa* at low concentrations (Brownlee *et al.*, 1990). Scalbert (1991) studied the antimicrobial properties of tannins and found that they were toxic towards filamentous fungi, yeasts, and bacteria. Methanolic extracts from the bark of *Terminalia alata* in Nepal were antibiotic in nature (Taylor *et al.*, 1996). In ripe fruits, antimicrobial properties of tannins are due to hydrolysis of the ester linkage between gallic acid and polyols and this serves as a natural defence mechanism (Samy & Gopalakrishnakone, 2010). Traditional medicine practitioners commonly use tannins to treat mouth ulcers, catarrh, wounds, haemorrhoids and diarrhoea (Ogunleye & Ibitoye, 2003).

2.4.2 Terpenoids and Essential oils

Essential oil or *quinta essentia* are compounds responsible for the fragrance in plants. Essential oil comprises of phenolic compounds with a C_3 side chain and has a low level of oxidation state in the absence of oxygen. Oils with enriched isoprene structure are identified as terpenes (Figure 1-8A), having a general chemical formula of $C_{10}H_{16}$. Terpenes exist as di (C_{20}), tri (C_{30}), tetra (C_{40}), hemi (C_5) or sesquiterpenes (C_{15}). Compounds with additional elements, usually oxygen are termed as terpenoids.

Terpenoids share a common origin with fatty acids but differ from fatty acids due to extensive branching and they possess cyclic ring structure. They actively inhibit bacteria (Amaral *et al.*, 1998; Ahmed *et al.*, 1993; Habtemariam *et al.*, 1993), fungi (Ayafor *et al.*, 1994; Harrigan *et al.*, 1993; Kubo *et al.*, 1993), viruses (Sun *et al.*, 1996; Xu *et al.*, 1996; Pengsuparp *et al.*, 1994), and protozoa (Ghoshal *et al.*, 1996; Vishwakarma, 1990). Examples of terpenoids include menthol (Figure 1-8B), camphor (monoterpenes) and artemisin (sesquiterpenoids) (Cowan, 1999; Ahmed *et al.*, 1993).

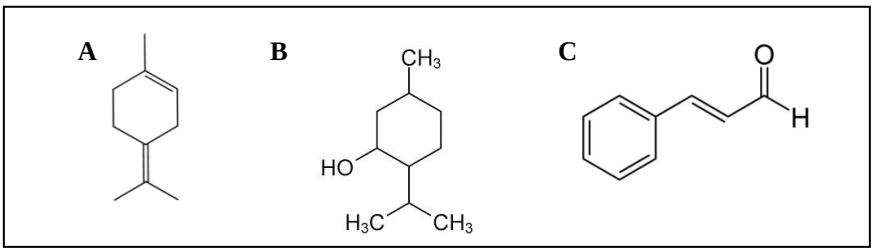

Figure 1- 8: Examples terpenoids and essential oils
Figure displays the molecular structure of limonene (monoterpene), a constituent of lemon and orange oil (**A**), menthol (**B**) and cinnamaldehyde (**C**).

Artemisin and its derivative α-arteether, also known as quinghasu possess antimalarial properties (Vishwakarma, 1990). Chaurasia and Vyas (1977) reported that about 60% of essential oil derivatives inhibited fungi whereas about 30% inhibited bacteria. An example of bioactive component from essential oil with antimicrobial properties is cinnamaldehyde (Figure1-8C) from *Cinnamomum osmophloeum*. It actively inhibited *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* (including methicillin resistant *S. aureus*) and *Vibrio parahaemolyticus*. Cinnamaldehyde also inhibited oral bacteria and was used in antiseptic mouthwashes (Wallace, 2004). Other bioactive compounds isolated from essential oils includes thymol, carvacol, camphor and terpinene-4-ol (Acamovic & Brooker, 2005).

An example of the terpenoid constituent is capsaicin, from *Capsicum annuum* used in many cuisines across the world (Vishwakarma, 1990). Capsaicin was found to inhibit *Helicobacter pylori* and was also reported to be analgesic (Cordell & Araujo,

1993) as it affects the nervous, cardiovascular and digestive systems in humans (Virus & Gebhart, 1979).

2.4.3 Alkaloids

Alkaloids are heterocyclic nitrogen compounds such as nicotine derived from amino acids (Figure 1-9A). Structures of the alkaloid ring includes pyridines, pyrroles, indoles, pyrrolidines, isoquinolines, and piperidines (Bennett & Wallsgrove, 1994) Morphine (Figure 1-9B) was the first medically useful alkaloid isolated from the flower of *Papaver somniferum* (opium poppy) in 1805 (Fessenden & Fessenden, 1982).

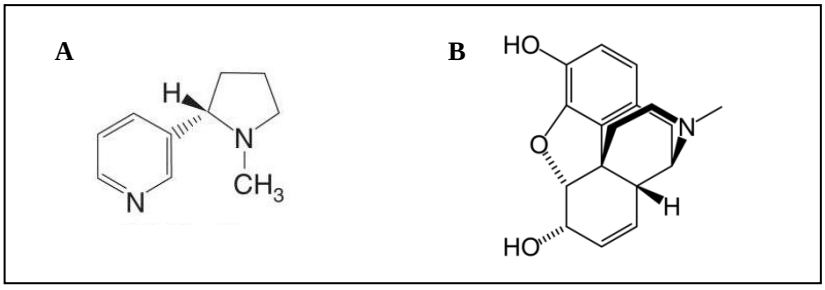

Figure 1- 9: Examples of alkaloids
Figure displays the molecular structure of nicotine (**A**) and morphine (**B**).

Pharmacologically active compounds isolated in the early years were mostly identified as alkaloids (Das *et al.*, 2010) such as (i) diterpenoid isolated from the Ranunculaceae (buttercup) family which displayed antimicrobial activities (Omulokoli *et al.*, 1997; Atta & Choudhary, 1995; Jones Jr & Luchsinger, 1979); (ii) solamargine, a glycoalkaloid from *Solanum khasianum* berries which inhibits HIV and intestinal infections associated with AIDS (McDevitt *et al.*, 1996; McMahon *et al.*, 1995); and (iii) quinine which inhibits malarial parasites (Iwu *et al.*, 1999).

2.4.4 Lectins and Polypeptides

Antimicrobial peptides (AMPs) are a positively charged group of molecules that provides fast and effective means of defences against invading pathogens in the majority of living organisms. The characteristic properties of AMPs include small size (less than 10kDa), cationic, amphipathic and vary in length, sequence and structure. AMP provides defence against a wide range of microorganisms such as gram-positive and gram-negative bacteria, protozoa, yeast, fungi and viruses (Reddy *et al.*, 2004). The first AMP discovered was from wheat flour in 1942 (Balls *et al.*, 1942).

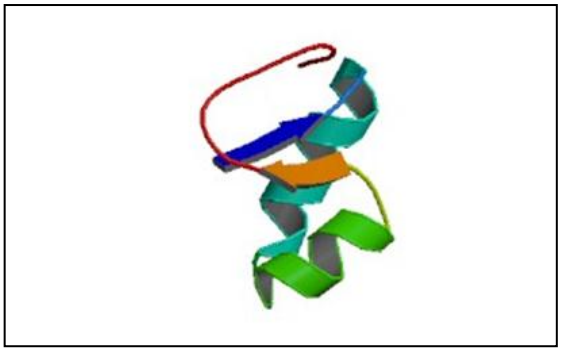

Figure 1- 10: Example of peptide
Figure displays the molecular structure of purothionin from *Hordeum vulgare* (barley).

AMPs are classified into five main groups. The five groups comprises of peptides (i) that form α-helical structure; (ii) β-sheet; (iii) rich in cysteine residues; (iv) rich in regular amino acids (mostly histatin, arginine and proline); and (v) composed of rare and modified amino acids. Mode of action is by selective destruction of cell membranes and the amphipathic structural arrangement by the AMPs. Examples of the mode of action includes the Barrel stave and carpet model (Reddy *et al.*, 2004).

Various plants have been discovered to possess low molecular mass peptides (Ye & Ng, 2002; Huynh *et al.*, 1996; Osborn *et al.*, 1995). These peptides are tissue specific (Price *et al.*, 1987), located in the external layers of plant tissues and act as the first line of defence against microorganisms (Samy & Gopalakrishnakone, 2010).

Purothionin is an example of peptide isolated from barley and wheat, comprising of 47 amino acids and is effective against yeast and bacteria (De Caleya *et al.*, 1972). Another antimicrobial peptide isolated from fava beans, identified as fabatin was found to inhibit *E. coli, P. aeruginosa,* and *Enterococcus hirae* (Zhang & Lewis, 1997).

2.5 Antioxidant Activity of Plant Phenols

The body generates free radicals through metabolic pathways and immune functions. Apart from that, the level of free radicals is also elevated in the body through environmental pollution, pesticides, radiation and ageing (Adedapo *et al.*, 2009; Gulcin *et al.*, 2007). Free radicals are categorized as reactive oxygen species (ROS) which includes radical super oxide anion (O_2^-), hydroxyl (OH·) and non-radicals such as hydrogen peroxide (H_2O_2). ROS target cellular and extracellular components such as lipids, proteins, enzymes, DNA, and RNA resulting in cell death by necrosis or apoptosis (Evans, 1993; Ames *et al.*, 1993).

Imbalance between production of ROS in the cell and enzymatic (superoxide dismutase, glutathione peroxidase, and catalase) and non-enzymatic (ascorbic acid, vitamin E, glutathione, carotenoids, and flavonoids) antioxidants brings about oxidative stress (Peuchant *et al.*, 2004; Gilgun-Sherki *et al.*, 2002). Oxidative stress may lead to degenerative diseases including diabetes, cancer, cardiovascular diseases and inflammatory diseases (Calir *et al.*, 2009; Que *et al.*, 2007; Sharififar *et al.*, 2007; Gerber *et al.*, 2002; Anderson *et al.*, 2001a; Shahidi *et al.*, 1992; Cerutti, 1991).

Antioxidants present in food are able to scavenge ROS and prevent the onset and progression of degenerative diseases (Knekt *et al.*, 1996). Medicinal plants were found to possess antioxidant activity due to the content of phenolic components such as flavonoids (Pietta, Simonetti, Gardana, *et al.*, 1998), phenolic acids, and phenolic diterpenes (Shahidi *et al.*, 1992) in the plants.

The antioxidant activity of phenolic compounds is attributed to (i) scavenging free radicals via donating a hydrogen atom to reduce ROS; (ii) their high tendency to chelate metal ions due to the presence of hydroxyl and carboxyl groups; and (iii) ability to inhibit enzyme involved in free-radical production (Hensley *et al.*, 2004; Aruoma, 2003; Yang *et al.*, 2001). Antioxidant activity of medicinal plants against hydroxyl radicals, superoxide anions, singlet oxygen and lipid peroxides have been investigated (Yen & Chen, 1995; Masaki *et al.*, 1995) and are associated to preventing occurrence of the above mentioned diseases.

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) used in the food industry to prolong shelf life of food may cause liver damage and carcinogenesis (Wichi, 1988; Witschi, 1986). Therefore antioxidants from natural sources are preferred to synthetic ones.

2.6 Selection of Plant Species for Investigation

Plants were selected based on several factors: (i) ethnobotanical approach which is local traditional folklore medical knowledge information (Anbarashan *et al.*, 2011); (ii) chemotaxonomic approach where the relatives of plants known to produce useful compounds were selected; and (iii) by random selection (Jantan, 2004). In the chemotaxonomic approach, plants are selected based on the correlation between taxonomy and occurrence of plant secondary metabolites. The same active biological compounds may be present in related species, genera or families (Eloff & McGaw, 2006). Based on the above criteria's *Aloe vera, Azadirachta indica, Carica papaya, Centella asiatica, Hymenocallis speciosa* and *Vernonia amygdalina* were chosen for investigation.

Traditional medicine practitioners have little knowledge on the scientific rationale of their medicines and their knowledge is purely based on experience (Gurib-Fakim, 2006). However, better knowledge on the therapeutic function of medicinal plants on treatment and prevention of diseases is required for more rational prescription.

2.6.1 *Aloe vera* (L.) *Burm. f.* (*A. vera*)

A. vera is a short-stemmed succulent perennial herb growing at 80 to 100 cm tall, spreading by offsets and root sprouts. The plant consists of elongated, pointed, thick and fleshy, green leaves with a serrated margin. The spike which is about 90 cm tall produces flowers. Each flower pendula has a yellow tubular corolla that is 2 to 3 cm long. The tissue in the centre of the aloe leaf contains a bitter, yellow latex and a transparent mucilaginous gel which is responsible for most of the bioactive compounds in the plant (Kumar *et al.*, 2010; Chow *et al.*, 2005).

A. vera comprises of over 400 different species and belongs to the Liliaceae family (Grover *et al.*, 2011) formerly known as the Asphodelaceae family. Another well-known member of this family is *A. ferox*. The characteristic medicinal feature of the genus aloe are polysaccharides, antharanoids and anthraglycosides (aloe-emodin) that accumulates in the leaves (Gurib-Fakim, 2006).

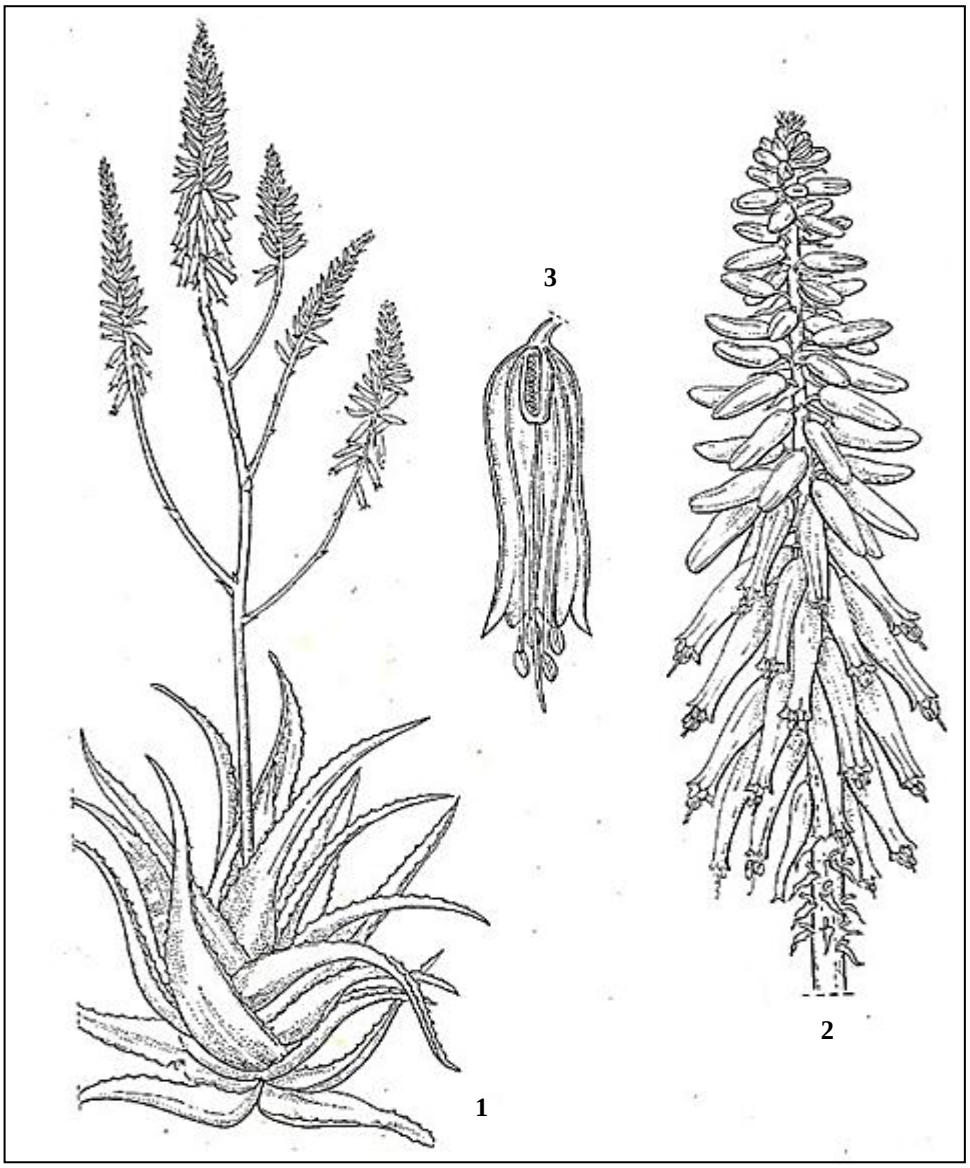

Figure 1- 11: *Aloe vera* (L.) *Burm. f.*
Figure displays the morphology of *A. vera* where **1**- plant habitat; **2**- part of inflorescence; **3**- flower in longitudinal section.

Image adapted from de Padua et al. (1999)

A. vera is native to North Africa, the Mediterranean region of South Europe and the Canary Islands. Now, it is widely cultivated in West Indies, tropical America and in tropical regions (Ross, 1999a). *A. vera* pulp is made up of 98.5% water, whereas the gel is made up of 99.5% water (Eshun & He, 2004). The remaining percentages of materials are fat soluble vitamins, minerals, enzymes, polysaccharides, phenolic compounds and organic acids (Boudreau & Beland, 2006).

This plant has many healing properties such that it relieves burning sensations, and blisters, heals ulcers, wounds on the skin and gastrointestinal lining through the release of interleukin-1, interleukin-6, tumour necrosis factor and interferon (Kumar *et al.*, 2010; Yates *et al.*, 1992; Peng *et al.*, 1991). The fresh leaf juice is used to treat rashes, vaginal infection, eczema, foot sores, fungus attacks, eye infections and fever (Anbarashan *et al.*, 2011; Kumar *et al.*, 2010). The leaf sap or juice is applied externally to treat pimples, blackheads and cuts.

In Indonesia, the locals mix the sap with other ingredients to mask the bitter taste to cure asthma and cough. In Malaysia, aloe drug 'jadam' is used as an aperient, to heal wounds and swelling and daubed in the abdomen when fever and after confinement (de Padua *et al.*, 1999). It also aids digestion, blood circulation and the lymph system and improves the function of the liver and gall bladder (Kumar *et al.*, 2010). *A. vera* is a good absorbent and penetrates into tissues of the skin four times faster than water making it a good moisturizer, cleanser, exfoliator and it relieves joint and muscle pains (Lawrence *et al.*, 2009).

The bioactive compound, anthraquinone isolated from *A. vera* displays antimicrobial activity against gram-negative and positive bacteria (Habeeb *et al.*, 2007). It inhibits *Shigella flexneri, Streptococcus progenies, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli, Propionibacterium acne, Helicobacter pylori and Salmonella typhi* (Ferro *et al.*, 2003;

Pugh *et al.*, 2001; Lawless & Allan, 2000; Reynolds & Dweck, 1999; Urch, 1999). *A. vera* also inhibits oral bacteria which cause gum diseases and therefore it was incorporated into toothpaste and rubbed on the gums (Kumar *et al.*, 2010).

2.6.2 *Azadirachta indica* A. Juss (*A. indica*)

A. indica or better known as "neem" was discovered more than 2000 years as the most versatile medicinal plant with an array of biological activities. Every part of the plant is useful in treating various diseases. Hence it was given the Sanskrit name 'arisjtha' which means 'reliever of sicknesses'(Biswas *et al.*, 2002). In 1992 this plant was recognized by the US National Academy of Sciences which published a report "Neem – A tree for solving Global Problems" (Schmutterer, 1995).

A. indica is a tropical evergreen plant growing up to 25 m high. The bark is rough, dark brown with flat ridges separating the wide longitudinal fissures. Leaves are compound and imparipinnate, comprising of 5 to 15 leaflets arranged with the terminal leaflet in alternate pairs. Each leaf is about 6 cm long and 2 cm wide. Flowers have oblanceolate petals and are sweet scented with yellow, ellipsoid and gabrus drupes measuring about 12 to 20 cm long. The sepals are ovate, about 1 cm long. Most flower panicles are arranged in the leaf axils (Ross, 1999a).

A. indica belongs to the Meliaceae family and is native to East India and Burma. It is widely distributed in Southeast Asia and West Africa. Recently it was found in the Caribbean and in South and Central America (Ross, 1999a).

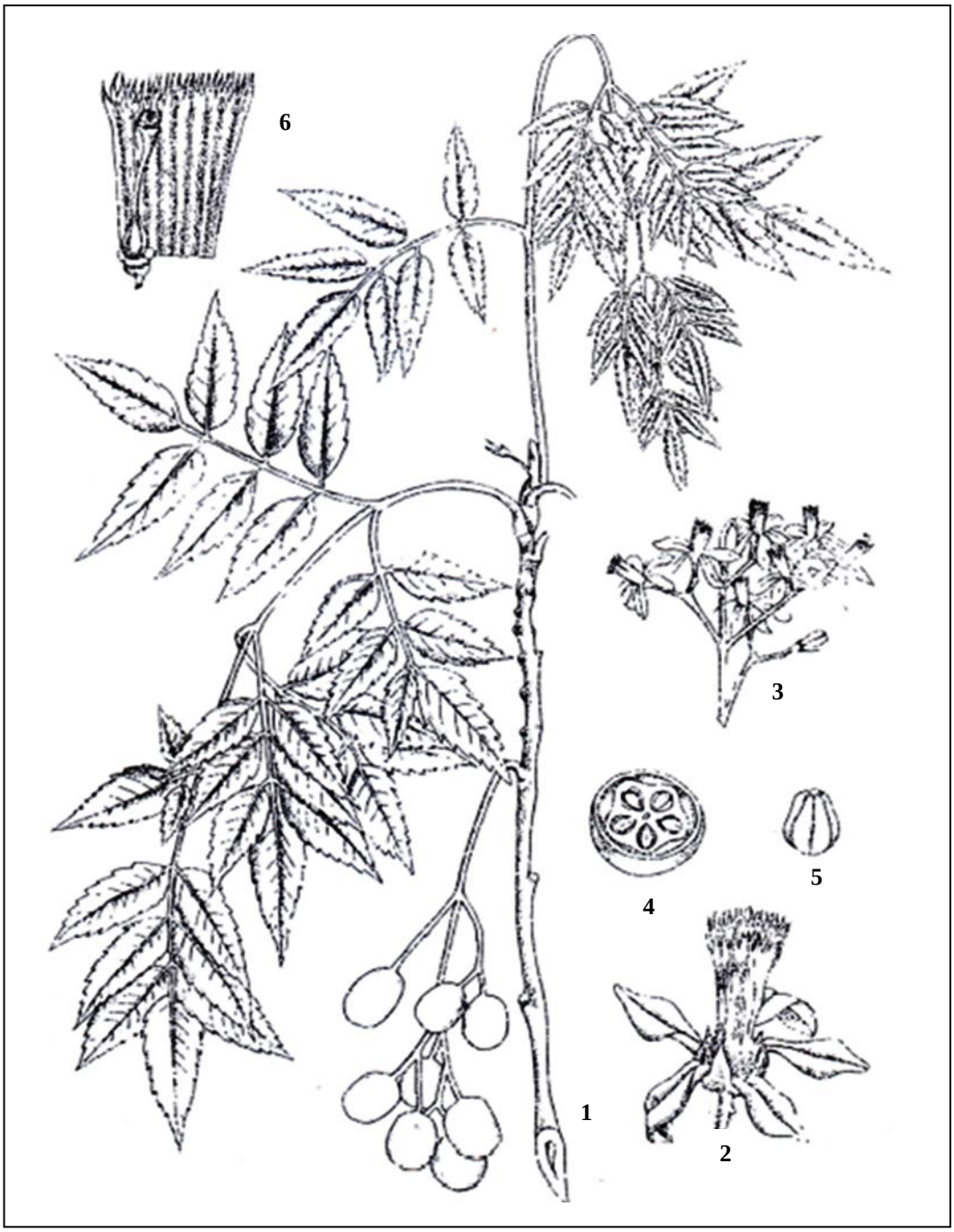

Figure 1- 12: *Azadirachta indica* A. Juss
Figure displays the morphology of *A. indica* where **1**- plant bark with leaves and fruits clustered towards ends of branches; **2**- flower; **3**- twig with flowers; **4**- fruit cross section; **5**- seed; **6**- flower cross section.

Image source from Kapoor (1990)

Both the crude extracts and fractions of the leaf, root, seed, oil, fruit and bark of the neem tree display biological activities. In folk medicine, the natives use neem oil, bark and leaf extracts to treat leprosy, intestinal helminthiasis, respiratory disorders, constipation and to improve health (Kirtikar & Basu, 1935). The oil is used in skin

infections and to kill head lice. Mixtures containing bark, leaf, root, flower and fruit extracts are used to treat blood morbidity, biliary afflictions, itching, skin ulcers, burning sensations and pthysis (Biswas *et al.*, 2002). Dried flowers are consumed orally for diabetes and hot water extracts of the dried fruit is used to treat piles, skin diseases and ulcers (Ross, 1999a). Leaf extracts cure ringworms, eczema and scabies. The bark is used to cure malarial fever and leaf paste was to sooth mumps (Anbarashan *et al.*, 2011).

A. indica showed various pharmacological activities such as reported by Murty *et al.* (1978) where the blood glucose level significantly decreased and adrenaline and glucose-induced hyperglycaemia was prevented by aqueous extracts of neem leaves. Another study by El-Hawary and Kholief (1990) revealed that normal rats fed orally with aqueous leaf extracts developed hypoglycaemia whereas blood glucose level dropped in diabetes induced rats. Anti-acid secretory and antiulcer activity was observed in glycosides isolated from aqueous extracts of neem (Biswas *et al.*, 2002). Bandyopadhyay *et al.* (2004) revealed that aqueous bark extracts inhibited gastric acid and pepsin secretion and was effective in healing duodenal ulcer that was both *H. pylori* mediated and non- *H. pylori* mediated with no significant side effects even at high dosages.

Seed extracts and purified fractions inhibited the growth and development of malarial parasite *P. falciparum* (Dhar *et al.*, 1998). Fungi such as *Trichophyton, Epidermophyton, Microsporum, Trichosporon, Geotricum* and *Candida* were inhibited by leaf, oil and seed extracts (Schmutterer & Ascher, 1983). *In vitro* application of the oil extracted from neem leaf, seed and bark inhibited Gram-negative and Gram-positive bacteria such as *Mycobacterium tuberculosis, Mycobacterium pyogenes, Streptococcus mutans, Streptococcus faecalis, Vibrio cholerae, Klebsiella pneumoniae* and streptomycin resistant strains (Almas, 1999; Satyavati *et al.*, 1976; Chopra *et al.*, 1952).

2.6.3 *Carica papaya* Linn. *(C. papaya)*

C. papaya belongs to the Caricaceae family. It is a perennial herbaceous plant of 10 metres tall. The stem is about 25 cm thick and may be simple or branched above the middle. Leaves have a cylindrical stalk, clustered around the apex of the stem with branches that are 25 to 100 cm long. Each leaf blade has lobes and prominent veins. Flowers are hermaphrodite and the male plant may convert to female once beheaded. Otherwise male (with drooping peduncles) and female (short stalked) flowers are borne on separate plants. Flower consist of 5 white petals, are oblong, recurved and emerge singly or clustered from the main stem among the lower leaf. The fruit comes in various forms and sizes with a smooth and thin skin, deep yellow to orange colour when ripe. The flesh is red, juicy and sweet, with a central cavity containing a mucous pulp which holds numerous black seeds.

C. papaya originated from Southern Mexico and Central America. Currently this plant is widely cultivated in all tropical and subtropical countries, utilized as a commercial crop. Parts of the plant that are beneficial to humans are the leaves, fruit, seed, latex and root (Ross, 1999b). In Burma, the latex is used externally to cure diphtheria and tapeworms. In China, the pulp is utilized to reduce swelling and inflammation of the feet (Wiart, 2002). In Malaysia the fresh unripe fruit juice and hot water extract of flowers are consumed as an emmenagogue (Ross, 1999b). In the Peninsular the root paste is rubbed on the body during confinement; seeds are consumed to induce abortion; latex is used to remove patches on skin. The latex of unripe fruit was used to poison criminals in Kelantan (Wiart, 2002).

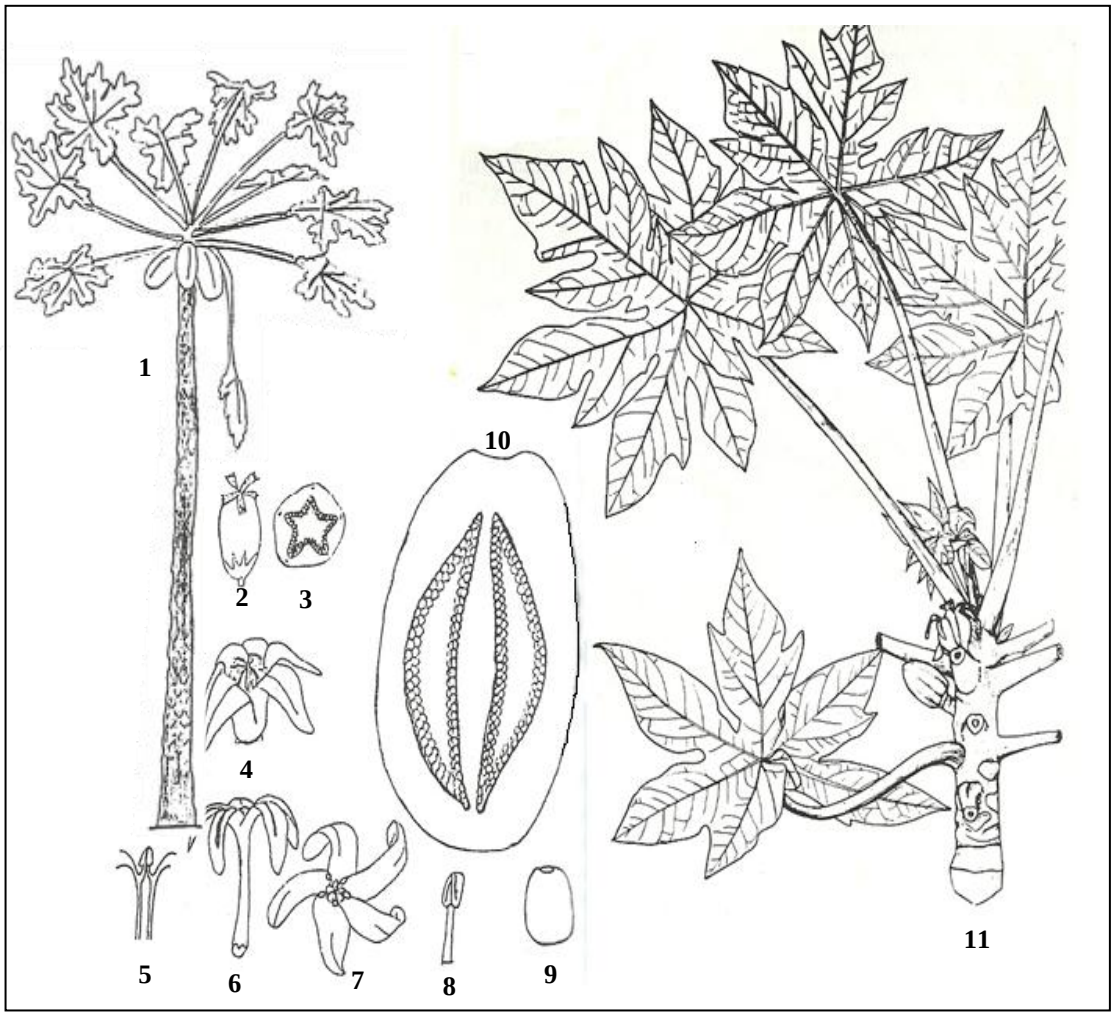

Figure 1- 13: *Carica papaya* Linn.
Figure displays the morphology of *C. papaya* where **1**- whole plant; **2**- flower bud; **3**- fruit cross section; **4**- female flower; **5**- male reproductive part; **6**- male flower; **7**- flower; 8- female reproductive part; **9**- seed; **10**- fruit; **11**- plant stem with leaves clustered around apex of stem.

Image adapted from Wiart (2002) and Duke (1985)

Natural compounds possessing high antitumor and pesticide properties are produced in the leaf, bark and twig. The tree has an array of natural self-defence compounds making it highly resistant towards pathogens (Baskaran *et al.*, 2012). Ahmad *et al.* (2011) proved that *C. papaya* leaf extract could cure dengue fever when a 45 year old patient who was bitten by a carrier mosquito was administered with aqueous leaf extract twice daily for five consecutive days. Based on the patient's condition and blood reports aqueous extracts of *C. papaya* leaves exhibited activity against dengue fever.

Plant latex was applied to the teeth to relieve inflammatory pain (Anbarashan *et al.*, 2011). Leaf extracts possesses antitumor agents and the juice is consumed to treat warts, cancer, tumour, and induration of the skin (Hartwell, 1969). Aqueous leaf extracts inhibited *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Suresh *et al.*, 2008). Ethanolic extract of the leaf and stem inhibited *E. coli*, *S. flexnert*, *S. paratyphi* A, *S. typhi*, *P. aeruginosa*, *M. luteus*, *P. mirabilis*, *B. subtilis* and *S. aureus* (Rahman *et al.*, 2011). This plant is anthelminthic as aqueous extract inhibited *Heligmosomoides polygyrus* parasite infection in mice (Satrija *et al.*, 1995).

2.6.4 *Centella asiatica* Linn. *(C. asiatica)*

C. asiatica (syn *Hydrocotyle asiatica*), locally known as 'pegaga' is one of the miracle 'elixirs of life', and is an important herb. It is used as a cover crop in tea and rubber plantations, incorporated into food where locals eat it raw or cooked, in salads or curries, and leaves are used as a fish poison (Duke, 1985).

C. asiatica belongs to the Apiaceae family, previously known as the Umbelliferae family, comprising of over 3000 herbaceous species. This family consists of many of the common spices and herbs used today. Some of the common herbs with medicinal properties belonging to this family are caraway (*Carum carvi*) used against bloats, coriander (*Coriander sativum*) and fennel (*Foeniculum vulgare*) used as a carminative and anis (*Pimpinella anisum*) (Gurib-Fakim, 2006).

Centella is made up of about 40 species that are found widely growing only in South Africa. Exception is made for the species *C. asiatica* which is distributed throughout South-East Asia and some subtropical regions (Hargono *et al.*, 1999). Parts

of the plant that are useful to humans are the whole plant, leaves, fruits, root and seed (Kapoor, 1990).

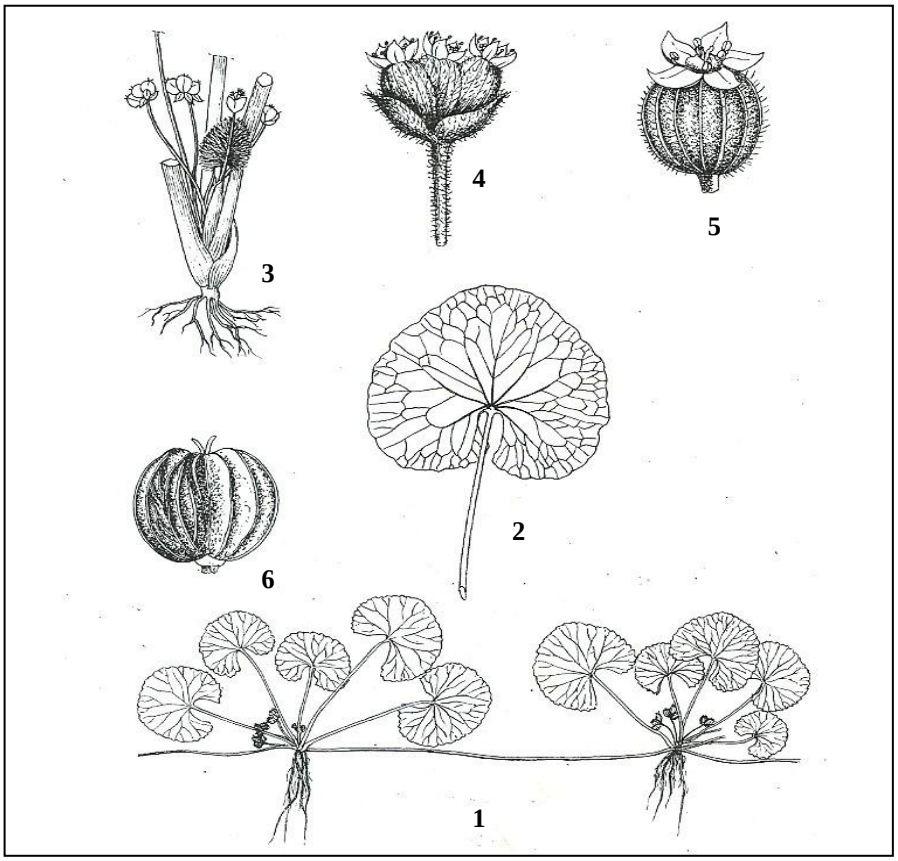

Figure 1- 14: *Centella asiatica* Linn.
Figure displays the morphology of *C. asiatica* where **1**- whole plant; **2**- leaf; **3**- stem base with young leaf, flowers and fruits; **4**- inflorescence; **5**- flowers; **6**- fruit.

Image taken from de Padua et al. (1999)

This plant has been around since prehistory to treat a wide range of health problems in southern-Asia, China and India. In China, this plant is known for its cooling properties and is used as a tonic. In India it is used as a tonic, to treat dysentery and as a non-alcoholic anti-epileptic medicine. In Sri Lanka, extracts are taken as galactagogue. In Vietnam, it is used for the treatment against senility (Hargono *et al.*, 1999). In Malaysia, according to Azmah (1989), *C. asiatica* was used to cure dizziness, hypertension, to improve memory, and relieve indigestion. It is also used to cure malaria and sore eyes in Kelantan (Sabariah, 1987). And in Sabah it is used to reduce fever, cool the body and to soothe toothaches (Ajik, 1990; Amandus, 1989).

This plant is popular amongst traditional medicine practitioners. The whole plant is used to cure skin related diseases. Plant extracts are used to heal surgical wounds minor burns, keloids, leg ulcers, slow healing wounds, lupus, scleroderma, leprosy and cellulitis. Pure extracts accelerate skin grafting and cicatrizing (Hargono *et al.*, 1999). Seeds were used to cure dysentery, headache and fever. A small quantity of the plant stimulates appetite, aids indigestion and alleviates bowel trouble in children. Decoctions of young roots are administered for haemorrhoid (Duke, 2010).

Triteropenoid isolated from *C. asiatica* such as asiaticosida, madecassoside, asiatic acid and madecassic acid were identified as biologically active compounds responsible for the medicinal properties of the plant. Aqueous extracts was discovered to inhibit *Herpes simplex* II virus and asiaticosida isolated from the plant accelerated the recovery of guinea-pigs induced with tuberculosis (Hargono *et al.*, 1999).

2.6.5 *Hymenocallis speciosa* (Salisb.) (*H. speciosa*)

H. speciosa is from the Amaryllidaceae family, genus Amaryllis and monocotyledon order Asparagales. It is a herbaceous, perennial, bulbous flowering plant (Stevens, 2001; Meerow & Snijman, 1998). The plant has 7 to 9 evergreen, rosulate leaves that lasts more than a year and are long, broad, elliptic blades with a distinct petiole. Each plant produces about 7 to 12 white flowers that are wide-spreading with pedicels of 1 cm long. The stamina cup is funnel shaped, placed between the erect filaments (Sealy, 1954).

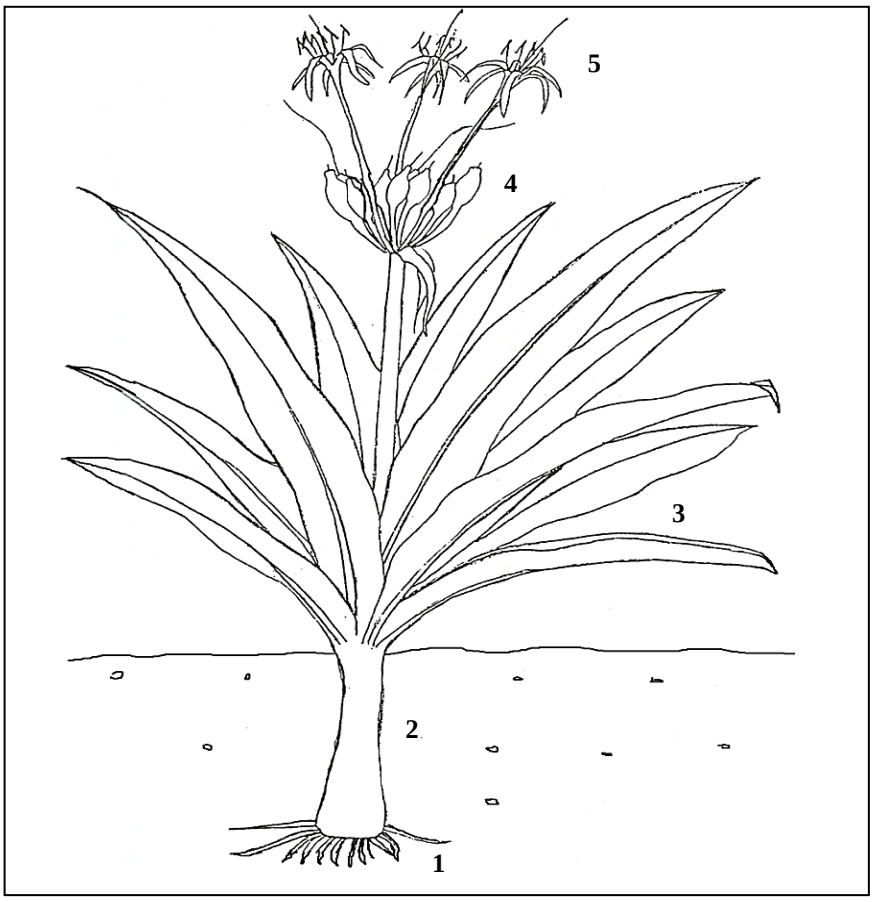

Figure 1- 15: *Hymenocallis speciosa* (Salisb.)
Figure displays the morphology of *H. speciosa* where **1**- roots; **2**- tuber; **3**- leaf blade; **4**- flower buds; **5**- flowers; **1,2**- parts underground; **3,4,5**- parts above the ground.

Image adapted from Wiart (2002)

Plants belonging to the Amaryllidaceae family were used as folk medicine to cure various diseases since thousands of years. Many bioactive compounds such as alkaloids, phenolics, flavonoids and glycosides were isolated from this family. Amongst these compounds, alkaloids isolated and identified as amaryllidaceae were associated with the pharmacological effects of the plant (Jin & Xu, 2013). A study by Şener *et al.* (2003) proved that four groups of amaryllidaceae alkaloids isolated from plant extracts of Amaryllidaceae plants had antimalarial activity by inhibiting the growth of *Plasmodium falciparum in vitro*. In Malaysia the leaves of *H. speciosa* which were locally known as 'pokok demam panas' were used to cure jaundice. The leaf extract was prepared as a decoction and was administered to the patient by bath (Azliza *et al.*, 2012).

2.6.6 *Vernonia amygdalina* Del. (*V. amygdalina*)

V. amygdalina, also known as "bitterleaf" belongs to the Asteraceae family, previously known as the Compositae family. It is a tropical plant growing in warmer regions, dominating South Africa and is popular as a leafy vegetable amongst the Ibos clan in Nigeria (Sule & Agbabiaka, 2008). About 35 species are distributed in Malaysia (Chuakul *et al.*, 1999).

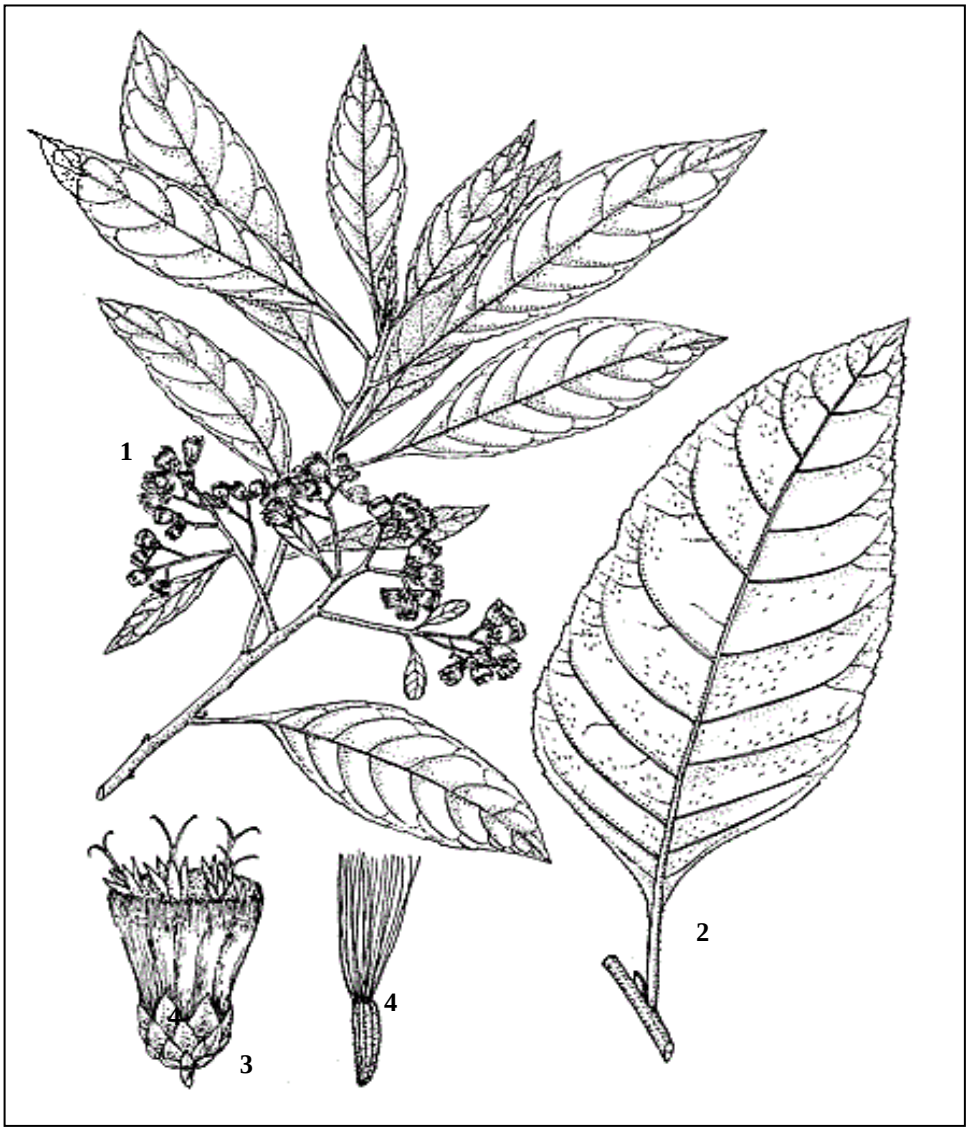

Figure 1- 16: *Vernonia amygdalina* Del.
Figure displays the morphology of *V. amygdalina* where **1**- branch with leaves and flowers; **2**- leaf; **3**- flower; **4**- flower head.

Image adapted and redrawn by Iskak Syamsudin

V. amygdalina is a perennial, evergreen shrub, climbers and small sized trees growing at 2 to 5 m in height. The petiolate leaves are about 6 mm in diameter, elliptic with a characteristic odour and bitter taste. Bark is rough with dense black straits (Sule & Agbabiaka, 2008; Owolabi *et al.*, 2008). Flowers are white or purple coloured, bisexual with a funnel shaped limb and fused anthers (Chuakul *et al.*, 1999).

The Asteraceae family comprises of 25000 species and 1400 genera, well distributed in most ecosystems. Some members of this family produce sesquiterpene lactones that are important bioactive compounds for example *Chrysanthemum parthenium* and *Arnica montana*. Some produce pyrrolizidine alkaloids responsible for hepatotoxic activity in *Senecio* species. Others produce diterpene glycoside stevioside which has intense sweetness (Gurib-Fakim, 2006).

The leaves of *V. amygdalina* are extremely bitter due to the presence of bioactive compounds such as alkaloids, saponins, tannins and glycosides. Leaves are added into the famous 'bitterleaf soup' and the water extract are consumed as a tonic to promote health (Farombi & Owoeye, 2011). It was also consumed by wild chimpanzees to cure parasitic infection (Huffman & Seifu, 1989). In Nigeria and other African countries, the leaf is used to treat fever, emesis, nausea, dysentery, gastrointestinal disorders, diabetes mellitus and sexually transmitted diseases (Aregheore *et al.*, 1998).

Bioactive compounds isolated from the plant were saponins, sesquiterpene lactones (vernodalin and vernoamygdalin), flavonoids, tannins and glycosides (Audu *et al.*, 2012; Igile *et al.*, 1994). Flavonoids were responsible for antioxidant properties in plant and saponins stimulate antitumor activity in leukaemia cells (Jisaka *et al.*, 1993). *V. amygdalina* possess anticancer, antidiabetic, antioxidant, antimalarial and antimicrobial activities.

Anticancer activity was observed in the treatment of breast cancer where low doses of aqueous leaf extracts inactivated the human breast cancer cells (MCF-7) *in vitro* (Gresham *et al.*, 2008). Antidiabetic activity was observed by Ekpenyong *et al.* (1999) when aqueous leaf extracts of *V. amygdalina* lowered blood glucose levels in diabetic induced rabbits. The reduction of blood glucose levels in normal and diabetic rats were significant when compared to the diabetic drug chlorpropamide (Osinubi, 2008).

Aqueous extracts of the plant exhibited antioxidant activity (Adesanoye & Farombi, 2010; Iwalokun *et al.*, 2006) due to the presence of flavonoids in the plant (Igile *et al.*, 1994). Leaf extracts inhibited *Plasmodium berghei* in mice, with antimalarial activity (Audu *et al.*, 2012). The plant also possessed amoebicidal activity in treating amoebic dysentery (Huffman & Seifu, 1989).

Antimicrobial activity of *V. amygdalina* was detected in the leaf sap against *Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* (Ijeh *et al.*, 1996). Methanolic extract of leaves inhibited *Bacillus subtilis, Klebsiella pneumonia, P. aeruginosa, Proteus vulgaris, Shigella dysenteriae and S. aureus* (Akinpelu, 1999). Erasto *et al.* (2006) isolated vernolide and vernodal from *V. amygdalina* which successfully inhibited *Bacillus cereus, S. epidermidis, S. aureus, Micrococcus kristinae, Streptococcus pyrogens,* and *Salmonella pooni*. The stems inhibit oral bacteria and are used as chew sticks to treat dental problems (Ijeh & Ejike, 2011).

2.7 General Extraction of Bioactive Compounds from Plants

Extraction of plants involves the use of selective solvents and extraction techniques to isolate biologically active compounds of plant tissue. The solvents diffuse into the solid plant tissues and solubilize compounds of similar polarity (Green, 2004). Quality and efficiency of plant extracts depends on plant material (wet or dry), type of solvent used and extraction technique (Das *et al.*, 2010).

2.7.1 Collection and drying of plant materials

Firstly, plant materials are carefully selected for collection. Leaves that were infested with insect or fungus are discarded because these contaminants could affect the chemical composition and biological activity. Underground parts of a plant such as the roots, tuber, rhizome, and bulb are preferred to isolate bioactive compounds possessing antimicrobial properties compared to aerial parts of plants.

The source for extraction of secondary metabolites in plant tissues could be either fresh or dried materials. Dried plant materials were favoured based on several factors. Firstly, bioactive compounds are more prominent in extracts from dry material because drying lyses the membranes of plant organelle containing different secondary metabolites making the extraction more efficient. However, liable compounds may be destroyed during the drying process (Eloff & McGaw, 2006).

Secondly, traditional medicine practitioners commonly use dried material or aqueous extracts. Thirdly, fresh plant materials are difficult to work with as there is time delay between plant collection and processing (Angeh, 2006). Fourthly, during

separation by liquid-liquid extraction, the solubility will be affected by the differences in water content making the secondary metabolites unstable (Ncube *et al.*, 2008).

Before extraction, plants are air dried (Baris *et al.*, 2006; Dilika *et al.*, 1997) to a constant weight or dried in an oven at 40°C for 72 hours (Salie *et al.*, 1996). Plant materials are dried at a low temperature to prevent the loss of volatile bioactive compounds (Wang *et al.*, 2002). Different drying techniques may produce different results. An example is the efficiency of antimicrobial activity in *Combretum erythrophyllum* leaf extracts. The lowest antibacterial activity was observed in lyophilized materials because volatile antibacterial compounds were lost. The highest activity was observed when plant materials were dried slowly at a low temperature (Martini *et al.*, 2004).

2.7.2 Choice of Solvent

The type of solvent used in extraction of medicinal plants determines the different bioactive compounds isolated. Solvents are chosen based on the yield of extracts, rate of extraction, toxicity of the solvents in bioassay, ease of evaporation at low heat, physiological absorption of extracts, ability to preserve plant materials and inability to cause the extract to complex or dissociate (Hughes, 2002). Traces of residual solvent may be present in the final product of extract even after evaporation, thus the solvent chosen should be non-toxic, non-hazardous to health and should not affect bioassays used to isolate bioactive compounds (Ncube *et al.*, 2008).

Another important factor in the choice of solvent is the type of bioactive compound that needs to be isolated from the plant such as phenolic compounds or antimicrobial compounds. Crude or alcohol extracts are used for the initial screening of plants for antimicrobial activity which is then followed by organic solvent extraction.

Aqueous extracts are most commonly used because traditional medicine practitioners use water to make their concoctions and water is a universal solvent. However, it was discovered that plant extracts from organic solutions effectively isolated bioactive compounds and showed higher antimicrobial activity (Parekh *et al.*, 2005). For example water soluble phenolic compounds such as flavonoids (mainly anthocyanins), failed to exhibit antimicrobial activity and only exhibited antioxidant activity (Nang *et al.*, 2007; Yamaji *et al.*, 2005).

Most plant antimicrobial are aromatic/ saturated organic compounds, therefore the most effective solvents used for preliminary screening of plant antimicrobial activity are methanol, ethanol and water (Parekh *et al.*, 2006; Rojas *et al.*, 2006; Lourens *et al.*, 2004; Bisignano *et al.*, 1996; Salie *et al.*, 1996).

2.7.3 Extraction technique

Extraction technique differs by the extraction time, type of solvent, pH of solvent, temperature, size of the plant tissue particles and the solvent-to-sample ratio. The most important step in extraction is the wet or dry plant material must be homogenized to form finer tissue particles to increase the surface area for extraction thereby increasing the rate of extraction. This will also shorten the extraction time. Eloff (1998), proved that fine plant particles (10 µm diameter) produced higher extraction yield over a period of 5 minutes compared to coarsely ground materials that were placed in a shaker for 24 hours. Thus the rate of extraction is increased by grinding plant materials to finer particles, increasing the time of contact between material and solvent and shaking the plant material in the solvent (Ncube *et al.*, 2008).

The most commonly used extraction technique is maceration (Basri & Fan, 2005; Parekh *et al.*, 2005; Meyer & Dilika, 1996) whereby wet or dry plant parts are

homogenized to fine particles, placed in contact with a certain quantity of solvent in a stoppered bottle for a defined period of time (usually 24 hours) with vigorous shaking (Handa, 2008). The ideal solvent to sample ratio is 10:1 (v/w) (Green, 2004). The extract is filtered and the filtrate is dried down under reduced pressure. Then it is re-dissolved in solvent to a defined concentration. The extract is centrifuged for 30 minutes for clarification (Cichewicz & Thorpe, 1996; Taylor *et al.*, 1996).

Apart from that, soxhlet extraction technique is used whereby dried plant tissues are extracted by continually exposing the materials with organic solvent (Kianbakht & Jahaniani, 2003). This method is suitable for compounds that can withstand high temperatures, and is not appropriate for thermolabile compounds as continues heating may lead to degradation of bioactive components (de Paiva *et al.*, 2004).

Serial exhaustive extraction involves continual extraction with solvents of increasing polarity from non-polar (hexane) to polar solvents (methanol) to ensure that compounds of all polarity are extracted (Green, 2004). Other methods are used to isolate particular compounds such as sequential grinding to isolate alkaloids, steroids and triterpenoids; gradial centrifugation to isolate lectins and polypeptides; and acid hydrolysis to isolate phenols (Eloff, 1999).

Specific solvent is used for the screening of specific secondary metabolites such as methanol or ethanol are used to isolate alkaloid; acetone for flavonoids and steroids; hexane, diethyl ether and chloroform for fat soluble oils, wax, lipids and esters; dichloromethane for terpenoids; ethyl acetate for esters; ethanol for sterols, polyphenols, and tannins; and water for the water soluble components such as glycosides, polysaccharides, polypeptides and lectins (Harborne, 1998).

2.8 Test Microorganism Used in the Study

Over the years, bacteria have become increasingly resistant towards antibiotics. Genetically, bacteria are able to transmit and develop resistance towards antimicrobial drugs (Cohen, 1992). Resistance towards antimicrobial drugs are developed through (i) reduction in bacteria efficiency to bind with the drug by modification of targeted active site; (ii) bacteria destructs or modifies the drug by producing enzymes or; (iii) the drug is efflux from the cell (Sheldon, 2005).

Five test microorganisms were selected in this study: Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus mutans*). Both Gram-positive and Gram-negative bacteria were chosen because the latter is more resistant to antimicrobial agents due to the presence of an outer membrane layer (Alberts *et al.*, 2002).

Most plant pathogens belong to the Gram-negative strains which are mostly resistant to the bioactive compounds isolated from plants. Whereas Gram-positive bacteria are usually susceptible to plant bioactive compounds suggesting that the difference in cell wall and outer membrane layer morphology of the two bacteria strains determine the susceptibility towards plant secondary metabolites (González-Lamothe *et al.*, 2009).

2.8.1 *Bacillus cereus* (*B. cereus*)

B. cereus belongs to the genus Bacillus, is a Gram-positive, motile, spore-forming rod. It is about 1 - 1.2 µm by 3 – 5 µm with square ends. This bacterium has the ability to grow both aerobically at 37 °C and anaerobically at 45 °C and exists as spore and vegetative cells. Spores are the infective agent for the bacterium and are 1 - 1.5 µm, ellipsoidal shaped, formed in aerobic conditions and are able to withstand extreme environmental conditions, such as heat, freezing, drying and radiation (Jensen *et al.*, 2003; Davenport & Smith, 1952). Spores germinate when contacted with organic matter or within a host cell (Arnesen *et al.*, 2008). The vegetative cells contain an outermost crystalline surface protein (S layer) which is formed during the cell colonization stage of the bacteria lifecycle (Kotiranta *et al.*, 2000; Kotiranta *et al.*, 1998). They can grow at optimal temperatures ranging from 25 °C – 37 °C (Drobniewski, 1993).

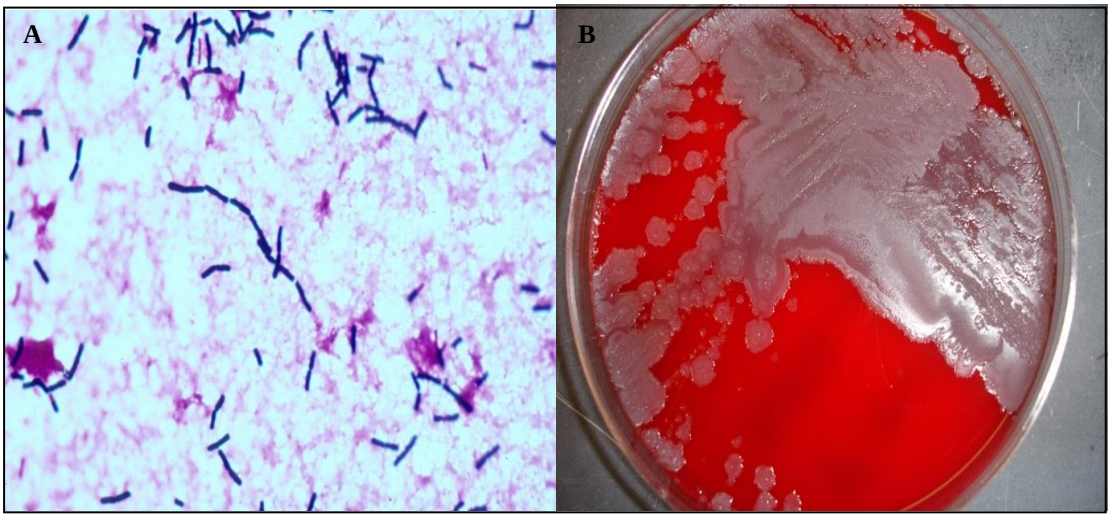

Figure 1- 17: Morphology of *B. cereus*
Figure displays the Gram staining of blood culture showing Gram-positive bacilli with rounded ends, found singly, in pairs and short chains (**A**) and grey, opaque, granular spreading colonies with irregular perimeters growing on 5% sheep blood agar at 37°C under aerobic conditions. The smaller smooth colonies admixed among spreading growth (**B**).

Image retrieved from Bottone (2010)

B. cereus is known as a soil bacterium and is found in decayed organic matter, fresh and marine waters, vegetable and the intestinal tracts of invertebrates. It can germinate, grow and sporulate in the soil contaminating soil and food products causing infection to the human intestine (Vilain *et al.*, 2006; Jensen *et al.*, 2003; Ghosh, 1978).

B. cereus is commonly associated with two types of food poisoning diseases, the diarrhoeal and emetic type (Gaur *et al.*, 2001). The diarrhoeal type is caused by enterotoxins produced by vegetative cells in the small intestine which are ingested. Protein rich food such as meat, vegetables and milk products are the source. After ingestion, the incubation time is between 8 to 16 hours or longer, while symptoms such as abdominal cramps, diarrhoea, nausea and vomiting usually last for 12 to 24 hours or several days (Kotiranta *et al.*, 2000).

The emetic type is caused by a plasmid encoded cyclic peptide (cerulide) produced by growing cells in the food before ingestion (Bottone, 2010). Food sources are starch rich foods such as fried and cooked rice, pasta and noodles. This type was first identified by (Mortimer & McCann) in 1974 following several outbreaks of the disease in the United Kingdom. Symptoms such as nausea and vomiting occur 30 minutes to 6 hours after ingestion and may last up to 24 hours (Ehling-Schulz *et al.*, 2004).

B. cereus can be transmitted through food sources that are heat treated because the spores can withstand high temperatures. Temperature of about 48 °C, achieved after cooking and cooling of food facilitates the germination of spores and with the absence of other competing flora results in the healthy growth of *B. cereus*. It is also easily spread through food sources of plant origin because it is a soil saprophyte (Drobniewski, 1993).

Non-gastrointestinal diseases related to *B. cereus* are burns; traumatic or postsurgical wounds; ocular infections; bacteraemia and septicaemia; infections of the central nervous system such as meningitis and brain abscesses; and respiratory infections such as pneumonia, endocarditis and pericarditis (Bottone, 2010; Drobniewski, 1993).

In the antibiotic resistance test, it was discovered that most *B. cereus* strains were susceptible towards aminoglycosides, clindamycin, vancomycin, chloramphenicol, erythromycin and tetracycline (Drobniewski, 1993). Resistance was also discovered towards clindamycin, cefazolin, cefotaxime, cephalosporin and penicillin due to the production of β-lactamase (Bottone, 2010; Drobniewski, 1993).

2.8.2 *Escherichia coli (E. coli)*

E. coli is a Gram-negative bacilli that are approximately 1.0 – 3.0 μm long with a diameter of 0.5 μm. They are non-sporus and a single layer of peptidoglycan is found in the periplasm. They have peritrichous flagella, making them motile in liquid and the most abundant facultative anaerobes in nature (Harshey, 1994).

The first *E. coli* was isolated by Escherich (1885) from the feces of a child in Austria and was named as the "Bacterium coli commune". *E. coli* colonizes the gastrointestinal tract of infants within hours after birth and coexists in mutualism with humans for their entire life as a harmless commensal of the intestinal tract. However, some strains developed virulence forming pathotypes, adapting to new niches as fecal contaminant of soil and water causing intestinal and extra-intestinal diseases (Kaper *et al.*, 2004).

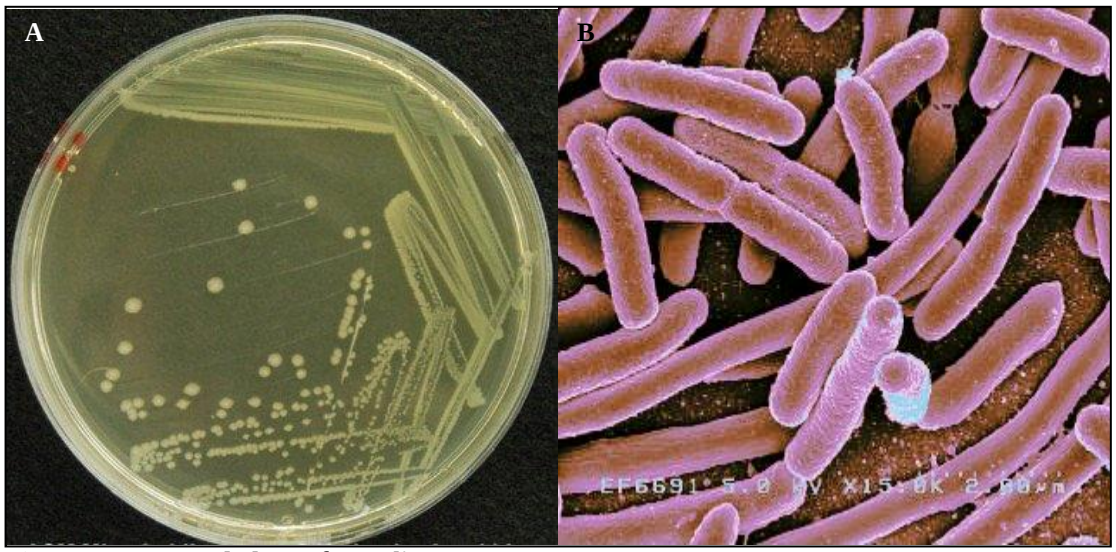

Figure 1- 18: Morphology of *E. coli*
Figure displays the Scanning electron microscopy of *E. coli*, grown in culture and adhered to a cover slip (**A**) and *E. coli* streaked onto Luria agar and incubated at 37°C for 24 hours (**B**).

Image retrieved from: http://en.wikipedia.org/wiki/Image:EscherichiaColi_NIAID.jpg and http://www.microbelibrary.org/images/atlas_lb/escherichia%20coli%20topview.jpg

Infection of these pathotypes caused three clinical diseases such as enteric or diarrhoeal disease, urinary tract infection (UTI) and sepsis or meningitis. There are six types of intestinal pathogens such as enterotoxigenic *E. coli* (ETEC); enteropathogenic *E. coli* (EPEC); enterohaemorrhagic *E. coli* (EHEC); enteroaggregative *E. coli* (EAEC); enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Extraintestinal types include uropathogenic *E. coli* (UPEC) and meningitis associated *E. coli* (MNEC) (Kaper *et al.*, 2004).

Enterotoxigenic *E. coli* (ETEC) causes diarrhoea, and usually infects children under the age of 5. Symptoms of the infection are mild watery diarrhoea or in some cases severe, cholera-like illness which can rapidly dehydrate the body and may lead to death (Welch, 2006). Enteropathogenic *E. coli* (EPEC) is a diarrhoeagenic *E. coli* strain that lack Shiga-toxins (verocytoxin). EPEC causes watery diarrhoea with mucus, vomiting, fever and dehydration which lasts for several days (Welch, 2006).Enterohaemorrhagic *E. coli* (EHEC) are Shiga-toxic producing bacteria such as *E. coli* 0157. The disease is transmitted to humans who consume beef contaminated with cattle feces. However in cattle's this strain is a member for the intestinal microflora

thus are not harmful to the animals. Pathogenic strains that do not possess enterotoxins or Shiga-toxins and self-aggregate are known as Enteroaggregative *E. coli* (EAEC) (Nataro *et al.*, 1987). Symptoms include chronic watery diarrhoea and abdominal cramps (Nataro *et al.*, 1995).

Diffusely adherent *E. coli* (DAEC) causes diarrhoea in infants and is different from the other pathogenic strains because it possesses a special adhesion phenotype. Enteroinvasive *E. coli* (EIEC) are phylogenetically related to *Shigella* species and infect the large intestine. Patients infected experience inflammatory colitis, dysentery, bloody diarrhoea, severe cramps and fever similar to infection caused by *Shigella* (Goldberg & Theriot, 1995).

An extraintestinal pathotype of *E. coli* is the uropathogenic *E. coli* (UPEC), also known as the urinary tract and bloodstream *E. coli*. About 60% of women acquire urinary tract infection (UTI) in their lifetime (Kunin, 1994), where a large percentage is associated with infection by UPEC (Haley *et al.*, 1985). Another is the meningitis associated *E. coli* (MNEC) which is the most common cause of neonatal meningitis. New-borns are infected with the strain during birth when passing through the birth canal which will cross the endothelial surface into the brain through the bloodstream. It is a severe disease where the infected patient faces death and survivors possibly face long term neurological problems (Stoll *et al.*, 2002; Unhanand *et al.*, 1993).

E.coli is used as a test microorganism for determining antimicrobial resistance towards antimicrobial agents because it is easily cultured in clinical laboratories, biochemically, physiologically and genetically well characterized (Welch, 2006), found frequently in a wide range of hosts, and acquires resistance easily (Erb *et al.*, 2007).

2.8.3 *Pseudomonas aeruginosa (P. aeruginosa)*

P. aeruginosa is a motile, rod-shaped, Gram-negative bacterium from the Pseudomonadaceae family (Balasubramanian *et al.*, 2013). It comprises of an outermost slime layer, an outer membrane, peptidoglycan layer and inner cytoplastic membrane. The bacteria contains pathogenic components such as (i) the cell envelope that controls adhesion, formation of microcolony, and the transport of antibacterial agents across the cell (Peterson, 1980), (ii) pili which enables the bacterium to adhere to surfaces and for conjugation (Weppelman & Brinton, 1971) and (iii) exotoxins (Liu, 1974). Gram-staining of clinical specimen produces slender Gram-negative bacilli arranged singly, in pairs or in short chains. The bacterial colony is identified by the presence of a blue-green pigment with a grape-like odour (Stratton, 1990).

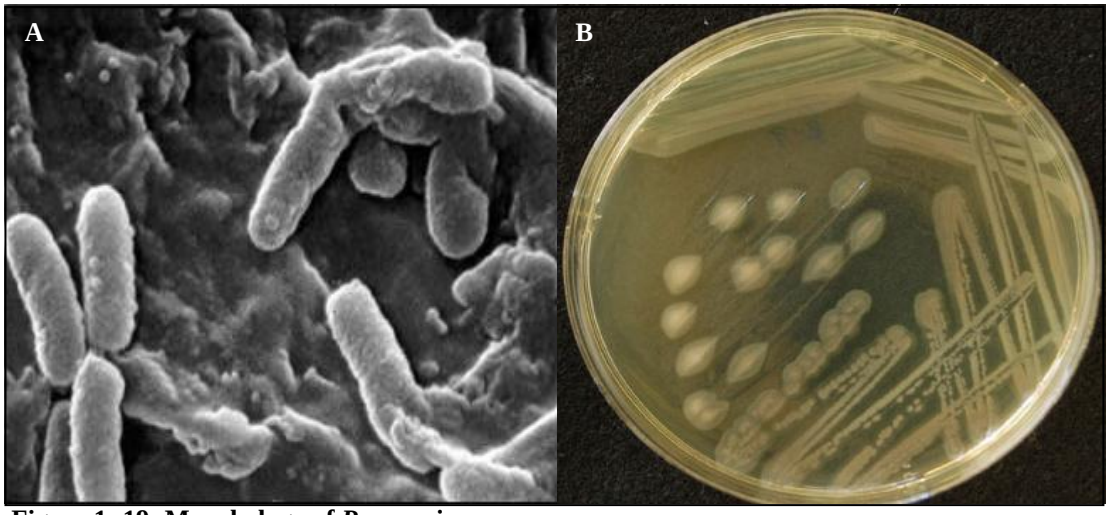

Figure 1- 19: Morphology of *P. aeruginosa*
Figure displays the Scanning electron microscopy of *P. aeruginosa* (**A**) and *P. aeruginosa* streaked onto Luria agar and incubated at 37°C for 48 hours (**B**).

Image retrieve from: CDC/ Janice Haney Carr and http://www.microbelibrary.org/images/atlas_lb/pseudomonas%20aeruginosa%20topview(48).jpg

P. aeruginosa exists widely in nature, thriving in aqueous environments and moist soil (Stanley, 1947). It is a nosocomial pathogen infecting immune compromised patients (Balasubramanian *et al.*, 2013). The microorganism infects patients with burn

wounds, cystic fibrosis (CF), acute immunodeficiency syndrome (AIDS), organ transplant, acute leukaemia and cancer (Morita *et al.*, 2014; Bodey *et al.*, 1983). It causes catheter-related bloodstream infection in patients with a device inserted in the body (Maki *et al.*, 1973) and 'ventilator-associated pneumonia' in patients receiving mechanical ventilation via an endotracheal tube (Kollef, 2000).

P. aeruginosa also causes urinary tract infection in structurally abnormal urinary tracts and corneal ulcer through the use of contaminated contact lenses (Wilson *et al.*, 1981). Intravenous drug abusers are at risk of *P. aeruginosa* endocarditis infection (Jackson, 1994). It infects areas in the skin that are constantly exposed to moisture such as the nails leading to the "green nail syndrome" where the nails develops greenish discolouration (Goldman & Fox, 1944).

A common factor amongst patients infected by *P. aeruginosa* is their host defence mechanism is disrupted which in some cases may lead to death. However, recovery from infection depends to the severity of the patients underlying disease (Bodey *et al.*, 1983). *P. aeruginosa* is a prominent pathogen because it possesses innate and acquired resistance towards antimicrobials and disinfectants such as 0.25% acetic acid, phenolic disinfectant, chlorhexidine, isopropyl alcohol, tetracycline and chloramphenicol (Morita *et al.*, 2014; Stratton, 1990). The resistant characteristic is due to the outer membrane barrier, multi drug efflux transporters, endogenous antimicrobial inactivation and their ability to form biofilm (Poole, 2011; Lewis, 2007; Mah *et al.*, 2003).

2.8.4 *Staphylococcus aureus (S. aureus)*

S. aureus belongs to the Staphylococcaceae family. It's a Gram-positive strain, with spherical shape of 0.5 – 1.0 µm in diameter, immobile and growing in grape-like clusters. Colonies formed are yellow, growing on rich nutrient medium (Foster, 1996). *S. aureus* are facultative anaerobes and common human commensal, however some strains are pathogenic. They exist asymptomatically in the nasal cavity of about 30% of the human population (Kluytmans *et al.*, 1997) and are transmitted through skin-to-skin contact and contaminated objects (Lowy, 1998).

S. aureus develops the ability to infect humans when the skin barrier is disrupted, presence of immunodeficiency syndrome and presence of underlying diseases such as diabetes (Grundmann *et al.*, 2006). The medical problems caused by *S. aureus* includes (i) superficial lesion such as boils, abscesses and wound infections; (ii) deep-seated, nosocomial infections such as osteomyletis, endocarditis, pneumonia, surgical wound infection and bacteremia; (iii) toxemic syndromes such as toxic shock, scarlet fever and food poisoning; and (iv) infections associated with indwelling medical devices such as joint prostheses, cardiovascular devices and artificial heart valves (Jarraud *et al.*, 2002; Foster, 1996).

An infection by *S. aureus* starts by the colonization of the bacteria in the anterior nares which is carried by the host for weeks or months which is then followed by infection which spreads locally or to the blood. Once in the blood, it moves to distant organs causing sepsis which could lead to death if left untreated (Archer, 1998). Virulence factors include surface proteins that colonize tissues; capsule and immunoglobulin binding protein A that inhibits phagocytosis; and toxins that damages host tissues and promotes disease symptoms (Foster, 1996).

S. aureus is an alarming pathogen in the community because it acquires resistance towards most antibiotics (Chambers & DeLeo, 2009). In 1940s penicillin was able to treat *S. aureus* infections. However, the bacteria developed resistance towards penicillin in 1942 (Rammelkamp & Maxon, 1942). In 1961, methicillin was discovered to inhibit *S. aureus*, which later developed resistance forming methicillin resistant *S. aureus* (MRSA). MRSA acquired resistance to all β-lactam antibiotics (Knight *et al.*, 2012). *S. aureus* developed resistance to almost all antimicrobial agent due to its commensal nature. Hence antibody based vaccines fail to immunize patients (Verkaik *et al.*, 2010) and this bacterium evolved to escape the host immune system (Daum & Spellberg, 2012).

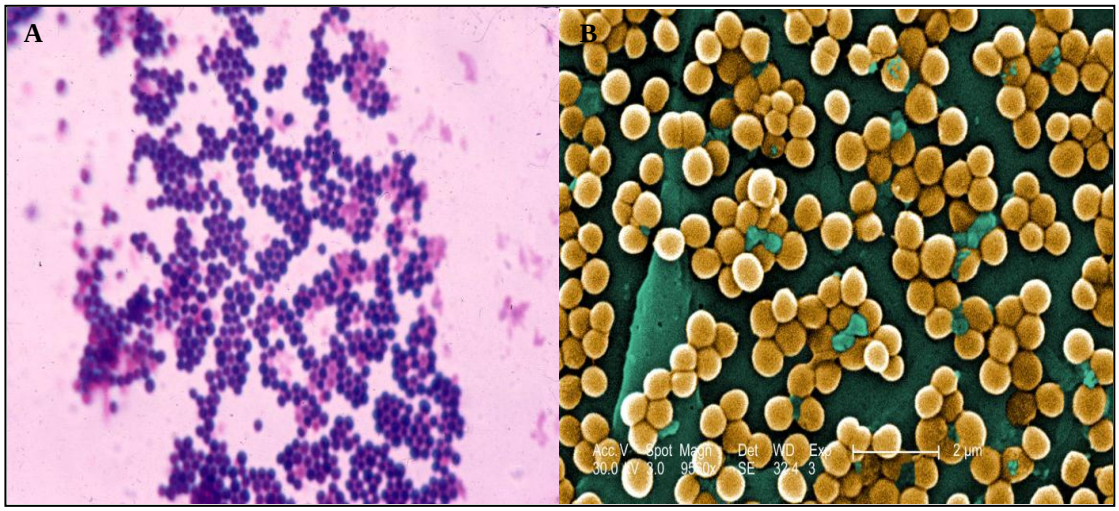

Figure 1- 20: Morphology of *S. aureus*
Figure displays the Scanning electron microscopy of *S. aureus* (**A**) and Gram staining of *S. aureus* (**B**).

Image retrieved from http://microbewiki.kenyon.edu

2.8.5 *Streptococcus mutans (S.mutans)*

The oral *S. mutans* is a non-motile, negative-catalase, Gram-positive streptococci that infects the oral cavity (Hamada & Slade, 1980). The first bacterial species was isolated from carious lesions in 1924 by Clarke. The name *Streptococcus mutans* was designated when Gram staining of the bacterium showed oval structures which appeared to resemble a mutant form of a coccus (Clarke, 1924). Clarke associated this organism with dental decay. However researchers later failed to isolate this bacterium. It was rediscovered later in the 1960s in rodents (Carlsson, 1968; Guggenheim, 1968; Larson & Fitzgerald, 1968).

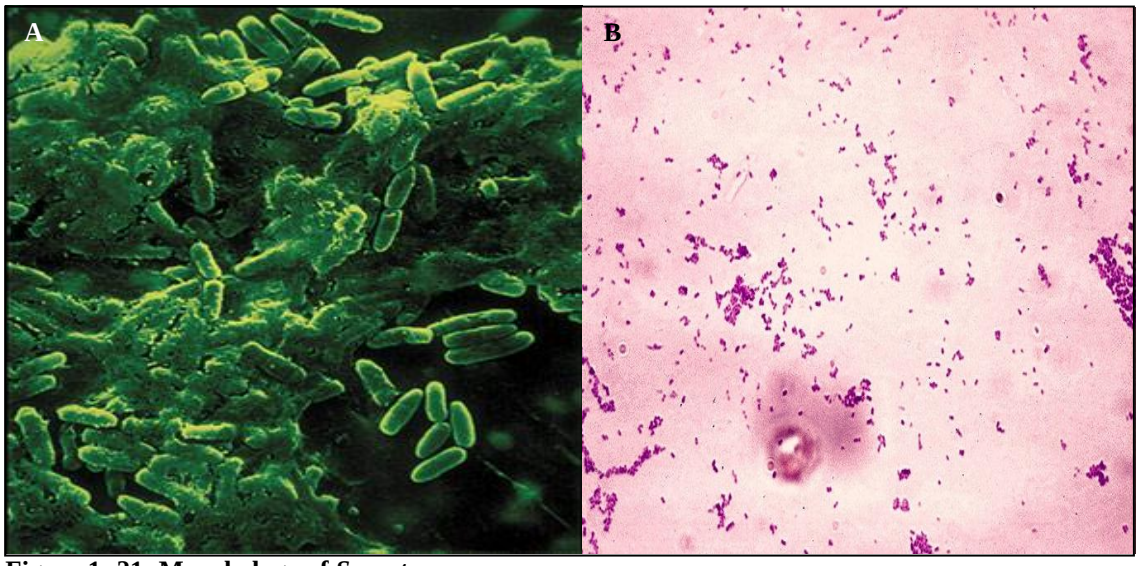

Figure 1- 21: Morphology of *S. mutans*
Figure displays the Scanning electron microscopy of *S. mutans* biofilm present on toothbrush bristles (**A**) and Gram staining of *S. mutans* (**B**).

Image retrieved from http://www.msi-lab.com/tl_files/msi/images/bacteries.jpg and http://www.publicdomainfiles.com/show_file.php?id=13528513816662

Bacterial isolates that exhibited similar characteristic to *S. mutans* species were collectively known as mutans streptococci (MS) (Coykendall, 1989). MS that ferment mannitol, sorbitol and various other sugars and synthesize extracellular water soluble glucans from sucrose were identified as *S. mutans* (Hamada & Slade, 1980; Stiles *et al.*, 1976; Ferretti & Ward, 1976). *S. mutans* was found to be widely distributed in animals as it was isolated from Patas monkeys, rhesus monkeys, wild rats, and indian fruit bats (Dent *et al.*, 1978; Coykendall *et al.*, 1976; Lehner *et al.*, 1975). This microorganism was known to be the most cariogenic oral Streptococci (Ophori *et al.*, 2013).

The cell wall of *S. mutans* is made up of four antigenic polymers such as peptidoglycan; group and type-specific polysaccharides; protein; and teichoic and lipoteichoic acids in the glycerol form. The virulence factors include the ability of *S. mutans* to adhere to smooth surfaces and the acidogenic properties exhibited by the bacteria. These factors are responsible for the bacterium's cariogenicity in both humans and animals. Plaque formation by *S. mutans* is caused by the adherence of the bacterium to smooth tooth surfaces along with the formation of glucose from the intake of sucrose in the diet (Hamada & Slade, 1980; Ikeda *et al.*, 1980). Plaque formation due to dietary intake was supported by scientific findings whereby high dental decay was observed shortly after sucrose was introduced into the diet (Loesche, 1986).

3.0. MATERIALS AND METHODS

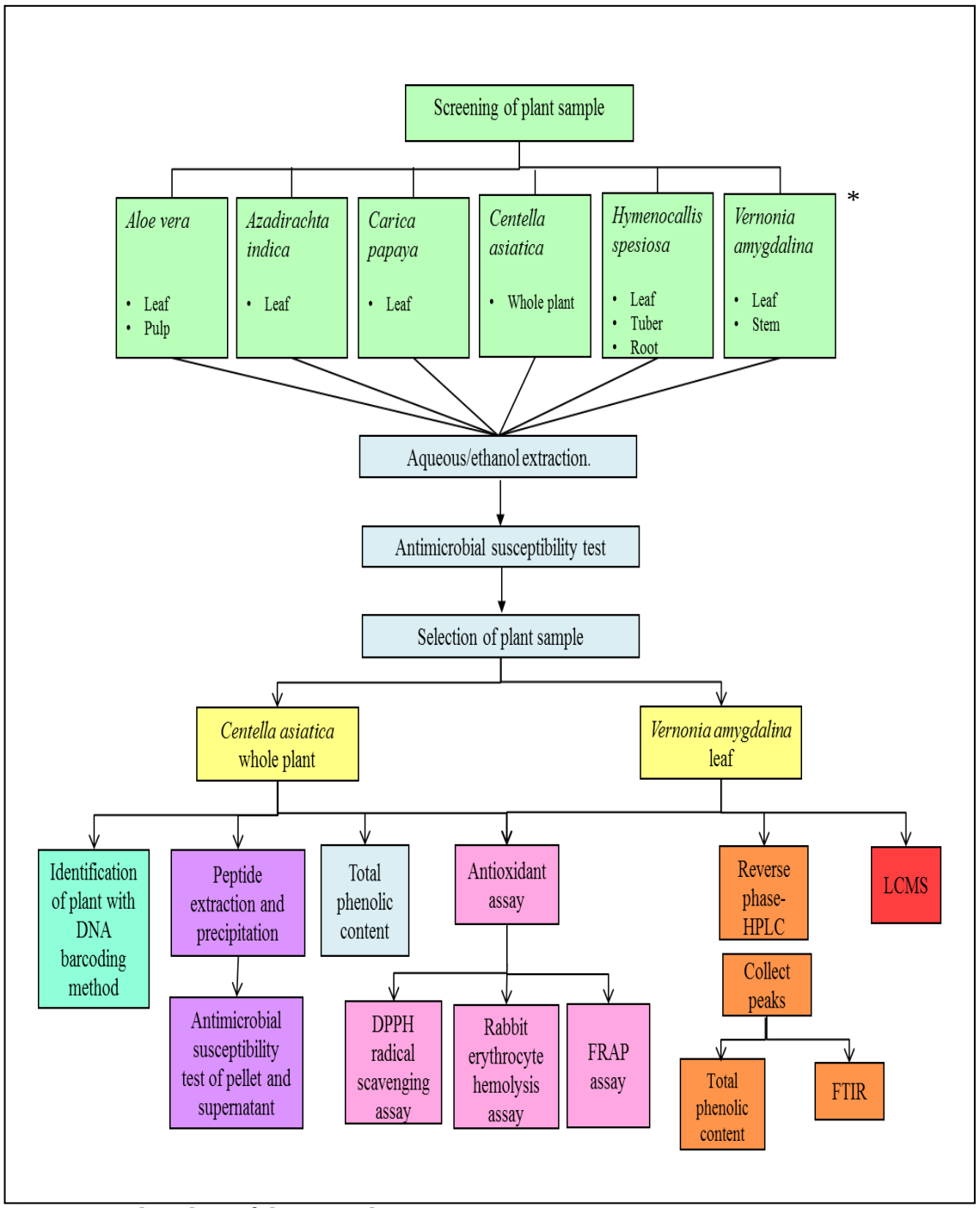

Figure 3- 1: Flow chart of the research

Figure displays the flow chart describing the research outline and steps taken

* the choice of plant parts used in the study are discussed in pg 138 and 139

3.1. Selection of Plant Samples

Six medicinal plants commonly found in Malaysia were selected for this study (**Appendix A**). The plant samples were collected from the housing area of Seksyen 4 Petaling Jaya, Selangor, Malaysia in May 2012. Plants were chosen based on folklore medicinal values and were identified by Dr. Sugumaran Manickam of Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. The voucher specimens were prepared and deposited at the herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia (Table 3-1, **Appendix B**).

Table 3- 1: Plant species screened for antimicrobial activity

Scientific name	Common name	Family	Voucher number	Part of plant used
Aloe vera	Aloe	Asphodeloideae	47777	leaf pulp
Azadirachta indica	Neem	Meliaceae	47778	leaf
Carica papaya	Papaya	Caricaceae	47779	leaf
Centella asiatica	Pegaga	Umbeliferae	47781	whole plant
Hymenocallis spesiosa	Lily	Amaryllidaceae	47783	leaf root tuber
Vernonia amygdalina/ *Gymnanthemum amygdalinum*	Bitterleaf	Asteraceae	47784	leaf stem

Table displays the details of the selected medicinal plants and parts of plant used with voucher deposited at the Herbarium of University of Malaya.

3.2. Preparation of Plant Materials

3.2.1. Preparing plant leaf, root and tuber

The plant parts were cleaned under tap water and air-dried to a constant weight (Das *et al.*, 2010) at room temperature away from the exposure to sunlight to prevent the loss of active components in the plant (Thakare, 2004). Dried plant parts were pulverized to produce fine homogenous powder using an electrical blender (National, Panasonic) and sifted to obtain fine powder and then stored in sterile 50 ml centrifuge tubes at 4 °C until required (Rafat *et al.*, 2010).

3.2.2. Preparation of *Aloe vera* (*A. vera*) pulp

The outermost whorls of leaves of mature *A.vera* were selected from the plants, and washed with distilled water. The yellow liquid was drained off before removing the rind from the leaves. The leaves were sliced across the width with a sharp knife. The inner exposed surfaces revealed a transparent gooey pulp without the addition of sap. The pulp was scooped with a spatula and homogenized using a blender (National Panasonic). At this stage, the homogenized pulp was lyophilized (Labconco®, UltraScientific Sdn. Bhd.) before being processed and stored at 4 °C until required (Ni & Tizard, 2004).

3.3 Extraction Procedure of Plant Samples

3.3.1 Aqueous extraction

Plant sample powder was weighed and placed in a 100 ml Schott bottle. Double distilled water was measured and poured into the Schott bottle following a solvent to dry weight ratio of 10:1 (v/w) (Das *et al.*, 2010). The plant samples were placed on an orbital shaker (Edmund Bühler, Germany) for 24 hours at room temperature. The extract was filtered through Whatman no.1 filter paper. The filtrate was lyophilized and then re-dissolved in distilled water to make up a stock solution of 100 mg/ml concentration and stored at 4˚C. Working solution was prepared at a desired concentration by diluting the stock solution with double distilled water (Busani *et al.*, 2012).

3.3.2 Ethanolic and methanolic extraction.

Fine plant powder was weighed and placed in a 100 ml Schott bottle. 95% analytical grade ethanol (Friendemann Schmidt Chemicals, USA) or HPLC grade methanol (Tedia Company Inc, USA) was measured and poured into the Schott bottles following a solvent to dry weight ratio of 10:1 (v/w) (Das *et al.*, 2010). The plant sample was placed on a shaker for 72 hours at room temperature in the dark. The solution was then filtered through Whatman no. 1 filter paper and evaporated to dryness using a rotary evaporator (Buchi Rotarvapor, Switzerland) at a temperature between 36 ˚C to 40 ºC.

A stock solution of 100 mg/ml of the plant extracts was prepared in 5% Polyoxyethylene sorbitan mono-oleate (Tween 80, Hopkins & Williams, London) dissolved in isotonic phosphate buffer (IPB) pH 7.4 (bioWorld, USA) and stored at 4 ºC

until required for experiments. The working solution was made by diluting the stock solution with IPB (Rafat *et al.*, 2010).

3.3.3 Determination of percentage of yield for plant extracts

The percentages of yield of the extracts were based on dry weight (d.w.) sample and were calculated as follows:

$$\text{Yield of extracts (\%)} = \frac{\text{Weight of extracts (g)}}{\text{Weight of dry plant powder/ Weight of freeze dried } \textit{A. vera} \text{ gel (g)}} \times 100\%$$

The percentage of yield for *A. vera* gel was calculated based on the weight of the freeze dried gel before extraction. The yield of extract for *A. indica* (leaf); *A. vera* (leaf and gel); *C. papaya* (leaf); *H. spesiosa* (leaf, root, and tuber) and *V. amygdalina* (stem) was determined with no replicates. The yield of extracts for the selected plants *C. asiatica* (whole plant) and *V. amygdalina* (leaf) was determined in trplicate.

3.4. Authentication of Selected Medicinal Plant with DNA Barcoding Method

Medical plants used in the study, *C. asiatica* and *V. amygdalina* were identified using ITS2 region as a DNA barcode based on the findings of Chen and colleagues (2010).

3.4.1 DNA extraction

Genomic DNA of plant samples *C. asiatica* and *V. amygdalina* was extracted using a commercially available DNeasy® Plant Mini Kit (Qiagen®, USA, **Appendix L1**) as follows:

The first step was tissue dissociation, where 100 mg of fresh plant leaves were cut and weighed. Next, the plant sample was ground with pestle and mortar under liquid nitrogen to obtain a fine powder and then transferred into a 1.5 ml microcentrifuge tube (Eppendorf® GmbH, Germany).

The second step was DNA lysis. AP1 buffer, 400 μl and RNase A, 4 μl were added into the sample tube and mixed by vortexing with a vortex machine (Grant-bio, England). The sample was incubated in a dry bath (Thermoline, Australia) at 65 ºC for 10 minutes and during incubation, the tubes were inverted 2-3 times. Next, Buffer P3, 130 μl was added into the sample and mixed. The tubes were incubated on ice for 5 minutes. The lysate was centrifuged for 5 minutes at 20,000 x g. Then, the lysate was pipetted into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged with a refrigerated centrifuge (ThermoScientific Inc, USA) at 20,000 x g for 2 minutes. The flow-through was transferred into a new tube without disturbing the pellet (if present).

The third step was DNA binding where 1.5 volumes of Buffer AW1 was added to the tubes and mixed by pipetting. The mixture, 650 μl was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube. The tube was centrifuged at ≥6000 x g for 1 min. The flow-through was discarded and this step was repeated for the remaining samples.

The fourth step was washing. The spin column was placed back into a new 2 ml collection tube. Buffer AW2, 500 μl was added and centrifuged at ≥ 6000 x g for 1 min. The flow-through was discarded and another 500 μl Buffer AW2 was added. The tube was centrifuged at 20,000 x g for 2 minutes.

The fifth and final step was DNA elution. The spin column was transferred into a new 1.5 ml microcentrifuge tube. The spin column was carefully removed from the collection tube so that the column did not come into contact with the flow-through. Finally 100 μl of Buffer AE was added for elution. The tube was incubated at room temperature for 5 minutes and then centrifuged at at ≥ 6000 x g for 1 min. The genomic DNA extracted was kept at -20 °C until further analysis.

3.4.2 Polymerase Chain Reaction (PCR) amplification

The concentration of the genomic DNA obtained was checked with nanodrop (Thermo Scientific™, USA). The primers used in the research for ITS2 region were forward primer (5'–ATG CGA TAC TTG GTG TGA AT–3') and reverse primer (5'-GAC GCT TCT CCA GAC TAC AAT-3') (Chen *et al.*, 2010) supplied by (First BASE Laboratories, Malaysia). Both the forward and reverse primers, 100 μM were diluted to 1.0 μM with nuclease-free water (Promega, USA). PCR amplification was conducted in a 25 μl reaction mixtures containing the following:

Table 3- 2: PCR Master Mix

Component	Volume (μl)	Final concentration
PCR Master Mix (Promega©, USA), 2X	12.5	1X
Forward primer, 100 μM	2.5	1.0 μM
Reverse primer, 100 μM	2.5	1.0 μM
DNA template, 30 ng/ml	5.0	16.39 ng/ml
Nuclease-free water	2.5	-
TOTAL	25.0	-

Table displays the components added into the 0.5 ml microcentrifuge tube for PCR.

Modified from Chen et al. (2010)

Next, the PCR Mastercycler Personal (Eppendorf® GmbH, Germany) was set with the program for 40 cycles as follows:

Table 3- 3: PCR Thermocycler program

Temperature (ºC)	Time
94	5 min
94	30 sec
56	30 min
72	45 min
72	10 min
4	hold

Table displays the thermocycler program followed for 40 cycles for ITS2 region of plant genomic DNA

Adapted from Chen et al. (2010)

3.4.3 Agarose gel electrophoresis

Firstly, the electrophoresis buffer, 5 X Tris-Borate-EDTA (TBE) buffer was prepared where 13.5 g of tris (First BASE Laboratories, Malaysia), 6.875 g of boric acid (Promega, USA) and 5 ml of EDTA were measured and added into a beaker and distilled water was added up to 250 ml. The mixture was stirred with a stirrer. Then the 5 X TBE buffer was diluted to 0.5 X where 100 ml of 5 X TBE buffer was diluted with 900 ml of distilled water.

Secondly 1% (w/v) agarose gel was prepared for separating the particular size fragments expected in the DNA sample by dissolving 0.2 g of agarose (First BASE Laboratories, Malaysia) in 20 ml of 0.5 X TBE buffer. The slurry was heated in a microwave oven until the agarose dissolved. The molten gel was left to cool on the bench for about 5 minutes to 60 ºC. Next, 1 μl of 10 mg/ml ethidium bromide (GibcoBrl Life Technologies, Japan) was added to a final concentration of 0.5 μg/ml. The gel solution was mixed thoroughly by gentle swirling.

Thirdly, the gel was slowly poured into the casting tray preventing the formation of bubbles. A comb was positioned about 0.5 - 1.0 mm above the casting tray so that a complete well was formed when the molten agarose was added to the mold. A small toothed comb allowed 15 μl of sample per well. The gel was allowed to set completely for 30 minutes at room temperature. Once set, the comb was carefully removed and the gel was mounted into the electrophoresis tank (Bio-Rad, USA). 0.5 X TBE running buffer was poured into the tank to submerge the gel so that it is completely covered in buffer.

Next, 1 μl of 6 X DNA loading dye (Promega, USA) was pipetted onto the parafilm. Pipetting one sample at a time, firstly 2 μl of DNA sample from the PCR reaction was mixed with DNA loading dye by resuspending three times with a micropipette on the parafilm. Next the sample was loaded into the appropriate well carefully. This step was repeated with 1 μl of 100 bp DNA ladder (Promega, USA).

The lid of the gel tank was closed, and the electrical leads were attached so that the DNA migrated towards the positive anode (red lead). A voltage of 1 – 5 V/cm (measured as the distance between the positive and negative electrodes) was applied. The gel was run at 90 V for 35 minutes until the blue dye migrated to an appropriate distance (75%) through the gel. When the DNA samples / dyes have migrated to a sufficient distance through the gel, the power was turned off and the leads and lid were

removed from the gel tank. The gel was examined under ultraviolet (UV) light and photographed using a Gel Documentation System Biospectrum 410, UVP (Fischer Scientific, USA). The gels were analysed for presence of a band at 500 bp which was the ITS2 region.

3.4.4 PCR product purification

The PCR product was purified with MEGAquick-spin™ Total Fragment DNA Purification Kit (Intron Biotechnology Inc, Korea, **Appendix L2**). Firstly, 5 volumes of BNL Buffer was added to the PCR reaction product and mixed well by vortexing. For 20 μl of PCR product, 100 μl of BNL Buffer was added to the tube directly. Secondly, one MEGAquick-spin™ column was placed in a Collection Tube for each DNA sample.

Thirdly the DNA sample was transferred to the MEGAquick-spin™ column assembly and centrifuged at 13,000 rpm for 1 minute to bind the DNA. The flow-through was discarded and the MEGAquick-spin™ column was placed back into the same 2 ml collection tube. Fourthly, 700 μl of Washing Buffer was added to the column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the MEGAquick-spin™ column was placed back into the same 2 ml collection tube and centrifuged at 13,000 rpm for 1 minute to dry the spin membrane.

Fifthly, the MEGAquick-spin™ column was placed in a clean 1.5 ml microcentrifuge tube. Elution Buffer, 30 μl was applied directly to the centre of the column without touching the membrane with the pipette tip and the tube was incubated at room temperature for 1 minute before it was centrifuged at 13,000 rpm for 1 minute. Finally the MEGAquick-spin™ column was discarded and the microcentrifuge tube containing the eluted purified PCR product was stored at -20 °C.

3.4.5 DNA sequencing

The purified PCR product was sent for sequencing to FirstBase Sdn. Bhd. (Malaysia), and was sequenced in both directions with the same primers used for PCR amplification. The sequence alignment was conducted with Mega 5.2 software. Firstly the forward and reverse sequence files were opened with Mega 5.2 software. Next one of the sequences was converted to reverse complement sequence. Then, the sequences were aligned by ClustalW. Both ends of the sequences were trimmed to remove the gaps.

The sequence was then compared with a known sequence in GenBank (www.ncbi.nlm.nih.gov/BLAST). An unknown sample was identified if the ITS2 region matched that of a reference species with ≥ 99% identity.

3.5 Antimicrobial Susceptibility Test

3.5.1 Test microorganisms used in the study

Antibacterial activity of plant extracts was investigated against five test microorganisms. Three Gram-positive test bacteria *Staphylococcus aureus*, *Bacillus cereus* and *Streptococcus mutans* and two Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Table 3-4) were used. The registered bacterial isolates were obtained from the American Type Culture Collection (ATCC) maintained in the Fermentation Laboratory, Microbiology Division, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. The test bacteria were cultured on Nutrient Agar (Difco™, USA) at 37 °C for 24 hours. The cultures were sub cultured regularly (every 30 days) and stored at 4 °C.

Table 3- 4: Test microorganism in study

Bacteria Name	Type	Strain
Bacillus cereus	Gram positive	ATCC 14579
Escherichia coli	Gram negative	UT189
Pseudomonas aeruginosa	Gram negative	PA7
Staphylococcus aureus	Gram positive	RF 122
Streptococcus mutans	Gram positive	GEJ 11

Table displays the strain for the five test microorganism obtained from the Fermentation Laboratory in the University of Malaya.

3.5.2 Preparation of Muller Hinton agar medium

Difco™ Muller Hinton Agar, 38.0 g (Becton, Dickson, USA) medium was weighed and mixed thoroughly in one litre of distilled water. The dissolved medium was then autoclaved at 121°C for 15 minutes. After autoclaving, the medium was cooled to about 50 °C. Next, the melted, sterile agar was poured into a series of sterile petri dishes 90 × 15 mm (Axygen Biotechnology, China) following strict aseptic conditions. The plates were filled to about one-third capacity of the molten agar that is about 10 ml per plate. Next, the plates were allowed to cool in the laminar flow (Holten LaminAir, USA) for about fifteen minutes. The cooled, set agar medium was checked for contamination and the plates were sealed with parafilm and stored at 4 °C until required.

3.5.3 Preparation of inoculums

Two or three colonies of the test microorganism were picked with a sterilized wire loop from the original culture plate and introduced into a test tube containing 5 ml of sterile nutrient broth. Next, the tubes were incubated for 24 hours to produce a bacterial suspension of moderate cloudiness. After 24 hours, the turbidity of the suspension was compared to a 0.5 McFarland standard (10^8 CFU /ml) (bioMérieux Inc, USA) and the turbidity of this suspension was adjusted by adding more organism if the suspension was too light or diluting with sterile saline solution 0.85% sodium chloride (Merck, Germany) if the suspension was too heavy. This suspension was used to inoculate the Muller Hinton agar within 15 minutes of preparation.

3.5.4 Antimicrobial assay

Antimicrobial assay was carried out using the modified well diffusion assay method by Perez and team (1990) following an accepted standard (Clinical and Laboratory Standards Institute, 2007). Firstly, a sterile cotton swab was dipped into the tube containing the nutrient broth with inoculums. The dried surface of a Muller Hinton agar plate was inoculated with the test microorganism by streaking evenly on the plate in three even planes with the swab over the entire agar surface. The plate was rotated approximately 60° each time the streaking was done to produce an even distribution of the inoculum. Excess liquid was removed by running the swab along the rim of the agar plate and the swab was discarded.

Following this, wells with a diameter of 6 mm were bored with a sterile cork borer (**Appendix C**, Figure 3a) and filled with 50 μl of aqueous, ethanolic and methanolic plant extracts using four different concentrations (10, 25, 50 and 100 mg/ml), antibiotic tetracycline at a concentration of 2.5 mg/ml (positive control) and distilled water / IPB (pH 7.4, negative control). Finally the plates were sealed with parafilm and incubated in an incubator (WTC Binder, Germany) at 37 ºC. Results were read after 18 hours of incubation.

3.5.5 Measuring the zone of inhibition

The zone of inhibition was measured using a vernier caliper after 18 hours of incubation. Measurements were made with the unaided eye while viewing the back of the petri dish. The petri dish was held a few inches above a black, non-reflecting surface illuminated with reflected light. The diameter of the zone of inhibition was determined by measuring the radius of the zone. This was done by measuring the centre of the antibiotic well to a point on the circumference of the zone where a distinct edge is present which was multiplied by two (**Appendix C**, Figure 3b). If individual colonies were apparent across the plate the test was repeated.

3.5.6 Statistical analysis

Antimicrobial activity of different medicinal plant extracts using well diffusion assay was measured by mean ± SD (standard deviation), n = 3. The results were expressed in mm excluding the 6 mm well diameter. Mean changes between the samples/positive control were compared to the negative control (IPB/ distilled water) by one-way ANOVA followed by Dunnett's Multiple Comparison Test. The p value < 0.001 was considered highly significant***. All statistical analysis was performed using GraphPad Prism 5 software.

3.6 Saturated Ammonium Sulphate Precipitation of Plant Peptides

Firstly, saturated ammonium sulphate solution was prepared by dissolving anhydrous ammonium sulphate (BDH Limited, England) in distilled water. Secondly, 10 ml of ethanolic extract with a concentration of 100 mg/ml was centrifuged at 30,000 rpm for 20 minutes. The pellet was discarded and the supernatant was added into a 50 ml beaker. Thirdly, 6.97 g of ammonium sulphate to be added was weighed depending on the volume of the solution which was 10 ml and the percent saturation of the salt needed which was 100 % according to a nomogram (Table 3-5). The extract was stirred with a magnetic stirrer (Thermoline Scientific, Australia) and the entire procedure was performed on ice to maintain 0 °C temperature.

Ammonium sulphate was added bit by bit slowly with a spatula into the extract with constant slow stirring. A small amount of ammonium sulphate was added at a time and then allowed to dissolve before further addition. When the solution turned cloudy, the addition of saturated ammonium sulphate solution was paused to allow mixing of the solution. Rapid addition of saturated ammonium sulphate solution leads to the formation of precipitate trapped with unwanted soluble peptides. When all the weighed ammonium sulphate was dissolved, it was maintained on the stirrer for 1 hour to allow precipitation to occur on ice. Finally the solution was centrifuged at 10,000 g for 15 minutes at 4 ºC (Thermo Scientific, USA). The supernatant was carefully removed into a 15 ml centrifuge tube. The pellet containing the precipitated protein was dissolved in IPB pH 7.4 (Wenk & Fernandis, 2007). Both the pellet and supernatant were stored at -20 ºC for further analysis. Antimicrobial susceptibility tests by well diffusion assay were performed on both the pellet and supernatant.

Results were expressed in mm excluding the 7 mm well diameter and the mean changes between the samples / positive control were compared to the negative control (saturated ammonium sulphate solution).

Table 3- 5: Nomogram for ammonium sulphate precipitation at 0 °C

Initial concentration of ammonium sulfate	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Table shows the nomogram for determining the amount of solid ammonium sulphate in grams to be added in 1 litre of solution which will yield the desired percentage saturation at 0 ºC.

Modified from Kornberg et al. (1955)

3.7 Determination of Total Phenolic Content (TPC).

Total phenolic content of the plant was determined based on the Folin-Ciocalteu colorimetry assay method as deduced by Waterhouse (2001) using gallic acid as a standard.

3.7.1 Gallic acid calibration standard preparation

Gallic acid (Sigma Aldrich®, Germany), 0.5 g was dissolved in 10 ml of absolute ethanol and then diluted to 100 ml with distilled water to obtain a concentration of 5 g/l of gallic acid solution which can be stored at 4 ºC, up to 2 weeks. Standards (50, 100, 200, 300, 400, and 500 mg/l) concentrations were prepared by diluting the stock with distilled water (Table 3-6).

Table 3- 6: Concentration of gallic acid standard in Folin-Ciocalteu assay

Concentration (mg/l)	Volume of gallic acid stock (μl)	Volume of distilled water (μl)
50	10	990
100	20	980
200	40	960
300	60	940
400	80	920
500	100	900

Table shows the different concentration of gallic acid standard (50 – 500 mg/l) prepared from the stock solution of gallic acid which has a concentration of 5 g/l.

3.7.2 Sodium carbonate preparation

Anhydrous sodium carbonate (Merck, Germany), 20 g was dissolved in 80 ml of distilled water and was brought to a boil on a hotplate (Thermoline Scientific, Australia). The solution was left to cool to room temperature and a few crystals of sodium carbonate were added into the solution and the solution was left at room temperature for 24 hours. Finally, the sodium carbonate solution was filtered through Whatman no. 1 filter paper and water was added to make up to 100 ml. The solution was stored indefinitely at room temperature.

3.7.3 Folin-Ciocalteu assay

Firstly, 20 μl of plant sample (2.5 mg/ml), gallic acid standard or blank (distilled water) were added into a 2 ml plastic cuvette. Secondly, 1.58 ml of distilled water, followed by 100 μl of Folin-Ciocalteu's phenol reagent, 2N (Sigma Aldrich®, Germany) was added. The mixture was mixed thoroughly by re-suspending with a micropipette and was incubated for 1 - 8 minutes. Incubation time should never exceed 8 minutes. Thirdly, 300 μl of sodium carbonate solution was added and the mixture was mixed thoroughly. Finally, the mixture was left to incubate for 2 hours, at room temperature. All samples and standards were prepared in trplicate. Next, the absorbance of the sample, standards and blank were measured at 765 nm with a spectrophotometer (Varians Carry 50, Agilent Technologies, USA).

3.7.4 Calculation of total phenolic content of plant samples

The absorbance of the blank was subtracted from all the readings and a calibration curve was plotted with the standard values. The curve was used to determine the corresponding gallic acid concentration of all the samples. In order to obtain results based on the correct concentration of the sample, the dilution factor was multiplied. All determinations were carried out in triplicate, and the results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g d.w.).

The total phenolic content of the plant was calculated as follows:

$C = c \times V / m$

Where C = total phenolic content in gallic acid equivalent (mg GAE/g), c = concentration of gallic acid established from the calibration curve (mg/l), V = volume of extract (ml) and m = weight of pure plant extract (g).

3.7.5 Statistical analysis

The mean changes between the samples were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test. The p value $p < 0.05$ was considered statistically significant. The software GraphPad Prism 5 was used to analyse the data. Results were presented as mean ± standard deviation, where n = 3.

3.8 Antioxidant Assay

3.8.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay

The DPPH radical scavenging activity assay to determine amount of antioxidant present in the plant was performed based on a method by Razali and colleagues (2008). Initially, DPPH solution is dark violet in colour, but the colour fades when an antioxidant added donates hydrogen (Szabo *et al.*, 2007). The change in colour is measured through absorbance with a spectrophotometer.

3.8.1.1 Preparing standard, positive control and sample

Firstly 100 μM 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich®, Germany) was prepared by mixing 0.004 g of DPPH in 100 ml methanol, HPLC grade (Tedia Company Inc, USA) and was stored in the dark. DPPH was prepared freshly for the assay and was stored at 4 ºC. Secondly, the standard, Trolox (Sigma Aldrich®, Germany) was prepared. Thirdly, the positive control ascorbic acid, butylated hydroxytoluene (BHT) and quercetin (Sigma Aldrich®, Germany) were prepared.

The standard, positive controls and the sample were prepared by dissolving in HPLC grade methanol to obtain a stock solution with a concentration of 1 mg/ml. The stock solution of the sample and positive control were further dissolved in serial two-fold dilution in methanol to obtain the desired concentration 15.625 – 1 000 μg/ml (Table 3-7). The stock solution of the standard was diluted in methanol to obtain a concentration of 25 – 500 μg/ml (Table 3-8).

Table 3- 7: Concentration of sample and positive control in DPPH assay

Concentration (μg/ml)	Volume of stock solution (μl)	Volume of methanol (μl)
1000	200	0
500	100	100
250	100	100
125	100	100
62.5	100	100
31.25	100	100
15.625	100	100

Table shows the serial two-fold dilution of the sample and positive control in methanol to obtain concentration of (15.625 – 1 000 μg/ml) for DPPH assay.

Table 3- 8: Concentration of standard, Trolox in DPPH assay

Concentration (μg/ml)	Volume of stock solution (μl)	Volume of methanol (μl)
25	25	975
50	50	950
75	75	925
100	100	900
200	200	800
300	300	700
400	400	600
500	500	500

Table shows the different concentration (25 – 500 μg/ml) of the standard Trolox prepared from a stock solution with the concentration of 1 mg/ml for DPPH assay.

3.8.1.2 DPPH assay

Firstly, 20 μl of standard, positive control and sample was added to a 96 well plate. Secondly, the prepared DPPH reagent was added into the well and was re-suspended with micro-pipette. The mixture was left in the dark at room temperature for 10 to 20 minutes. The reactions were performed in triplicate and the entire assay was performed in the dark. The changes in absorbance were measured at 515 nm with a microplate reader (Bio-Rad, USA).

3.8.1.3 Calculation of antioxidant capacity in plant of study

Percentage of inhibition was calculated as follows:-

$$\% \text{ Inhibition} = \frac{A_0 - A_s}{A_0} \times 100\%$$

Where A_0 = absorbance of the blank, A_s = absorbance of the tested sample, standard or positive control.

The antioxidant activity of plant extracts, standards and positive controls were expressed as IC_{50}, which is defined as the concentration in μg/ml of plant extracts, standards, or positive controls required to inhibit the formation of DPPH radicals by 50%. IC_{50} was determined using a non-linear regression analysis computed using GraphPad Prism 5.

The antioxidant activity was based on the Trolox standard curve at concentration 25 – 500 μg/ml (Table 3-8) expressed as milimole Trolox equivalent antioxidant capacity per gram dried weight of plant sample (mmol TE/g d.w.). The assay was conducted in triplicate and results were presented as mean ± SD. The mean changes between the samples for each test were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test.

3.8.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Total antioxidant activity was measured by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain (1996). In the FRAP assay, the Fe^{3+}/tripyridyltriazine complex is reduced to a ferrous form by plant extracts with an intense blue colour and absorbance maximum at 593 nm (Benzie & Szeto, 1999).

3.8.2.1 Preparing reagent, standard, positive control and sample

The FRAP reagent consists of 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri (2-pyridyl)-S-triazine (TPTZ) (Sigma-Aldrich®, Germany) and 20 mM ferric (III) chloride ($FeCl_3.6H_2O$) in the ratio of 10:1:1.

Firstly, 300 mM acetate buffer, (pH 3.6) was prepared whereby 0.155 g of sodium acetate trihydrate (Fischer Scientific, UK) was dissolved in 0.8 ml of 100% acetic acid (Merck, Germany) and 49.2 ml of double distilled water. Secondly, 10 mM 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ) was prepared by dissolving 0.0156 g of TPTZ in 0.2 ml of 1N hydrochloric acid, HCl (Fischer Scientific, UK) and 4.8 ml of double distilled water. Thirdly, 20 mM ferric (III) chloride ($FeCl_3.6H_2O$) was prepared by dissolving 0.027 g of iron (III) chloride hexahydrate ($FeCl_3$) (Merck, Germany) in 5 ml of double distilled water.

The standard used was 10 mM ferrous sulphate ($FeSO_4.7H_2O$), and a stock solution 100 mmole/L consisting of 0.0139 g of ferrous sulphate (Fischer Scientific, UK) in 5 ml of distilled water was prepared. Six concentrations of standards were prepared (Table 3-9) for the standard curve. Ascorbic acid and butylated hydroxytoluene (BHT) (Sigma Aldrich®, Germany) were used as positive controls and

were prepared in two concentrations (1 mg/ml and 0.5 mg/ml) by dissolving each compound in distilled water. Sample was prepared in two concentrations (1mg/ml and 0.5 mg/ml) in distilled water.

Table 3- 9: Concentration of standard, ferrous sulphate $FeSO_4.7H_2O$ in FRAP assay

Concentration (µmol/L)	$FeSO_4.7H_2O$ (µl)	Double distilled water (µl)
100	100	900
200	200	800
400	400	600
600	600	400
800	800	200
1000	1000	0

Table shows the different concentration (100 – 1000 µmol/L) of the standard ferrous sulphate $FeSO_4.7H_2O$ prepared from a stock solution with the concentration of 100 mmol/L for FRAP assay.

3.8.2.2 FRAP assay

The working solution of the FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ) and 20 mM ferric (III) chloride ($FeCl_3.6H_2O$) in the ratio of 10:1:1 at the time of use. The FRAP reagent was incubated at 37 ºC for 5 minutes before the experiment. For the assay, 300 µl of FRAP reagent was mixed with 10 µl of sample, standard or positive control and the mixture was vortexed well. A mixture of FRAP reagent with distilled water was used to zero the machine. The absorbance of the mixture was read at 595 nm at 0 minutes and every 15 seconds for 4 minutes. The assay was carried out in triplicate and the results were presented as mean ± SD.

3.8.2.3 Calculation of antioxidant capacity

The antioxidant activity of plant extracts, and positive controls (1 mg/ml) in the reaction time 0 - 4 minutes were expressed as milimole ferrous sulphate per gram dried weight (mmol Fe^{2+}/g d.w.) and was determined based on the ferrous sulphate ($FeSO_4.7H_2O$) standard curve. One unit of FRAP is defined as the reduction of 1 mole of Fe (III) to Fe (II).

Results were presented as mean ± SD where n = 3. The mean changes between the samples for each test were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test.

3.8.3 Investigation of the Protective Effect of the Test Plant Against Hydrogen Peroxide (H_2O_2) Induced Red Blood Cell Lysis.

3.8.3.1 Preparation of rabbit erythrocyte suspension

Blood was withdrawn from the marginal vein of healthy normal white rabbits using a 27G × 1/2" sterile needle (TERUMO®, Belgium) and aspirated into silicone coated blood collection tubes (Vacutainer®, Becton Dickson, USA). Next, 5 ml of defibrinated blood was centrifuged in a 15 ml centrifuge tube at 1000 × g in a bench top clinical centrifuge at 4 ºC for 20 minutes. The buffy coat and plasma layer were removed with a pipette and discarded. Cold IPB, 5 ml (pH 7.4, bioWorld, USA) was added to the packed erythrocytes, mixed gently, and then centrifuged at 2400 rpm for 5 minutes at 4 ºC. The supernatant was discarded. This step of washing was repeated two more times as described. After the final wash, the volume of the packed erythrocytes in the centrifuge tube was noted. A 10% erythrocyte suspension in IPB was prepared where 1 ml of the pellet of packed erythrocytes was added to 9 ml of IPB. The suspension was stored at 4 ºC until required (Rose & Okrend, 1998).

3.8.3.2 *In vitro* anti-haemolysis assay

The protective effect of the plant leaf extracts on rabbit erythrocyte was performed according to the procedure described by Ajila and Rao (2008) with slight modifications. The free radical initiator used in the study was hydrogen peroxide (H_2O_2) (Merck, Germany). To evaluate haemolysis induced by plant extracts, the rabbit erythrocytes were pre-incubated with 50 μl of extracts at 1.0 mg/ml concentration for 1 hour to determine effect of extract on haemolysis. Next, 50 μl of plant leaf extracts with different concentrations (0.1-1 mg/ml) were added to 100 μl of 10% (v/v) erythrocyte suspension in IPB. Then, 100 μl of 10 mM H_2O_2 in IPB was added into the samples.

A standard was prepared by replacing the plant extract with ascorbic acid (Sigma Aldrich®, Germany). Complete haemolysis was achieved by treating the erythrocytes with distilled water (without H_2O_2 and plant extract). The reaction mixture was then incubated in a water bath at 37 ºC for 18 hours. Next, 1 ml of IPB was added to dilute the reaction mixture and then the mixture was centrifuged at 2000 × g for 10 minutes. The absorbance of the supernatant was measured at 540 nm with a spectrophotometer (Varians Carry 50, Agilent Technologies, USA) to determine haemolysis. The entire assay was performed in triplicate.

3.8.3.3 Calculation of anti-haemolytic activity

Percentage of haemolysis was based on the 100% haemolysis of distilled water and was calculated as follows:

$$\% \text{ Haemolysis} = \frac{A_s - A_0}{A_{dw} - A_0} \times 100\%$$

Where A_s = plant sample or standard, A_{dw} = distilled water and A_0 = absorbance of blank. Results were presented as mean ± SD.

The concentration (mg/ml) of plant extracts and positive control required to inhibit haemolysis of red blood cells by 50% (IC_{50}), whereby 10 mM and 50 mM H_2O_2 was used to induce haemolysis was determined by non-linear regression analysis computed using GraphPad Prism 5. Results were presented as mean ± SD for three replicate. The mean changes between the samples against 10 mM and 50 mM were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test separately.

Antioxidant capacity of extracts and positive control were determined by comparing with ascorbic acid standard curve and expressed as milimole ascorbic acid equivalent (AAE) per gram dried weight plant sample (mmol AAE/g d.w.). Results were expressed as mean ± SD where n = 3. The mean changes between the samples for each test were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test.

3.9 Correlation between the Phenolic Compounds with the Antimicrobial and Antioxidant Activity of *C. asiatica* and *V. amygdalina* Extracts

3.9.1 Correlation between the Total Phenolic Content (TPC) and Antimicrobial Activity of *C. asiatica* and *V. amygdalina* Extracts.

Regression analysis was performed to correlate TPC with antimicrobial activity in comparison between (i) aqueous, ethanolic and methanolic extracts of each plant and (ii) ethanolic extracts of both *C. asiatica* and *V. amygdalina*. Correlation was performed separately for each test microorganism in the study which were *B. cereus*, *E. coli*, *S. aureus*, *S. mutans* and *P. aeruginosa*. TPC was determined by Folin's Ciocalteu assay, presented as milligram gallic acid equivalent per gram dry weight (mg GAE/g d.w.). The antimicrobial activity was presented as diameter of the zone of inhibition (mm).

Statistical significance between correlation coefficient (p value) was determined by Pearson correlation analysis whereby $p < 0.05$ was statistically significant. Results were presented as mean ± SD where n = 3.

3.9.2 Correlation between the Total Phenolic Content (TPC) and Antioxidant Activity and between Antioxidant Assays of Extracts of *C.asiatica* and *V.amygdalina*

Regression analysis was performed to correlate TPC with antioxidant capacity and various antioxidant assays for (i) aqueous, ethanolic and methanolic extracts of each plant and (ii) both the plants (ethanolic extract). Correlation coefficient (r) between TPC and DPPH, TPC and FRAP, TPC and anti-haemolysis assay, DPPH and FRAP, DPPH and anti-haemolysis assay, and FRAP and anti-haemolysis assay was determined.

TPC was determined by Folin's Ciocalteu assay, presented as milligram gallic acid equivalent per gram dry weight (mg GAE/g d.w.). The antioxidant capacity was determined by (i) DPPH assay presented as milimole Trolox equivalent per gram dried weight (mmol TE/g d.w.); (ii) FRAP assay presented as milimole ferrous sulphate per gram dried weight (mmol Fe^{2+} /g d.w.); and (iii) anti-haemolysis assay presented as milimole ascorbic acid equivalent per gram dried weight (mmol AAE/g d.w.).

Statistical significant between correlation coefficient (p value) was determined by Pearson correlation analysis where $p < 0.05$ was statistically significant. Results were presented as mean ± SD where $n = 3$.

3.10 Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

3.10.1 Preparing the mobile phase

The mobile phase was a binary solvent system consisting of solvent A and solvent B. Solvent A was 0.1% of trifluoroacetic acid (TFA) (Sigma Aldrich®, Germany) in Milli-Q grade water (v/v) with pH 2.6 (Sartorius, Germany). Solvent B was HPLC grade methanol (Tedia Company Inc, USA). Both the solvents were passed through a vacuum degasser with a 0.45 µm pore size membrane filter (Fisher Scientific (M) Sdn Bhd).

3.10.2 Pre-treatment of plant crude extracts

Stock solution of plant extracts for HPLC analysis was prepared by re-dissolving the aqueous, ethanolic and methanolic extracts in HPLC grade methanol to obtain a final extract concentration of 100 mg/ml. Next, the respective extracts were pre-treated with ISOLUTE® C18 SPE Columns (Biotage, Sweden) before injection of samples into a HPLC system.

3.10.3 Preparing standards for comparison

Gallic acid, caffeic acid, *p*-coumaric acid, benzoic acid, chlorogenic acid, (+)-catechin, chloramphenicol, and quercetin were purchased from (Sigma Aldrich®, Germany). Stock solutions of the individual standards were prepared at a concentration of 1.0 mg/ml in methanol. Prior to injection, all the standard solution were filtered through a PTFE filter with 0.20 µm pore size (Waters, USA).

3.10.4 Instrumentation and analytical conditions

Phenolic compounds were evaluated with a reverse phase-high performance liquid chromatography (RP-HPLC; Shimadzu LC, Japan) from the Department of Molecular Medicine, Faculty of Medicine, University of Malaya. Detection and quantification was carried out with a CBM-20A system controller, a LC-20AD binary pump, a CTO-10ASvp oven and a SPD-20A ultraviolet detector with 280 nm detection wavelength. The column fitted was a Jones Chromatography Genesis C_{18} (150 × 4.6 mm, 4 µm) column (Jones Genesis®, UK).

Chromatography for *C. asiatica* was achieved at 30 ºC with a flow rate of 1 ml/min following the gradient profile displayed in Table 3-10. Chromatography for *V. amygdalina* was achieved at 30 ºC with a flow rate of 0.5 ml/min, following the gradient profile displayed in Table 3-11. Injection volume of the samples were 20 µl via a thin layer chromatography syringe (Hamilton®, USA). The data was integrated and analysed with the Shimadzu LCSolution Analysis Report Software system. The well separated, major peaks were collected manually several times and stored in -20 ºC for further analysis.

Table 3- 10: Solvent gradient profile in RP-HPLC for *C.asiatica*

Time (min)	Solvent A concentration (%)	Solvent B concentration (%)
0.01	90	10
10.00	75	25
20.00	40	60
30.00	30	70
45.00	0	100
55.00	90	10

Table shows the solvent gradient that was performed by varying the proportion of Solvent A (0.1% of trifluoroacetic acid in Milli-Q water v/v) to Solvent B (methanol) at respective time in minutes.

Method adapted from Mišan et al. (2010)

Table 3- 11: Solvent gradient profile in RP-HPLC for *V. amygdalina*

Time (min)	Solvent A concentration (%)	Solvent B concentration (%)
0.01	90	10
10.00	40	60
30.00	35	65
50.00	30	70
55.00	0	100
60.00	100	0

Table shows the solvent gradient that was performed by varying the proportion of Solvent A (0.1% of trifluoroacetic acid in Milli-Q water v/v) to Solvent B (methanol) at respective time in minutes.

3.10.5 Identification and Quantitation

Phenolic compounds were identified by comparing the retention times obtained for the plant extract with pure standards. To further confirm the phenolic compounds present in the samples, samples were spiked with pure standards. Data was analysed with Shimadzu LCsolution Software.

Concentration of unknown samples were determined by two methods. Firstly using response factor (RF) as shown by Ghafoor *et al.* (2012) as follows:

RF = Area of standard / Concentration of standard

Concentration of sample = sample area / RF.

Results were presented as mg per g dry weight (mg/g d.w.) of sample extract.

Secondly Folin Coicalteu's assay was performed on the major peaks collected and the total phenolic content of the peaks were expressed as mg gallic acid equivalent per 100g dry weight (mg GAE/100g d.w.).

3.11 Fourier Transform Infrared Spectroscopy (FT-IR)

Major peaks obtained from RP-HPLC of *C. asiatica* methanol extract were collected in 1.5 ml microcentrifuge tubes (Eppendorf® GmbH, Germany). The peaks were dried down with a centrifuge concentrator (Labconco®, USA) to a powder and weighed. Next, FT-IR spectra of the peaks were recorded on a Perkin Elmer Spectrum R x I at the Department of Chemistry, Faculty of Science, University of Malaya. The spectra of the peaks were scanned at room temperature in the 4000-400 cm^{-1} spectral range.

3.12 Liquid Chromatography – Mass Spectroscopy (LC-MS) Analysis

3.12.1 Preparing the mobile phase

For LC-MS, the mobile phase was a binary solvent system consisting of solvent A and solvent B. Solvent A was 0.1% of formic acid (Sigma Aldrich®, Germany) in Milli-Q grade water (v/v). Solvent B was 0.1% of formic acid in acetonitrile (Tedia Company Inc, USA). Both the solvents were passed through a vacuum degasser with 0.45 µm pore size membrane filter (Fisher Scientific (M) Sdn Bhd).

3.12.2 Pre-treatment of plant crude extracts

The stock solution of plant extracts for LC-MS analysis were prepared by re-dissolving the methanol extract in HPLC grade methanol to obtain a final extract concentration of 100 mg/ml concentration. Next, the extracts were pre-treated with ISOLUTE® C18 SPE Columns (Biotage, Sweden) and the concentration was adjusted to about 20 ppm before injection of the samples into the LC-MS system.

3.12.3 Instrumentation and analytical conditions

The separation of phenolic compounds was performed on an Agilent 6530 Accurate-Mass Q-TOF LC-MS system (Agilent Technologies, USA) at the Department of Chemistry, Faculty of Science, University of Malaya. Mass spectra were acquired with a TOF/Q-TOF mass spectrometer with gas temperature 250°C; gas flow 8 l/min; and nebulizer 35 psig. The mass spectrometer was operated in both negative and positive ion modes with a scanning range of 100 to 1000 m/z. Liquid chromatography separation was performed with a Hardware Kit ZORBAX Eclipse XDB-C18 column (150 × 4.6 mm; particle size, 5 μm; Agilent Technologies). The gradient profile for LC-MS was the same as HPLC. Data analysis performed with the Mass Hunter software.

4.0 RESULTS

4.1 Extraction Yield of the Selected Medicinal Plants

Extraction yields for the selected medicinal plants *A.vera* – leaf and gel, *A.indica* – leaf, *C. asiatica* whole plant, *C. papaya* leaf, *H. speciosa* leaf, tuber and root, and *V. amygdalina* leaf and stem were determined based on dry weight as described in Section 3.3.3 and presented in Figure 4-1. The ethanolic extract of *H. speciosa* root had the highest yield (66.67%) followed by the ethanolic extract of *A. vera* gel (35.14%). The lowest yield was observed for ethanolic extract of *H. spesiosa* tuber (2 %).

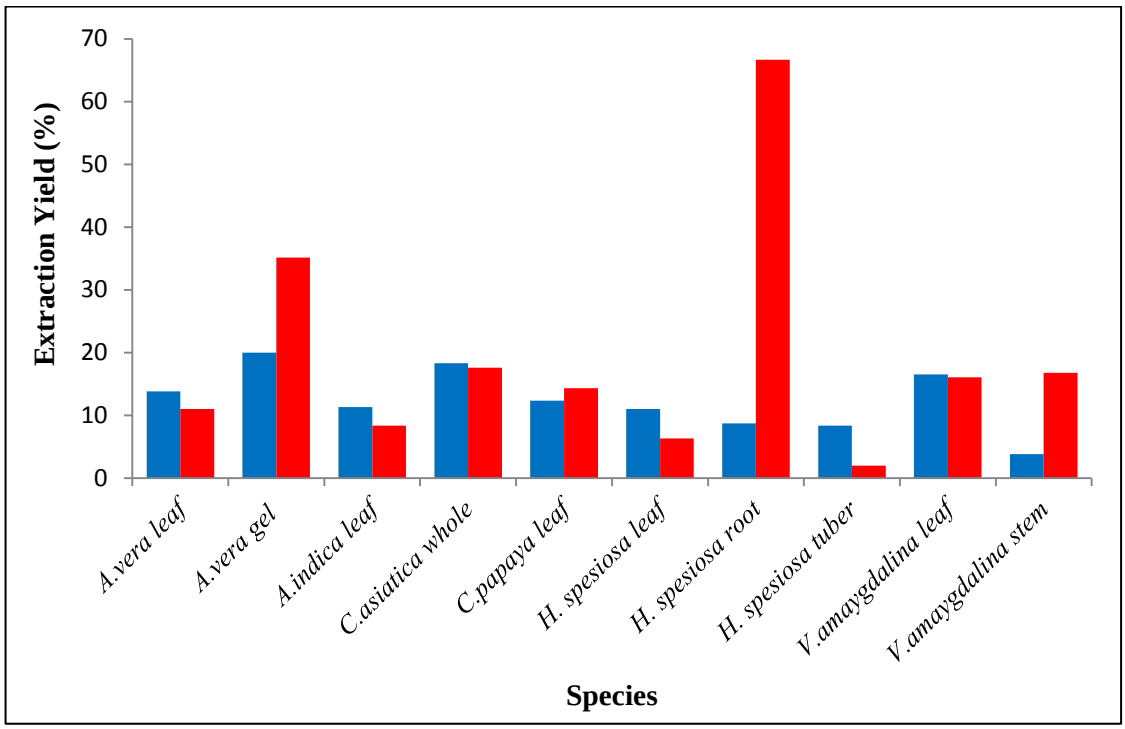

Figure 4-1: Extraction yield in percentage for medicinal plants screened in the study
Yield of in aqueous ( ) and ethanol ( ) extracts were determined based on the dry weight of the plant parts.

4.2 Preliminary Screening of Medicinal Plants for Antimicrobial Activity by Well Diffusion Assay

In this study, parts of medicinal plants (*A. vera* - leaf and gel, *A. indica* - leaf, *C. papaya* - leaf, *C. asiatica* - whole plant, *H. spesiosa* - leaf, tuber and root, and *V. amygdalina* - leaf and stem) were used. For antimicrobial activity, aqueous extracts (Table 4-1) and ethanolic extracts (Table 4-2) of the selected medicinal plants were tested against the five test microorganisms namely *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus mutans.* Assays were performed in triplicate and each well was filled with 50 µl of various concentrations of plant extracts (10, 25, 50 and 100 milligram extract per millilitre isotonic phosphate buffer, mg/ml). The positive control was 50 µl of antibiotic tetracycline at 2.5 mg/ml.

Plant extracts showed varying antimicrobial activity towards the test microorganisms compared to the control tetracycline which showed antimicrobial activity against test microorganisms except *P. aeruginosa* at 2.5 mg/ml concentration. For antimicrobial screening of the aqueous extract (Table 4-1) from the 6 plants studied, only *C. asiatica* showed significant antimicrobial activity ($p < .05$) against all the five test strains. The antimicrobial activity was in a dose dependent manner whereby the minimum inhibitory concentration (MIC) of the aqueous extract was 25 mg/ml for *B. cereus, E. coli* and *S. mutans* (3.00±0.00 mm, 6.00±0.00 mm, and 3.00±0.00 mm respectively). *P. aeruginosa* and *S. aureus* were inhibited at MIC 50 mg/ml of aqueous plant extract (6.33±0.58 mm and 10.0±0.00 mm respectively).

Antimicrobial screening of ethanolic extracts (Table 4-2) revealed that all five test microorganisms exhibited remarkable susceptibility towards *C. asiatica.* It was observed that the ethanolic extract of *C. asiatica* exhibited the highest antimicrobial activity towards *S. aureus* at 100 mg/ml concentration (13.00 ± 0.00 mm). The ethanolic

extract of *V. amygdalina* leaf exhibited significant antimicrobial activity ($p < .05$) towards *B. cereus* (4.00±0.00 mm and 6.67±0.58 mm at 50 mg/ml and 100 mg/ml respectively).

Slight antimicrobial activity was observed in ethanolic extract of *A. vera* leaf against *B. cereus* (MIC 25 mg/ml; 2.00±0.00 mm) and *E. coli* (MIC 100 mg/ml; 2.00±0.00 mm). *A. vera* gel showed insignificant activity ($p > .05$) towards *B. cereus* (3.33±0.58 mm) and *S. aureus* (2.00±0.00 mm). Apart from that, *H. spesiosa* leaf showed slight inhibition against *B. cereus* (2.33±0.58 mm) and *P. aeruginosa* (2.00 ± 0.00 mm respectively) which were insignificant ($p > .05$).

Overall comparison between the antimicrobial activity of aqueous and ethanolic extracts of the six medicinal plants revealed that ethanolic extracts possessed stronger antimicrobial activity compared to aqueous extracts. But the aqueous extracts of *C. asiatica* exhibited better antimicrobial activity compared to ethanol extract (**Appendix D**). Thus *C. asiatica* and *V. amygdalina* were selected for further tests based on the screening for antimicrobial properties.

Table 4-1: Antimicrobial Activity of Aqueous Plant Extracts

Plant Sample (aqueous)	Concentration (mg/ml)	Diameter of the zone of inhibition (mm)[a]				
		B. cereus	***E. coli***	***P. aeruginosa***	***S. aureus***	***S. mutans***
A. vera (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
A. vera (gel)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
A. indica (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
C. papaya (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
C. asiatica (whole plant)	10	NI	NI	NI	NI	NI
	25	$3.00\pm0.00^{**}$	$6.00\pm0.00^{***}$	NI	NI	3.00 ± 0.00^{ns}
	50	$5.67\pm0.58^{***}$	$7.33\pm0.58^{***}$	$6.33\pm0.58^{***}$	$10.0\pm0.00^{***}$	$15.33\pm1.15^{***}$
	100	$10.0\pm0.00^{***}$	$10.0\pm0.00^{***}$	$8.33\pm1.15^{***}$	$14.0\pm0.00^{***}$	$18.00\pm1.73^{***}$
H. spesiosa (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
H. spesiosa (tuber)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
Speciosa (root)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
V. amygdalina (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
V. amygdalina (stem)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
Positive control[b]	2.5	16.67±0.58 ***	22.33±0.58 ***	NI	27.33±1.15 ***	24.67±0.58 ***
Negative control[c]	-	<0.5±0.00	<0.5±0.00	<0.5±0.00	<0.5±0.00	<0.5±0.00

Table displays [a]diameter of the zone of inhibition (excluding 7 mm well diameter in mm) after 18 hours of incubation against 5 test microorganism in well diffusion assay. Each well was filled with 50 µl of extract. [b]Positive control was tetracycline (2.5mg/ml). [c]Negative control was double distilled water. 'NI' presents no inhibition zone observed. Assay was performed in trplicate and results were presented as mean ± SD. The mean changes between the extracts and positive control compared to the negative control were analysed by one-way ANOVA followed by Dunnett's Multiple Comparison Test. $p < .001$ - highly significant***; $p < .01$ -very significant**; $p < .05$ – significant*; ns - not significant.

Table 4- 2: Antimicrobial Activity of Ethanolic Plant Extracts

Plant Sample (ethanolic)	Concentration (mg/ml)	Diameter of the zone of inhibition (mm)[a]				
		B. cereus	***E. coli***	***P. aeruginosa***	***S. aureus***	***S. mutans***
A. vera (leaf)	10	NI	NI	NI	NI	NI
	25	2.00±0.00^{ns}	NI	NI	NI	NI
	50	3.00±0.58**	NI	NI	NI	NI
	100	5.33±0.58***	2.00±0.00^{ns}	NI	NI	NI
A. vera (gel)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	3.33±0.58^{ns}	NI	NI	2.0±0.00^{ns}	NI
A. indica (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
C. papaya (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
C. asiatica (whole plant)	10	NI	NI	NI	NI	NI
	25	3.00±0.00**	4.67±0.58**	3.00±0.00*	NI	4.00±0.00**
	50	5.33±0.58***	6.33±1.15***	6.00±1.00***	9.00±0.00***	7.00±0.00***
	100	8.33±0.58***	9.33±0.58***	8.67±0.58***	13.00±0.00***	9.67±1.15***
H. spesiosa (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	2.33±0.58^{ns}	NI	2.00±0.00^{ns}	NI	NI
H. spesiosa (tuber)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
H. spesiosa (root)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
V. amygdalina (leaf)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	4.00±0.00***	NI	NI	NI	NI
	100	6.67±0.58***	NI	NI	NI	NI
V. amygdalina (stem)	10	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI
Positive control[b]	2.5	16.67±0.58 ***	22.33±0.58 ***	NI	27.33±1.15 ***	24.67±0.58 ***
Negative control[c]	-	<0.5±0.00	<0.5±0.00	<0.5±0.00	<0.5±0.00	<0.5±0.00

Table displays [a]diameter of the zone of inhibition (excluding 7 mm well diameter in mm) after 18 hours of incubation against 5 test microorganisms in well diffusion assay. Each well was filled with 50 µl of extract. [b]Positive control was 2.5mg/ml of tetracycline. [c]Negative control was isotonic phosphate buffer, pH 7.4. 'NI' presents no inhibition zone observed. Assay was performed in trplicate and results were presented as mean ± SD. The mean changes between the extracts and positive control compared to the negative control were analysed by one-way ANOVA followed by Dunnett's Multiple Comparison Test. $p < .001$ - highly significant***; $p < .01$ -very significant**; $p < .05$ – significant*; ns - not significant.

4.3 Authentication of Selected Medicinal Plants by DNA Barcoding

Authentication of the selected medicinal plants *C. asiatica* and *V. amygdalina* was performed by DNA barcoding method using the well characterized internal transcribed spacer 2 (ITS2) region. The primers used in the study for ITS2 region were forward primer (5'–ATG CGA TAC TTG GTG TGA AT–3') and reverse primer (5'-GAC GCT TCT CCA GAC TAC AAT-3') (Chen *et al.*, 2010).

Agarose gel electrophoresis of the polymerase chain reaction (PCR) amplified DNA fragment extracted from dried leaf material for both the medicinal plants produced bands that were about 500 bp long when compared with a standard 100 bp DNA ladder (Figure 4-2). The numbers of nucleotide after multiple sequence alignment by ClustalW of the query sequence for CA and VA were 414 bp and 390 bp respectively.

The unknown sequences were BLAST searched with sequences in GenBank and CA was identified as *Centella asiatica* accession number AF 272352 (Figure 4-3) with 99% identity and E-value 1e-152 (**Appendix E** Figure 5a).VA was identified as *Gynthemum amygdalina*/*Vernonia amygdalina* accession number AY 504695 (Figure 4-4) with 99% identity and E-value 5e-141(**Appendix E** Figure 5c).

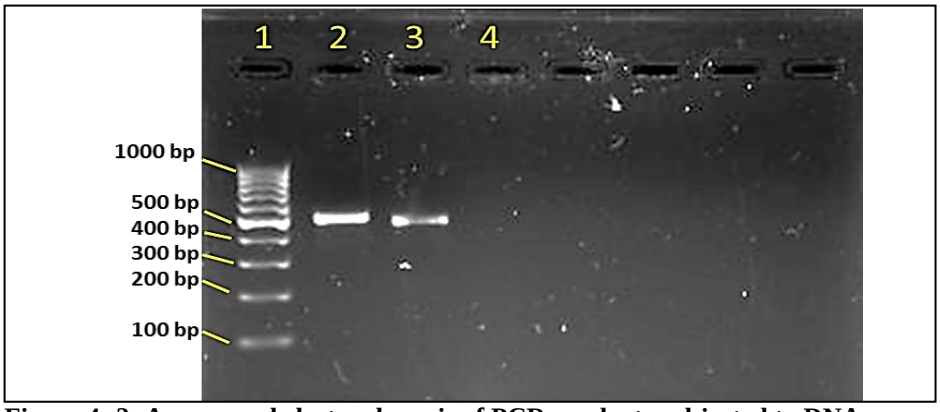

Figure 4- 2: Agarose gel electrophoresis of PCR products subjected to DNA barcoding with ITS2 primers
Figure illustrates PCR amplified DNA for each of the sample electrophoresed on 2.0% agarose gels, where **1** - 100 bp DNA ladder marker; **2 –** ITS2 region of CA**; 3 –** ITS2 region of VA and **4** – negative control.

```
CA     1    TCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCACTCGGCCGAGGGCACGTCTGCCTGGG 60
C.asi  322  TCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCACTCGGCCGAGGGCACGTCTGCCTGGG 381

CA     61   CGTCACGCATCGCGTCGccccccccCACCCGTCGACCTCGAAAGGGGTCGGGGCGGAGGG 120
C.asi  382  CGTCACGCATCGCGTCGCCCCCCCCC-ACCCGTCGGCCTCGAAAGGGGTCGGGGCGGAGGG 440

CA     121  GCGGAGAATGGCCTCCCGTGCCTCGGGGCGCGGTTGGCCCAAACGTCAGCCCGCGGCGAC 180
C.asi  441  GCGGAGAATGGCCTCCCGTGCCTCGGGGCGCGGTTGGCCCAAACGTCAGCCCGCGGCGAC 500

CA     181  GGACGTCACGACAAGTGGTGGTTTGACAAAGGCCCTCGCATGTTGTCGTGCGGTGATCCG 240
C.asi  501  GGACGTCACGACAAGTGGTGG-TTGTCAAAGGCCCTCGCATGTTGTCGTGCGGTGATCCG 559

CA     241  TCGTCGGCGTGAGCTCGTGCGACCCTGTTGCCACGCCGTGCTCGGCGCGCGCTCCGACCG 300
C.asi  560  TCGTCGGCGTGAGCTCGTGCGACCCTGTTGCCACGCCGTGCTCGGCGCGCGCTCCGACCG 619

CA     301  CGACCCC  307
C.asi  620  CGACCCC  626
```

Figure 4- 3: Sequence alignment of the ITS2 region for *C. asiatica*
ClustalW alignment of the ITS2 region for unknown (CA) sequence and sequence from GenBank *C. asiatica* (*C. asi* - AF 272352, BLAST).The ITS2 was PCR amplified with forward primer (5'–ATG CGA TAC TTG GTG TGA AT–3') and reverse primer (5'-GAC GCT TCT CCA GAC TAC AAT-3'). Red alphabets indicate differences in nucleotide.

```
VA     117  TCGAAGCGTCATCATAAGACGACTCATTAAGGGATTTTTACAAACCTTTCCTTACGGTTA 176
V.amy  645  TCGAAGCGTCATCATAAGACGACTCATTAAGGGATTTTTACAACCTTTCCTTACGGTTA 586

VA     177  ACGACACACAACTCCAAACGAAGGTCTTGTCAACCACCACTAGTCATGTATCCACCGAAA 236
V.amy  585  ACGACACACAACTCCAAACGAAGGTCTTGTCAACCACCACTAGTCATGTATCCACCGAAA 526

VA     237  GGGAGTTACGTTTAGGCCAACCACACCATCAGCATGGGAGACCAATCTCCGCCCCGAACA 296
V.amy  525  GGGAGTTACGTTTAGGCCAACCACACCATCAGCATGGGAGACCAATCTCCGCCCCGAACA 466

VA     297  ACAAGCCTACTAAGGAAGGAGGCATTGAAGGAGGCGATGCAATGCGTGACGCCCAGGCAG 356
V.amy  465  ACAAGCCTACTAAGGAAGGAGGCATTGAAGGAGGCGATGCAATGCGTGACGCCCAGGCAG 406

VA     357  ACGTGCCCTCGACCAAATGGCTTCGGGCGCAACT  390
V.amy  405  ACGTGCCCTCGACCAAATGGCTTCGGGCGCAACT  372
```

Figure 4- 4: Sequence alignment of the ITS2 region for *V. amygdalina*.
ClustalW alignment of the ITS2 region for unknown (VA) sequence and sequence from GenBank *V. amygdalina* (*V.amy* - AY 504695, BLAST).The ITS2 was PCR amplified with forward primer (5'–ATG CGA TAC TTG GTG TGA AT–3') and reverse primer (5'-GAC GCT TCT CCA GAC TAC AAT-3'). Red alphabets indicate differences in nucleotide.

4.4 Extraction Yield and Antimicrobial Activity in Aqueous, Ethanolic and Methanolic Extracts of *C. asiatica* and *V. amygdalina*

The extraction yields of aqueous, ethanolic and methanolic extracts of the selected medicinal plants were compared based on dry weight (Figure 4-5). For the medicinal plant *C. asiatica*, the highest yield was observed in aqueous extract (18.31%). Ethanolic extract and methanolic extracts had similar yields (16.83% and 16.80% respectively). The yield for *V. amygdalina* was highest in methanolic extract followed by ethanolic and aqueous extracts (16.71%, 16.26% and 14.61%) respectively, nevertheless not significantly different.

Antimicrobial activity in aqueous, ethanolic and methanolic extracts of the selected medicinal plants *C. asiatica* (whole plant) and *V. amygdalina* (leaf) was compared at various concentrations (10, 25, 50 and 100 mg/ml) against the five test microorganisms. Each well contained 50 µl of each extract.

For *C. asiatica*, the highest antimicrobial activity was observed in aqueous extract against *S. mutans* (18.00±1.73 mm at 100 mg/ml) while the lowest activity was observed in the methanolic extract against *S. mutans* (2.00±0.00 mm at 25 mg/ml). Generally aqueous extract showed greater antimicrobial activity against the tested strains compared ethanolic and methanolic extracts at corresponding concentrations. Antimicrobial activities for both ethanolic and methanolic extracts were similar for corresponding concentrations (Table 4-3).

The MIC value of aqueous extract was 25 mg/ml against *B. cereus*, *E. coli*, and *S. mutans* and 50 mg/ml against *P. aeruginosa* and *S. aureus*. For ethanolic extract, the MIC was 25 mg/ml against *B. cereus*, *E.coli*, *P. aeruginosa* and *S. mutans* and 50 mg/ml against *S. aureus*. Methanolic extracts exhibited MIC values of 25 mg/ml towards *B. cereus*, *E.coli*, and *S. mutans* and 50 mg/ml for *P. aeruginosa* and *S. aureus*.

Overall, the three extracts of *C. asiatica* inhibited all the test microorganisms at varying levels.

Ethanolic and methanolic extracts of *V. amygdalina* showed significant antimicrobial activity towards *B. cereus*. Slight antimicrobial activity was observed towards *S.mutans* in methanolic extract (Table 4-4). The highest antimicrobial activity was recorded in methanolic extract against *B. cereus* (10.00±0.00 mm at 100 mg/ml) whereas the lowest antimicrobial activity was recorded in methanolic extract against *S. mutans* (3.00 ± 0.00 mm at 50 mg/ml).

The MIC value for ethanolic extract was 50 mg/ml against *B. cereus*. For methanolic extract, the MIC values were 25 mg/ml against *B. cereus* and 50 mg/ml against *S. mutans*. Overall it was observed that antimicrobial activity was present in ethanolic and methanolic extracts but absent in aqueous extract of *V. amygdalina* (**Appendix D)**.

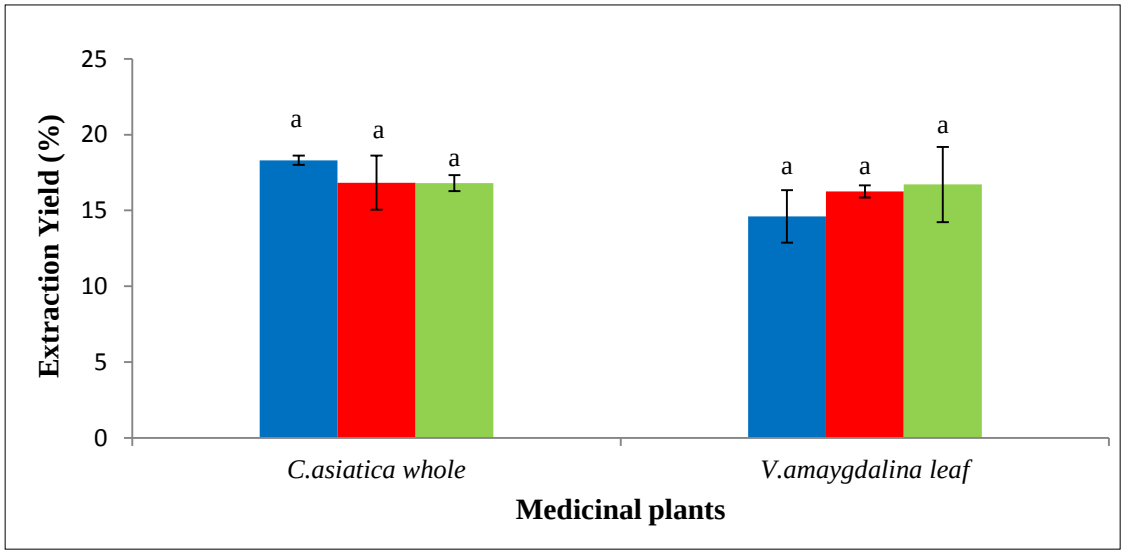

Figure 4- 5: The yield of extract in percentage for *C. asiatica* and *V. amygdalina*.
Yield of in aqueous ( ), ethanol ( ), and methanol ( ) extracts were determined based on the dry weight of the plant parts. Data was presented as mean ± SD, n = 3. The mean changes between the extracts for each plant were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test. Samples represented by same alphabet indicated statistical insignificant difference between samples ($p > .05$).

Table 4- 3: Antimicrobial Activity of *C. asiatica* using well diffusion assay

Test microorganism	Diameter of the zone of inhibition (mm)[a]															
	Positive control[b]	Negative control[c]	Ethanol (mg/ml)					Methanol (mg/ml)					Aqueous (mg/ml)				
			10	25	50	100	MIC[d] (mg/ml)	10	25	50	100	MIC[d] (mg/ml)	10	25	50	100	MIC[d] (mg/ml)
B. cereus	16.67±0.58 ***	<0.5±0.00	NI	3.00±0.00 **	5.33±0.58 ***	8.33±0.58 ***	25	NI	3.67±0.58 **	7.33±0.58 ***	9.00±0.00 ***	25	NI	3.00±0.00 **	5.67±0.58 ***	10.0±0.00 ***	25
E. coli	22.33±0.58 ***	<0.5±0.00	NI	4.67±0.58 **	6.33±1.15 ***	9.33±0.58 ***	25	NI	4.00±0.00 ***	6.00±0.00 ***	8.00±0.00 ***	25	NI	5.67±0.58 ***	7.33±0.58 ***	10.0±0.00 ***	25
P. aeruginosa	NI	<0.5±0.00	NI	3.00±0.00 *	6.00±1.00 ***	8.67±0.58 ***	25	NI	NI	5.00±0.00 ***	6.33±0.58 ***	50	NI	NI	6.33±0.58 ***	8.33±1.15 ***	50
S. aureus	27.33±1.15 ***	<0.5±0.00	NI	NI	9.00±0.00 ***	13.00±0.00 ***	50	NI	NI	5.00±0.00 ***	8.67±0.58 ***	50	NI	NI	10.0±0.00 ***	14.0±0.00 ***	50
S. mutans	24.67±0.58 ***	<0.5±0.00	NI	4.00±0.00 **	7.00±0.00 ***	9.67±1.15 ***	25	NI	2.00±0.00 ns	4.00±0.00 ***	7.33±0.58 ***	25	NI	3.00±0.00 ns	15.33±1.15 ***	18.00±1.73 ***	25

Table displays the antimicrobial activity of ethanolic, methanolic and aqueous extracts of *C. asiatica* whole plant against five test microorganism *B. cereus*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. mutans* in trplicate at different concentration of sample (10, 25, 50 100 mg/ml) as determined by well diffusion assay. Each well was filled with 50 µl of extract. [a]Diameter of the zone of inhibition in exclusion of 6 mm well diameter in mm; [b]Positive control - 2.5mg/ml tetracycline; [c]Negative control - isotonic phosphate buffer, pH 7.4 for ethanol and methanol extract and distilled water for aqueous extract; [d]MIC – Minimum Inhibitory Concentration (mg/ml); NI - no inhibition. Results were presented as mean ± SD. n = 3. The mean changes between the extracts and negative control were analysed by one-way ANOVA followed by Dunnett's Multiple Comparison Test.
$p < .001$ – highly significant***; $p < .01$ -very significant**; $p < .05$ – significant*; ns - not significant.

Table 4- 4: Antimicrobial Activity of *V. amygdalina* using well diffusion assay

Test microorganism	Diameter of the zone of inhibition (mm)[a]															
	Positive control[b]	Negative control[c]	Ethanol (mg/ml)					Methanol (mg/ml)					Aqueous (mg/ml)				
			10	25	50	100	MIC[d] (mg/ml)	10	25	50	100	MIC[d] (mg/ml)	10	25	50	100	MIC[d] (mg/ml)
B. cereus	16.67±0.58 ***	<0.5±0.00	NI	NI	4.00±0.00 ***	6.67±0.58 ***	50	NI	5.00±0.00 ***	8.33±0.58 ***	10.00±0.00 ***	25	NI	NI	NI	NI	-
E. coli	22.33±0.58 ***	<0.5±0.00	NI	NI	NI	NI	-	NI	NI	NI	NI	-	NI	NI	NI	NI	-
P. aeruginosa	NI	<0.5±0.00	NI	NI	NI	NI	-	NI	NI	NI	NI	-	NI	NI	NI	NI	-
S. aureus	27.33±1.15 ***	<0.5±0.00	NI	NI	NI	NI	-	NI	NI	NI	NI	-	NI	NI	NI	NI	-
S. mutans	24.67±0.58 ***	<0.5±0.00	NI	NI	NI	NI	-	NI	NI	3.00±0.00 **	4.00±0.00 ***	50	NI	NI	NI	NI	-

Table displays the antimicrobial activity of ethanolic, methanolic and aqueous extracts of *V. amygdalina* leaf against five test microorganism *B. cereus*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. mutans* in trplicate at different concentration of sample (10, 25, 50 100 mg/ml) as determined by well diffusion assay. Each well was filled with 50 µl of extract. [a]Diameter of the zone of inhibition is presented in exclusion of 6 mm well diameter in mm; [b]Positive control - 2.5mg/ml tetracycline; [c] Negative control - isotonic phosphate buffer, pH 7.4 for ethanol and methanol extract and distilled water for aqueous extract; [d]MIC – Minimum Inhibitory Concentration (mg/ml); NI is no inhibition. Results were presented as mean ± SD, n = 3. The mean changes between the extracts and negative control were analysed by one-way ANOVA followed by Dunnett's Multiple Comparison Test.
$p < .001$ - highly significant***; $p < .01$ -very significant**; $p < .05$ – significant*; ns - not significant.

4.5 Antimicrobial Activity after Ammonium Sulphate Precipitation of Ethanolic Extracts.

After 100% ammonium sulphate precipitation of ethanolic extracts, the pellet and supernatant were tested for their antimicrobial activity against the five test microorganisms (*B. cereus*, *E. coli*, *S. aureus*, *P. aeruginosa* and *S. mutans*, Figure 4-6). Results of the assay indicated that the supernatant for both the tested plant extract showed various level of antimicrobial activity while ammonium sulphate precipitates had relatively low or no antimicrobial activity.

For *C. asiatica*, it was observed that the supernatant showed the highest antimicrobial activity towards *B. cereus* (14.00±1.73 mm) and *S. aureus* (14.00±0.00 mm) followed by *E.coli* (13.33±1.53 mm), and *P. aeruginosa* (12.67±0.58 mm) and no activity towards *S. mutans*. It was interesting to note that there was significant antimicrobial activity ($p < .05$) of *C. asiatica* towards *P. aeruginosa* compared to the standard antibiotic, tetracycline that had no antimicrobial activity towards *P. aeruginosa* at 2.5mg/ml concentration (Figure 4-6A). The pellet showed low activity against *E. coli* (4.33±0.58 mm) followed by *P. aeruginosa* (3.67±0.58 mm) and no activity against *B. cereus*, *S. aureus* and *S. mutans*.

Supernatant of *V. amygdalina* showed antimicrobial activity towards *B. cereus* (5.00 ±0.00 mm) and pellet showed lower activity against the same test microorganism (4.67±0.58 mm). No antimicrobial activity was observed against the other 4 test microorganisms (Figure 4-6B). Figure 4-3C shows the zone of inhibition for the well diffusion assay.

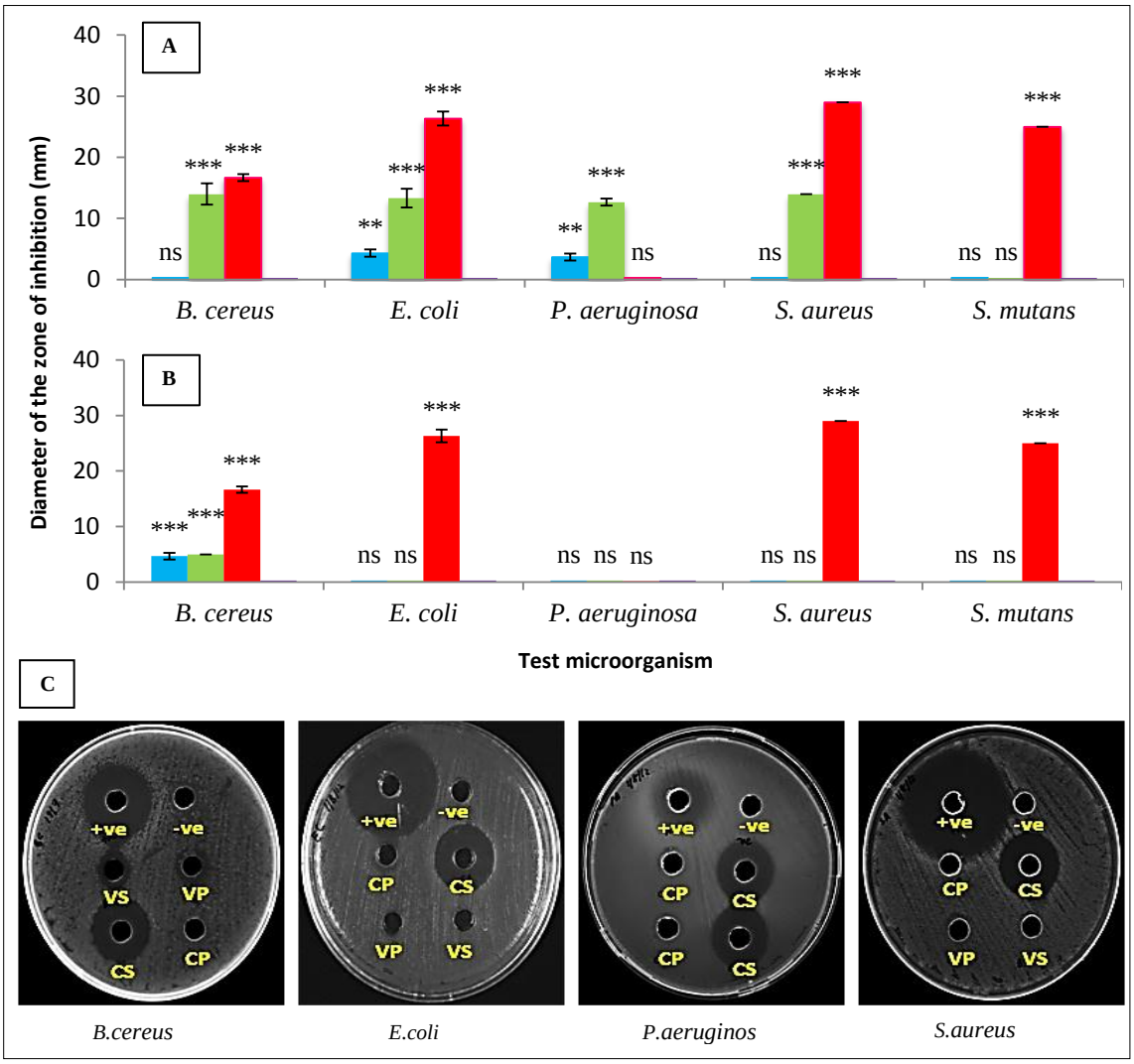

Figure 4- 6: Antimicrobial activity of pellet and supernatant obtained from ammonium sulphate precipitation by well diffusion assay of *C. asiatica* (A) and *V. amygdalina* (B) ethanolic extracts and their images (C).

Figures A and B displays the antimicrobial activity of pellet and supernatant for the ethanolic extract of *C. asiatica* and *V. amygdalina* against test microorganisms where diameter of the zone of inhibition for the pellet [ ], supernatant [ ] , positive control [ ] and negative control [ ] excluded 7 mm well diameter. Each well was filled with 50 µl of extract. Positive control - 2.5 mg/ml tetracycline. Negative control – saturated ammonium sulphate solution. Results were presented as mean ± SD, n = 3. The mean changes between the samples and negative control were analysed by one-way ANOVA followed by Dunnett's Multiple Comparison Test ($p < .001$ - highly significant***; $p < .01$ -very significant**; $p < .05$ – significant*; ns - not significant).

Figure C displays the zone of inhibition for the antimicrobial activity of pellet and supernatant of the selected medicinal plants. +ve – Positive control (tetracycline 2.5mg/ml); -ve- Negative control (saturated ammonium sulphate solution); CP- *C. asiatica* pellet; CS – *C. asiatica* supernatant; VP – *V. amygdalina* pellet and VS – *V. amaygdalina* supernatant.

4.6 Total Phenolic Content (TPC) in *C. asiatica* and *V. amygdalina*

TPC was determined by Folin-Ciocalteu's method (Section 3.7) for aqueous, ethanolic and methanolic extracts of the plant of study by reference to a gallic acid standard curve (y = 0.0006x + 0.0017, R^2 = 0.9961 **Appendix F** Figure 6). TPC was expressed as milligrams of gallic acid equivalent per gram of dry weight of extract (mg GAE/g d.w.).

Significant difference ($p < .05$) was observed in the TPC of *C. asiatica* extracted using different solvents. The ethanolic extract contained the highest amount of phenolics (4.006±0.032 mg GAE/g d.w.) followed by methanolic extract (3.346±0.029 mg GAE/g d.w.). The lowest amount of phenol was in aqueous extract (1.120±0.063 mg GAE/g d.w).

The TPC in *V. amygdalina* was lower than *C. asiatica* for all the extracts tested. The highest TPC was in the methanolic extract (1.168±0.101 mg GAE/g d.w) while the lowest TPC was observed in ethanolic and aqueous extract which showed no significance ($p > .05$) from each other (Figure 4-7 and **Appendix F** Table 1).

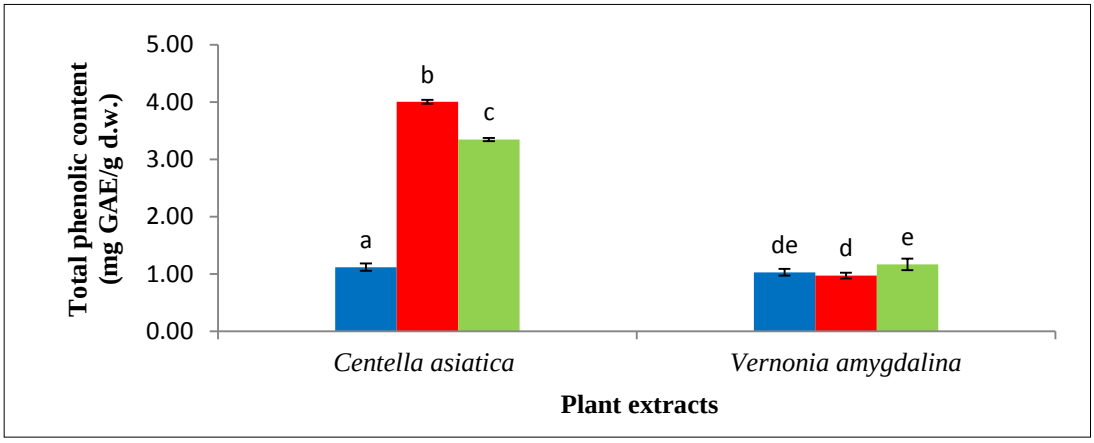

Figure 4- 7 : Total phenolic content of extracts determined by Folin-Coicalteu's method
TPC from aqueous ( ), ethanolic ( ) and methanolic ( ) extracts of *C. asiatica* and *V. amygdalina* based on the gallic acid standard curve. TPC was expressed as mg of gallic acid equivalence per gram dry weight of extract (mg GAE/g d.w). The tested concentration of each plant extract was 2.5 mg/ml. Data was presented as mean ± SD, n = 3. The mean changes between the extracts for each plant were analysed separately by one-way ANOVA followed by Tukey's Multiple Comparison Test. Samples represented by different alphabets indicated significant different ($p < .05$).

4.7 Antioxidant Activity in *C. asiatica* and *V. amygdalina*

4.7.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity

Figure 4-8 (**Appendix G** Table 2) shows the DPPH radical scavenging activity in *C. asiatica* and *V. amygdalina* extracts compared with standards BHT and ascorbic acid. Table 4-5 shows the antioxidant activity of the extracts expressed as the concentration required to inhibit the formation of DPPH radicals by 50% (IC_{50}). Non-linear regression curve (**Appendix G** Figure 7b) was plot to determine IC_{50}.

Table 4- 5: IC_{50} (µg/ml) of DPPH radical scavenging assay

Sample	Aqueous extract	Ethanol extract	Methanol extract	Ascorbic acid	BHT
C. asiatica	4039.00±0.03[a]	122.40±0.04[b]	210.70±0.03[c]	67.38±0.10[g]	1290.00±0.05[h]
V. amygdalina	2706.00±0.15[d]	1063.00±0.04[e]	1049.00±0.06[f]		

IC_{50} (µg/ml) of *C. asiatica* and *V. amygdalina* towards DPPH radicals were determined. Ascorbic acid and BHT were the standards used for comparison. Results were expressed as mean ± SD, where n = 3. The mean changes between the extracts and standards were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test. Samples presented by different alphabets were significantly different ($p < .05$) from each other.

Figure 4-8A depicts a steady increase in the inhibition of the formation of DPPH radicals by all the extracts of *C. asiatica* revealing a dose dependant increase in the radical scavenging activity. At the dosage of 400 µg/ml, the radical scavenging activity of *C. asiatica* extracts and standards towards DPPH radical was in the following order: ascorbic acid (90.49±0.60%) > ethanolic extract (88.83±0.23%) > methanolic extract (73.17±0.17%) > BHT (26.34±1.47%) > aqueous extract (8.28±0.74%) at 400 µg/ml dosage. The IC_{50} of ethanolic extract was the lowest (122.40±0.04 µg/ml), lower than standard BHT (1290.00±0.05 µg/ml). The IC_{50} of the aqueous extract was the highest (4039.00±0.03 µg/ml Table 4-5).

At the dosage of 400 µg/ml, the radical scavenging activity of *V. amygdalina* and the standards towards DPPH radical was as follows: ascorbic acid (90.49±0.60%) > BHT (26.34±1.47%) > ethanolic extract (26.07±5.32%) > methanolic extract

(25.97±4.39%) > aqueous extract (5.95±1.37%). The aqueous extract showed unstable scavenging activity towards DPPH radical. Ethanolic and methanolic extracts of *V. amygdalina* showed low DPPH radical scavenging activity compared to ethanolic and methanolic extracts of *C. asiatica*. The IC_{50} for all the tested extracts were relatively high (Table 4-5).

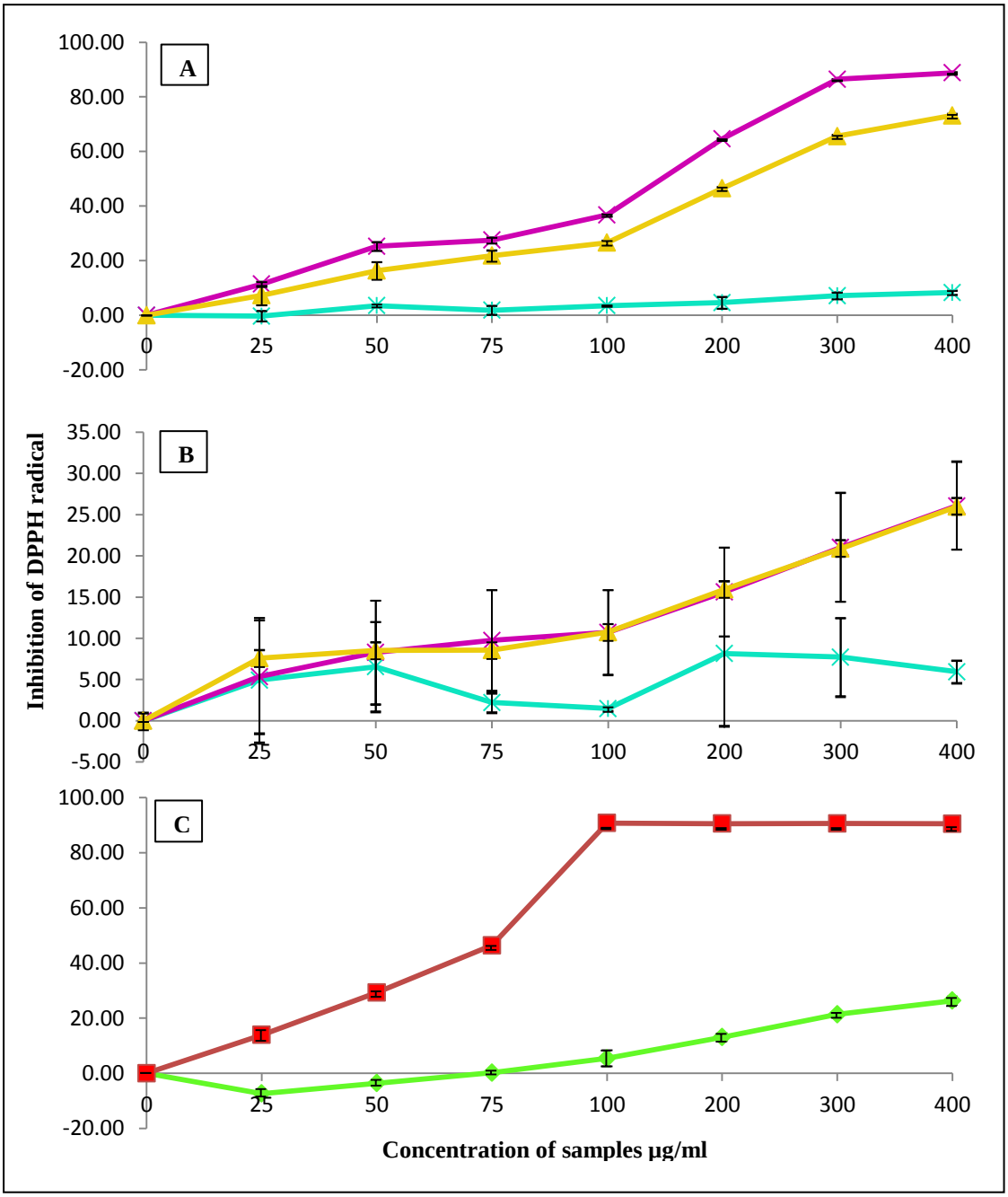

Figure 4- 8: Percentage inhibition of DPPH radical in *C. asiatica* (A), *V. amygdalina* (B) extracts and positive controls (C).

The percentage inhibition of DPPH radical with varying concentration (0-400µg/ml) of aqueous ( ), ethanolic ( ) and methanolic ( ) extracts of *C. asiatica*, *V. amygdalina* and the positive controls ascorbic acid ( ) and BHT ( ) were analysed by measuring the inhibitory effects on DPPH radical at 517 nm. Results are presented as mean ± SD, n = 3.

4.7.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assays were conducted on aqueous, ethanolic and methanolic extracts of *C. asiatica* and *V. amygdalina*. All assays were performed simultaneously and ascorbic acid and BHT were used as the positive controls. Each sample was tested at 0.5 mg/ml and 1.0 mg/ml (Figure 4-9 and 4-10). In the FRAP assay, reductants antioxidants in the sample reduces Fe^{3+}/tripyridyltriazine complex to the blue coloured ferrous form, with an increase in absorbance at 595 nm. The absorbance of the samples and positive control showed gradual increase and reached a plateau by the fourth minute of incubation. For all samples and positive control the absorbance doubled at 1 mg/ml compared to the rate at 0.5 mg/ml (Figure 4-9 and 4-10).

At 1.0 mg/ml concentration, *C. asiatica* methanol extracts and standards were able to reduce ferric ions efficiently with absorbance unit (AU) as follows: ascorbic acid (3.224±0.103 AU at 0 seconds to 3.318±0.021 AU at 4 minutes) > methanolic extract (0.466±0.012 AU at 0 seconds to 0.555±0.012 AU at 4 minutes) > ethanolic extract (0.421±0.011 AU at 0 seconds to 0.502±0.014 AU at 4 minutes) > BHT (0.048±0.017 AU at 0 seconds to 0.179±0.017 AU at 4 minutes) > aqueous extract (0.041±0.003 AU at 0 seconds to 0.096±0.006 AU at 4 minutes Figure 4-9).

Figure 4-10 generally depicts that *V. amygdalina* extracts had lower absorbance compared to *C. asiatica* extracts at the same concentration where only methanolic extracts showed reducing capabilities (0.142±0.020 AU at 0 seconds to 0.222±0.012 AU at 4 minutes) for the same concentration (1.0 mg/ml). Both ethanolic and aqueous extract showed low absorbance 0.093±0.006 AU at 0 seconds to 0.137±0.011 AU at 4 minutes) and (0.041±0.003 AU at 0 seconds to 0.096±0.006 AU at 4 minutes) respectively (**Appendix H** Table 3a and 3b).

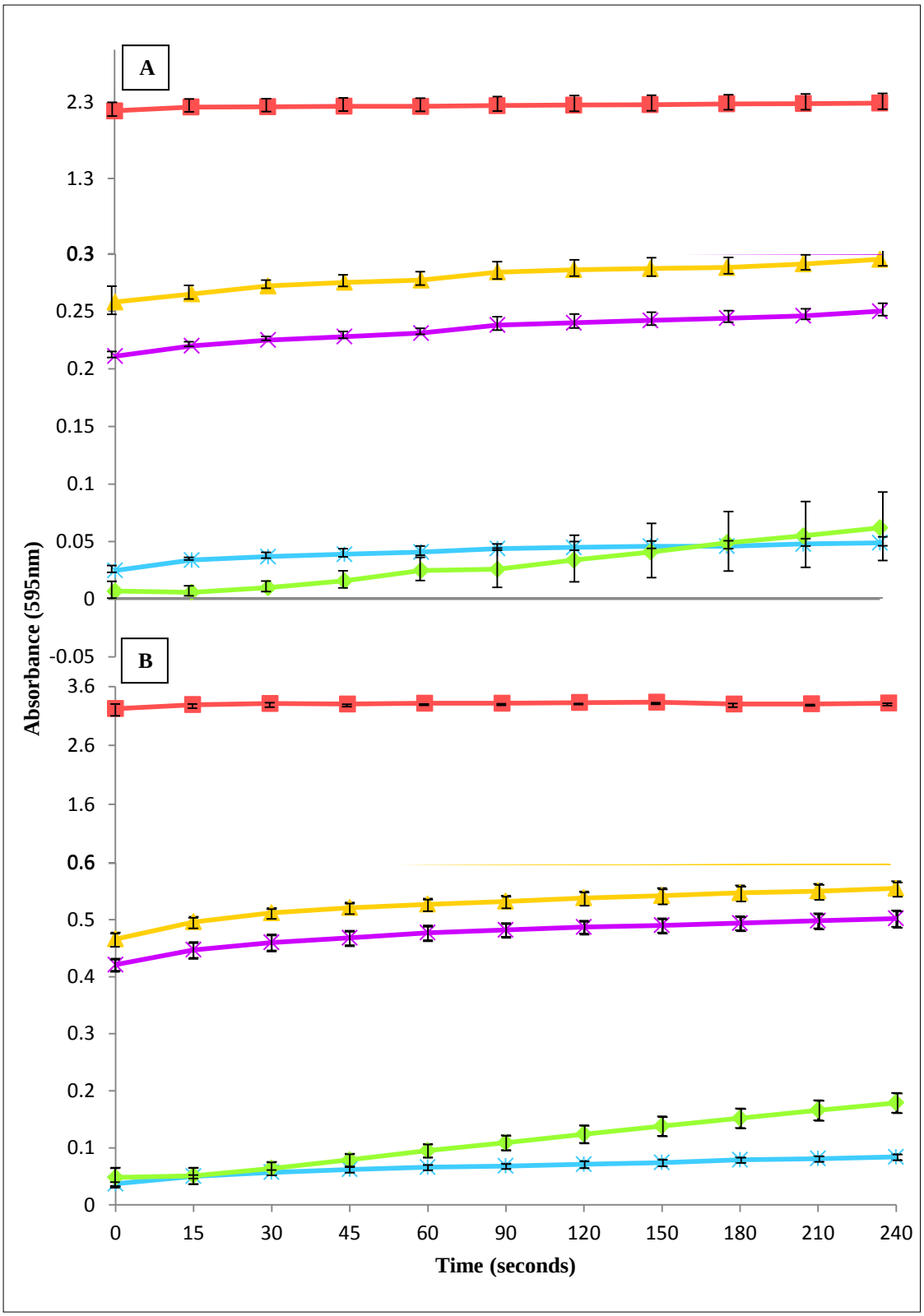

Figure 4- 9: Ferric Reducing Antioxidant Power (FRAP) activity in 0.5 mg/ml (A) and 1 mg/ml (B) *C. asiatica* extracts

Aqueous ( ), ethanol ( ) and methanol ( ) extracts of *C. asiatica* were tested. The reaction time was followed every 15 seconds for 4 minutes and the absorbance was recorded at 595 nm. Ascorbic acid ( ) and BHT ( ) were the standard antioxidant used. Results are an average of 3 readings ± standard deviation.

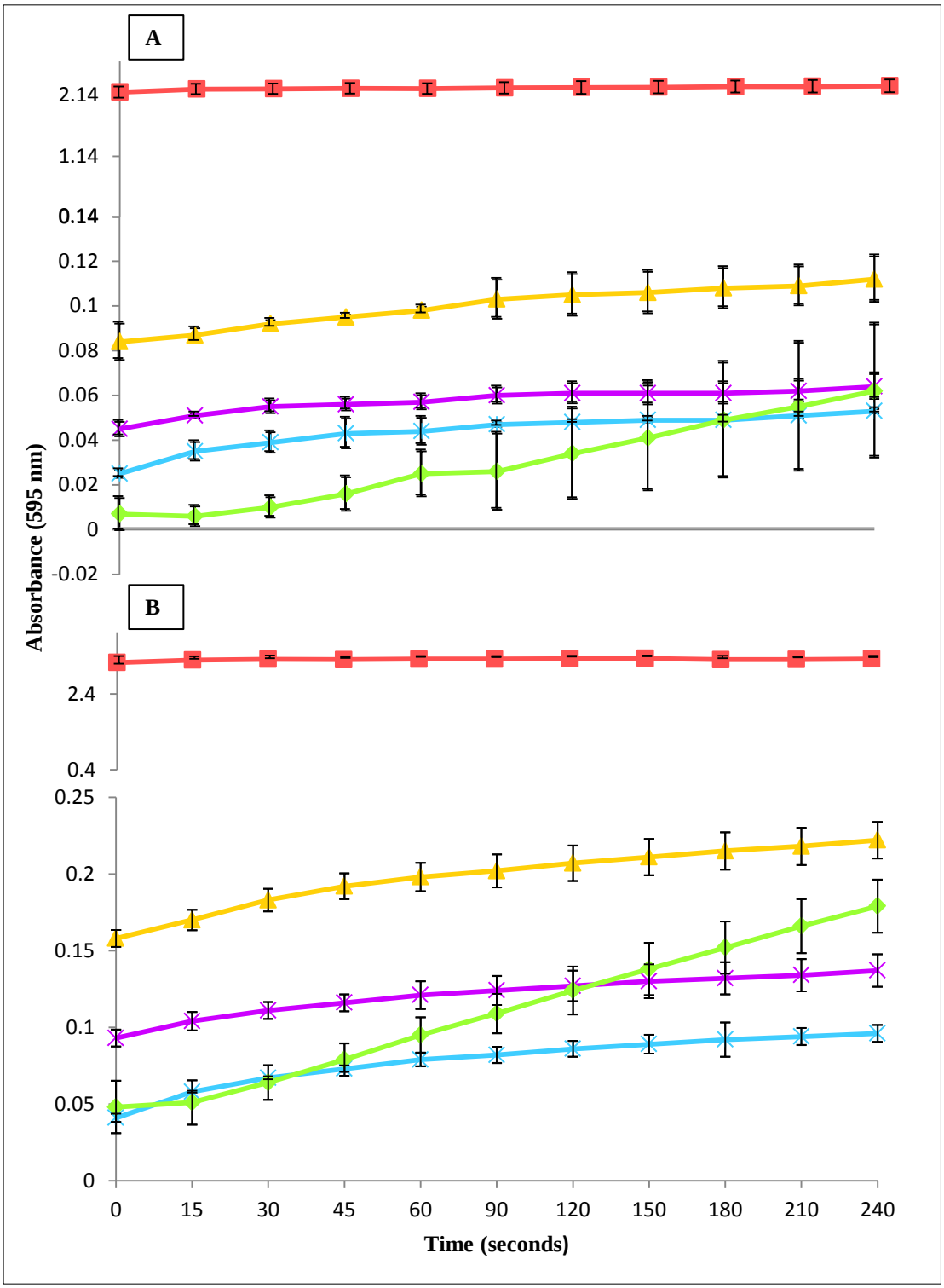

Figure 4- 10: Ferric Reducing Antioxidant Power (FRAP) activity in 0.5 mg/ml (A) and 1 mg/ml (B) *V. amygdalina* extracts.

Aqueous ( ), ethanol ( ) and methanol ( ) extracts of *V. amygdalina* were tested. The reaction time was followed every 15 seconds for 4 minutes and the absorbance was recorded at 595 nm. Ascorbic acid ( ) and BHT ( ) were the standard antioxidant used. Results are an average of 3 readings ± standard deviation.

4.7.3 Protective Effect of Extracts of *C. asiatica* and *V. amygdalina* Against Hydrogen Peroxide (H_2O_2) Induced Haemolysis.

It was observed that the aqueous, ethanolic and methanolic extracts of *C. asiatica* and *V. amygdalina* thereafter did not exhibit any harmful effect towards the rabbit erythrocytes after pre-incubation of the extracts with erythrocytes for 1 hour. Interestingly, it was found that the extracts inhibited haemolysis at various concentrations (0.1 – 1.0 mg/ml) through *in vitro* haemolysis assays. The assays were conducted for 18 hours to evaluate the protective effects of extracts against H_2O_2 (10 mM and 50 mM) induced haemolysis.

Table 4-6 shows the concentration (mg/ml) required to inhibit haemolysis of red blood cells by 50% (IC_{50}), whereby 10 mM and 50 mM H_2O_2 was used to induce haemolysis. Non-linear regression curves (**Appendix I** Figures 9a and 9b) were used to determine IC_{50}. The percentage of haemolysis was based on 100% haemolysis of distilled water. The negative control used was isotonic phosphate buffer which displayed 72.44±1.04% and 57.13±0.80% protective effect against 10 mM and 50 mM H_2O_2 induced haemolysis respectively.

Inhibition of haemolysis by the extracts of *C. asiatica* and standards at 1 mg/ml dosage against 10 mM H_2O_2 were as follows: methanolic extract (88.14±0.83%) > ascorbic acid (86.05±0.66%) > ethanolic extract (85.12±1.69%) > aqueous extract (76.47±0.79%). Inhibition of the extracts at the same dosage against 50 mM H_2O_2 was as follows: methanolic extract (91.81±0.55%) > ethanolic extract (84.73±0.65%) > ascorbic acid (71.97±0.76%) ≈ aqueous extract (71.61±0.53%; Figure 4-11 and **Appendix J** Tables 4a and 4b).

All extracts showed low IC_{50} values against 10 mM H_2O_2 and were compatible with ascorbic acid. IC_{50} value against 50 mM H_20_2 was lowest for both ethanolic and

methanolic extract (0.1 mg/ml) which was comparable with that of ascorbic acid. However, aqueous extract showed a higher value (0.29 mg/ml Table 4-6).

Table 4- 6: IC_{50} (mg/ml) values of the protective effect of extracts of *C. asiatica* and *V. amygdalina* against H_2O_2 induced red blood cell lysis.

Sample	IC_{50} against 10 mM H_2O_2 (mg/ml)	IC_{50} against 50 mM H_2O_2 (mg/ml)
C. asiatica aqueous extract	0.13 ± 0.08^{a}	0.29 ± 0.04^{cf}
C. asiatica ethanolic extract	0.11 ± 0.04^{a}	0.10 ± 0.04^{d}
C. asiatica methanolic extract	0.11 ± 0.03^{a}	0.10 ± 0.04^{d}
V. amygdalina aqueous extract	0.28 ± 0.04^{b}	0.31 ± 0.04^{ef}
V. amygdalina ethanolic extract	0.14 ± 0.06^{a}	0.11 ± 0.07^{d}
V. amygdalina methanolic extract	0.22 ± 0.03^{ab}	0.13 ± 0.08^{d}
Ascorbic acid	0.11 ± 0.04^{a}	0.12 ± 0.08^{d}

IC_{50} of *C. asiatica* and *V. amygdalina* towards 10 mM and 50 mM H_2O_2 were determined by non-linear regression curve. Ascorbic acid was the standard used for comparison. Results were presented as mean ± SD of three replicates. The mean changes between the extracts and standard were analysed separately against 10 mM and 50 mM H_2O_2 by one-way ANOVA followed by Tukey's Multiple Comparison Test. Values represented by different alphabets within columns indicated significant differences ($p < .05$).

Inhibition of haemolysis by the extracts of *V. amygdalina* and standards at 1 mg/ml dosage against 10 mM H_2O_2 were as follows: ascorbic acid (86.05±0.66%) > methanolic extract (79.25±0.64%) > ethanolic extract (78.46±0.35%) > aqueous extract (72.92±0.77%). At the same dosage, inhibition against 50 mM H_2O_2 were as follows: methanolic extract (81.77±0.23%) > ethanolic extract (78.10±0.26%) > ascorbic acid (71.97±0.76%) > aqueous extract (57.55±1.46% Figure 4-12 and **Appendix I** Tables 4a and 4b).

The IC_{50} against 50 mM H_2O_2 was lowest for the ethanolic extract (0.11±0.07 mg/ml) and was comparable with ascorbic acid (0.12±0.08 mg/ml) whereas the highest IC_{50} was recorded in the aqueous extract (0.31±0.04 mg/ml Table 4-6). Overall, for increasing concentration of extracts, an increasing pattern was observed in the protective effect of methanolic and ethanolic extracts whereas aqueous extract showed varying protective effect (Figure 4-12).

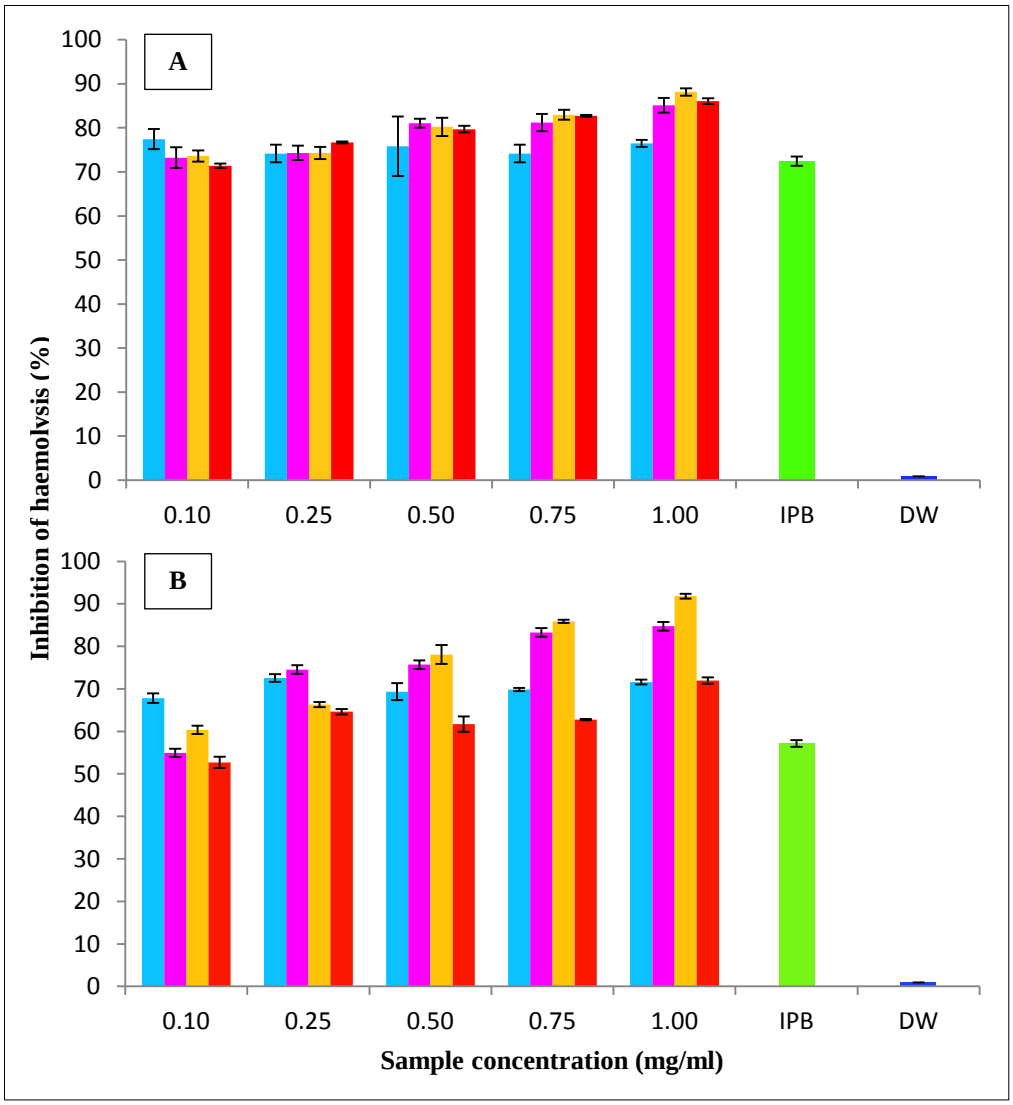

Figure 4- 11: *In vitro* protective effect of *C. asiatica* against 10 mM (A) and 50 mM (B) H_2O_2 induced haemolysis of rabbit erythrocytes.

Aqueous ( ), ethanol ( ) and methanol ( ) extract of *C. asiatica* and positive control - ascorbic acid ( ) were tested at (0.1, 0.25, 0.5, 0.75 and 1.0 mg/ml) concentration. Protective effect of distilled water ( ) and negative control – IPB ( ) were displayed. Absorbance was measured at 540 nm. Percentages of haemolysis were based on 100% haemolysis of water. Results were presented as mean ± SD whereby n = 3.

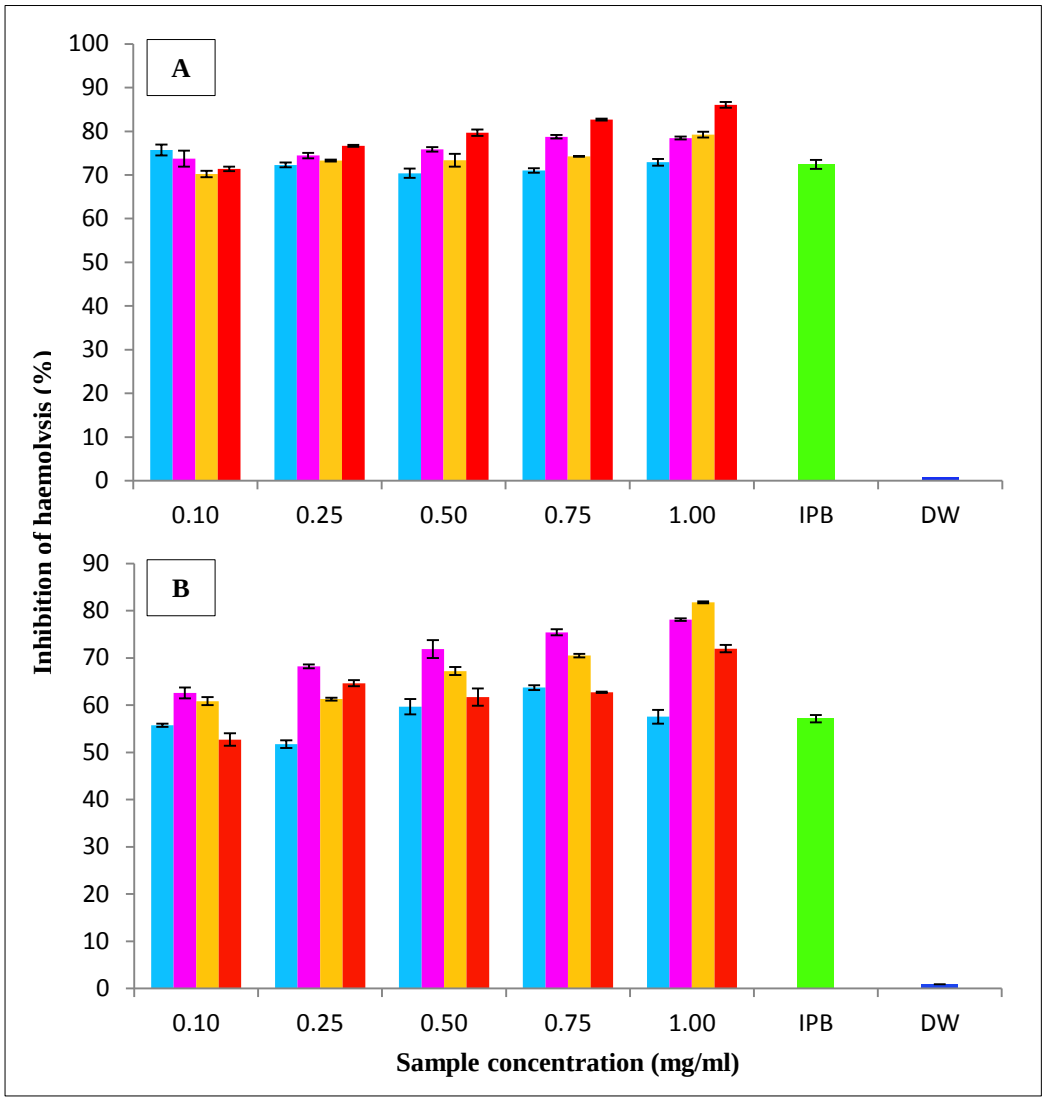

Figure 4- 12: *In vitro* protective effects of *V. amygdalina* against 10 mM (A) and 50 Mm (B) H_2O_2 induced haemolysis of rabbit erythrocytes.

Aqueous (■), ethanol (■) and methanol (■) extract of *V. amygdalina* and positive control - ascorbic acid (■) were tested at (0.1, 0.25, 0.5, 0.75 and 1.0 mg/ml) concentration. Protective effect of distilled water (■) and negative control – IPB (■) were displayed. Absorbance was measured at 540 nm. Percentages of haemolysis were based on 100% haemolysis of water. Results were presented as mean ± SD whereby n = 3.

4.7.4 Antioxidant capacity of *C. asiatica* and *V. amygdalina* extracts

Antioxidant capacity of extracts and positive control were determined by three methods. Firstly, using the DPPH assay by comparing with Trolox standard curve (**Appendix G** Figure 7a) and expressed as milimole Trolox equivalent (TE) antioxidant capacity per gram dry weight sample (mmol TE/g d.w.) as described in Section 3.8.1.3. Secondly, using the FRAP assay by comparing with ferrous sulphate (FeSO4.7H2O) standard curve in a 0 - 4 minute reaction time (**Appendix H** Figure 8) and expressed as milimole ferrous sulphate (Fe^{2+}) per gram of dry weight (mmole Fe^{2+}/g dry weight) as described in Section 3.8.2.3. Thirdly using anti-haemolysis assay by comparing with ascorbic acid standard curve (**Appendix I** Figure 9c) and expressed as milimole ascorbic acid equivalent (AAE) per gram dry weight plant sample (mmol AAE/g d.w.) described in Section 3.8.3.3.

The DPPH antioxidant capacity of C. asiatica displayed that ethanolic extract had the highest antioxidant activity (1.8824±0.0062 mmol AAE/g d.w.) being more potent than aqueous extract but had similar potency to methanolic extract. Ethanolic extract had a higher potency compared to BHT (Table 4-7).

In the FRAP assay, 1 mg/ml of samples and controls were chosen to determine the antioxidant capacity. The ferric reducing activity in methanolic extract of *C. asiatica* was the highest followed by ethanolic extract. The activity was higher compared to ascorbic acid and lower than BHT (positive controls). Aqueous extract had the lowest reducing activity (Table 4-7).

Results of anti-haemolysis antioxidant capacity in *C. asiatica*, proved that methanolic extract possess the strongest activity (13.7316±0.2052 mmol AAE/g d.w.) being stronger than the control ascorbic acid (6.3724±0.2806 mmol AAE/g d.w.). The weakest activity was observed in aqueous extracts (6.1151±0.4082 mmol AAE/g d.w. (Table 4-7).

Table 4- 7: Antioxidant Capacity of *C. asiatica* extracts and controls.

Samples	[A]DPPH (mmol TE/g d.w.)	[B]FRAP (mmol Fe^{2+}/g d.w.)	[C]anti-haemolysis (mmol AAE/g d.w.)
C. asiatica aqueous extract	0.0000±0.0000[a]	0.1567±0.0285[ab]	6.1151±0.4082[a]
C. asiatica ethanol extract	1.8824±0.0062[b]	0.2711±0.0482[ad]	11.1076±0.2394[b]
C. asiatica methanol extract	1.4582±0.0192[c]	0.2967±0.0731[d]	13.7316±0.2052[c]
Ascorbic acid	7.7364±0.0329[d]	0.0922±0.0367[b]	6.3724±0.2806[a]
BHT	0.1895±0.0398[e]	0.4356±0.0051[c]	-

Table displays the antioxidant activity of aqueous, ethanolic and methanolic extracts. Ascorbic acid and BHT were standards used for comparison. Results were expressed as mean ± SD where n = 3. The mean changes between the samples for each test were analysed separately by one-way ANOVA followed by Tukey's Multiple Comparison Test. Values represented by different alphabets within columns indicated significant different ($p < .05$). **[A]DPPH** presented as milimole Trolox equivalent per gram dry weight (mmol TE/g d.w.). **[B]FRAP** presented as milimole ferrous sulphate per gram dry weight (mmol Fe^{2+}/g d.w.). **[C]Anti-haemolysis** presented as milimole ascorbic acid equivalent per gram dry weight (mmol AAE/g d.w.).

Studies on the extracts of *V. amygdalina* through DPPH antioxidant capacity, revealed that ethanolic and methanolic extracts possess similar antioxidant potency. It was interesting to note that the antioxidant potency was also similar to that of the BHT. However, there was no antioxidant activity in aqueous extract (Table 4-8). Overall it was discovered that the antioxidant activity was lower in *V. amygdalina* extracts compared to *C. asiatica* extracts.

Through the FRAP assay, methanolic extract of *V. amygdalina* was able to reduce ferric ions effectively and was higher than ascorbic acid. Aqueous extract had low reducing capability whereas ethanolic extract had the lowest reducing capacity (Table 4-8). Overall the methanolic and ethanolic extracts of *V. amygdalina* had lower reducing capacity but aqueous extract had higher reducing capacity when compared to *C. asiatica*.

Antioxidant capacity in anti-haemolysis assay showed that methanolic extract of *V. amygdalina* was the most potent extract (10.0081±0.0845 mmol AAE/g d.w.) in inhibiting haemolysis. This was followed by the ethanolic extract (8.6454±0.0980 mmol AAE/g d.w.), while the lowest potency was observed in aqueous extract (1.0241±0.5429 mmol AAE/g d.w.), being lower than ascorbic acid (Table 4-8).

Table 4- 8: Antioxidant Capacity of *V. amygdalina* extracts and controls.

Samples	[A]DPPH (mmol TE/g d.w.)	[B]FRAP (mmol Fe^{2+}/g d.w.)	[C]anti-hemolysis (mmol AAE/g d.w.)
V. amygdalina aqueous extract	0.0000 ± 0.0000^{abd}	0.1822 ± 0.0255^{abc}	1.0241 ± 0.5429^{a}
V. amygdalina ethanol extract	0.1823 ± 0.1441^{aef}	0.1467 ± 0.0176^{ae}	8.6454 ± 0.0980^{b}
V. amygdalina methanol extract	0.1797 ± 0.1189^{beg}	0.2656 ± 0.0769^{b}	10.0081 ± 0.0845^{c}
Ascorbic acid	7.7364 ± 0.0329^{c}	0.0922 ± 0.0367^{ce}	6.3724 ± 0.2806^{d}
BHT	0.1895 ± 0.0398^{dfg}	0.4356 ± 0.0051^{d}	-

Table displays the antioxidant activity of aqueous, ethanolic and methanolic extracts. Ascorbic acid and BHT were standards used for comparison. Results were expressed as mean ± SD where n = 3. The mean changes between the samples for each test were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test. Values represented by different alphabets within columns indicated significantly different ($p < .05$). **[A]DPPH** presented as milimole Trolox equivalent per gram dry weight (mmol TE/g d.w.). **[B]FRAP** presented as milimole ferrous sulphate per gram dy weight (mmol Fe^{2+}/g d.w.). **[C]Anti-haemolysis** presented as milimole ascorbic acid equivalent per gram dry weight (mmol AAE/g d.w.).

4.8 Relationship between the Phenolic Compounds with the Antimicrobial and Antioxidant Activity of *C. asiatica* and *V. amygdalina* Extracts.

4.8.1 Relationship between the Phenolic Compounds and Antimicrobial Activity of Plant Extracts

Correlation analysis was performed to compare the relationship between total phenolic content (TPC) and antimicrobial activities between (i) aqueous, ethanolic and methanolic extracts of each plant and (ii) ethanolic extract of both the plants in this study using regression analysis.

Table 4- 9: Correlation coefficient (r) between [a]total phenolic content (TPC) and [b]antimicrobial activity of *C. asiatica* and *V. amygdalina*.

Plant of study	TPC vs Antimicrobial activity				
	B. cereus	***E. coli***	***P. aeruginosa***	***S. aureus***	***S. mutans***
[c]*C. asiatica*	-0.9151***	0.4978^{ns}	-0.0955^{ns}	0.2659^{ns}	-0.8876**
[c]*V. amygdalina*	0.4412^{ns}	-	-	-	0.7723*
[d]*C. asiatica* and *V. amygdalina*	0.8720*	0.9967***	0.9974***	0.9997***	0.7994^{ns}

Table displays the correlation coefficient, r between TPC and antimicrobial activity of *C. asiatica* and *V. amygdalina* between (i) extracts of each plant and (ii) both plants. Statistical significant between correlation coefficient was determined by Pearson correlation analysis for two-tailed p value. $p < .001$ - highly significant***; $p < .01$ -very significant**; $p < .05$ – significant*; ns - not significant. " – " indicated no antimicrobial activity. [a]Total phenolic content (TPC) - milligram gallic acid equivalent per gram dry weight (mg GAE/g d.w.). [b]Antimicrobial activity – Diameter of zone of inhibition (mm). [c]Correlation between aqueous, ethanolic and methanolic extracts of each plant. [d]Correlation between *C. asiatica* and *V. amygdalina ethanolic* extract.

C. asiatica displayed weak correlation between the antimicrobial activity and TPC of the three aqueous, ethanolic and methanolic extracts against each bacteria. The r values were between 0.4978 and -0.0955 and decreased in the following order: *E. coli* (r = 0.4978) > *S. aureus* (r = 0.2659) > *B. cereus* (r = -0.9151) > *S. mutans* (r = -0.8876) > *P. aeruginosa* (r = -0.0955). The negative correlation coefficient indicated inverse relationship between phenolic content and antimicrobial activity whereby extracts with the highest phenolic content displayed lowest antimicrobial activity (Table 4-9 and Figure 4-13).

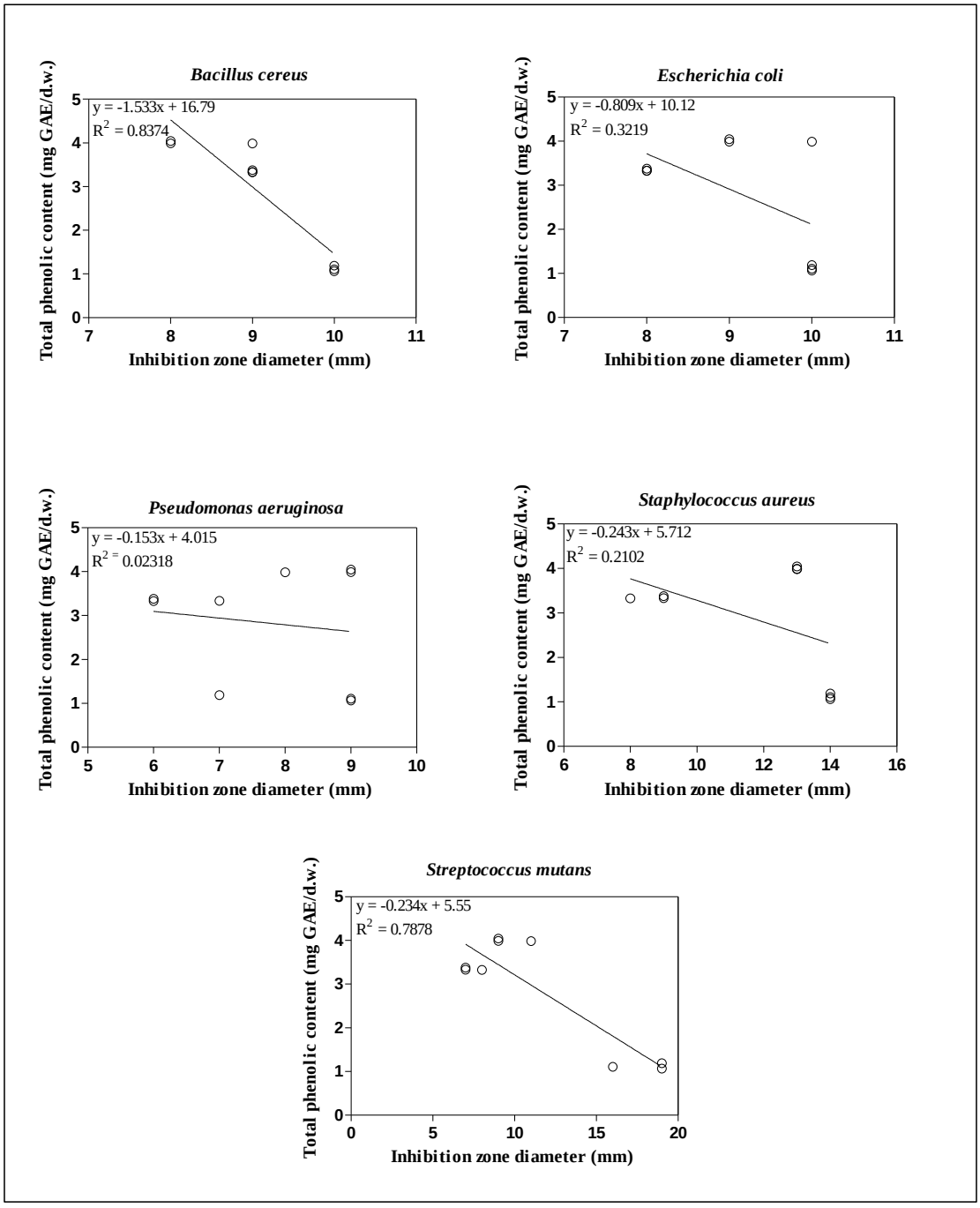

Figure 4- 13: Relationship between total phenolic content and antimicrobial activity of *C. asiatica* extracts

Graph displays the linear correlation between diameter of the zone of inhibition (mm) of the five test microorganisms (*B. cereus*, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. mutans*) and total phenolic content (milligram gallic acid equivalent per gram dry weight mg GAE/g d.w) of aqueous, ethanolic and methanolic extracts of *C. asiatica*. Results were displayed as mean ± SD, n = 3. Coefficient of determinant (R^2) - measures how well the regression line represents the data.

Higher correlation between the antimicrobial activity and TPC was detected in extracts of *V. amygdalina*. However, antimicrobial activity was displayed only towards *B. cereus* and *S. mutans* and not towards *E. coli*, *P. aureus* and *S. aureus*. The correlation coefficients obtained were (r = 0.7723; R^2 = 0.5964) and (r = 0.4412; R^2 = 0.1946) for *S. mutans* and *B. cereus* respectively (Table 4-9 and Figure 4-14).

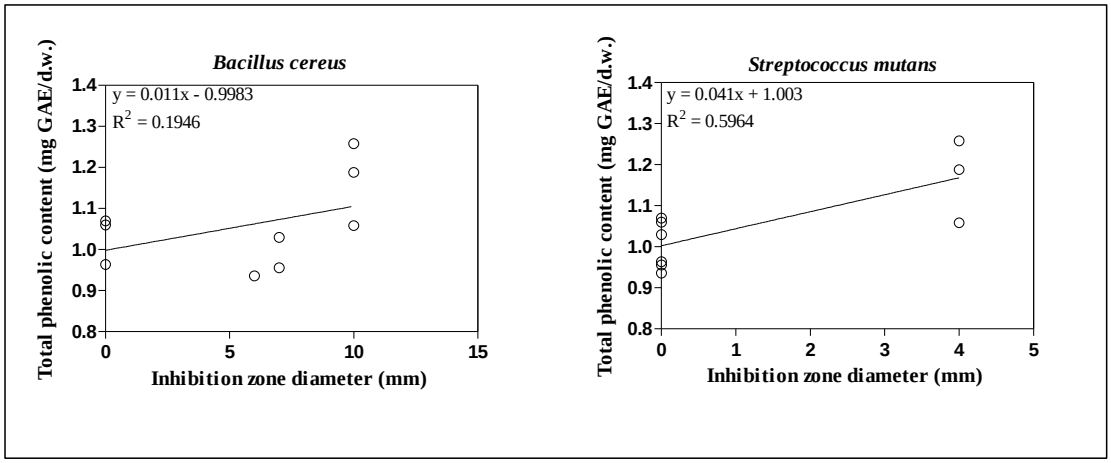

Figure 4- 14: Relationship between total phenolic content and antimicrobial activity of *V. amygdalina* extracts
Graph displays the linear correlation between diameter of the zone of inhibition (mm) of the two test microorganisms (*B. cereus* and *S. mutans*) and total phenolic content (milligram gallic acid equivalent per gram dry weight mg GAE/g d.w) of aqueous, ethanolic and methanolic extracts of *V. amygdalina*. Results were displayed as mean ± SD, n = 3. Coefficient of determinant (R^2) - measures how well the regression line represents the data.

Strong correlations were observed between the antimicrobial activity TPC when compared between ethanolic extract of *C. asiatica* and *V. amygdalina*. The r values obtained were between 0.9997 and 0.7994 and decreased in the following order: *S. aureus* (r = 0.9997) > *P. aeruginosa* (r = 0.9974) > *E. coli* (r = 0.9967) > *B. cereus* (r = 0.8720) > *S. mutans* (r = 0.7794; Table 4-9, Figure 4-15).

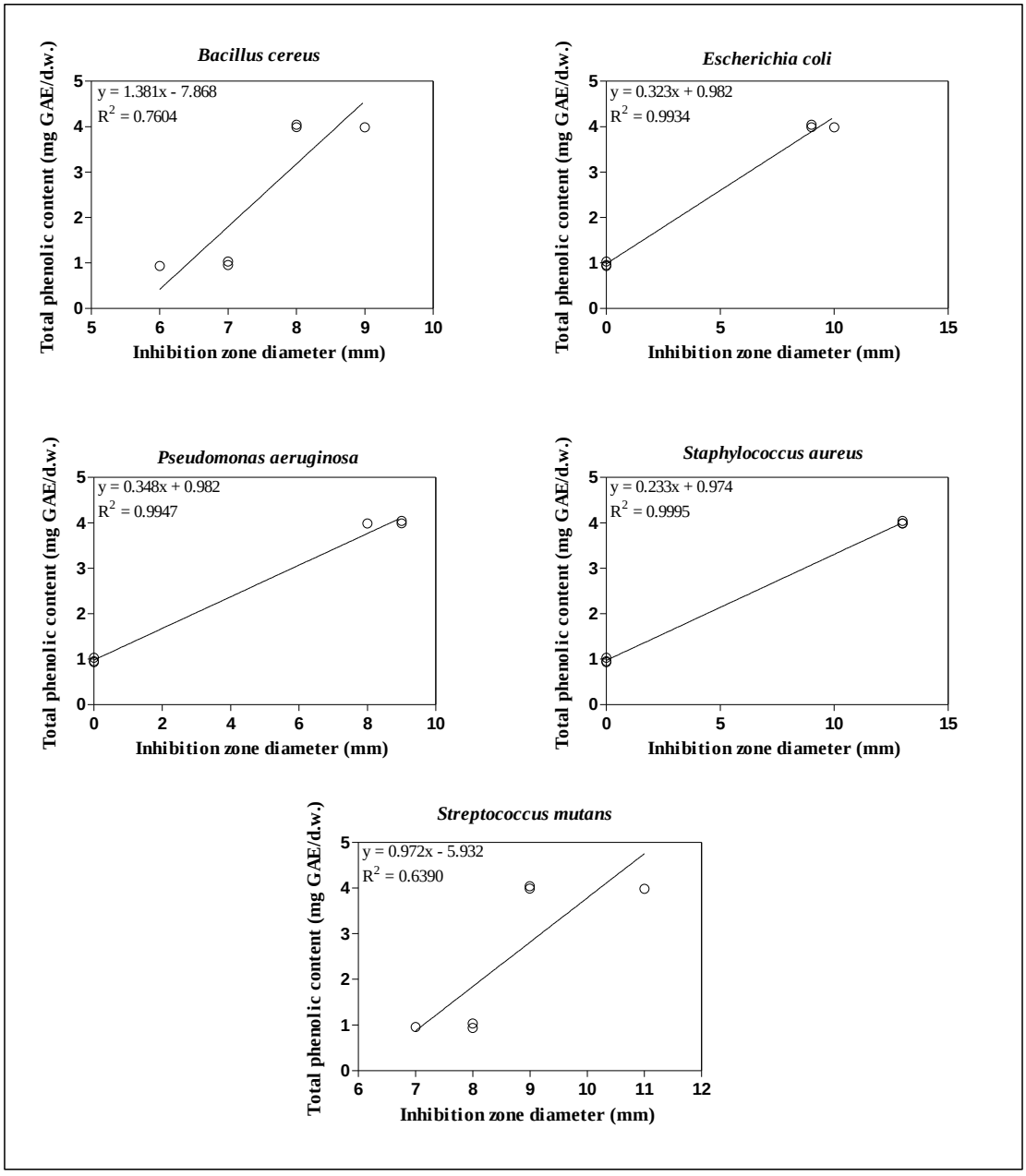

Figure 4- 15: Relationship between total phenolic content and antimicrobial activity of *C. asiatica* and *V. amygdalina* ethanolic extract

Graph displays the linear correlation between diameter of the zone of inhibition (mm) of the five test microorganisms (*B. cereus*, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. mutans*) and total phenolic content (milligram gallic acid equivalent per gram dry weight mg GAE/g d.w) of ethanolic extract of *C. asiatica* and *V. amygdalina*. Results were displayed as mean ± SD, n = 3. Coefficient of determinant (R^2) - measures how well the regression line represents the data.

4.8.2 Relationship between the Phenolic Compounds and Antioxidant Activity and Relationship between the Antioxidant Assays of the Plant of Study.

Regression analysis was performed to correlate total phenolic content (TPC) determined by the Folin-Ciocalteu method with antioxidant capacity determined by DPPH, FRAP and anti-haemolysis assay in order to correlate antioxidant assays. The correlation analysis was performed to determine the relationship of the various assays between aqueous, ethanolic and methanolic extracts of each plant and between ethanolic extract of both plants.

Significantly high correlations were found between the TPC and antioxidant capacity of *C. asiatica* extracts. The strongest positive correlation was observed between TPC and DPPH radical scavenging activity ($r = 0.9996$; $R^2 = 0.9991$; $p < 0.001$) and the lowest correlation was between TPC and FRAP ($r = 0.7624$; $R^2 = 0.5813$; $p < 0.05$). Likewise, significant correlation was observed between various assays used to determine antioxidant capacity especially between DPPH and anti-haemolysis assay ($r = 0.8435$; $R^2 = 0.7115$; $p < .01$; Table 4-10, Figure 4-16).

Table 4- 10: Correlation coefficient (r) between total phenolic content and antioxidant capacities and between antioxidant assays of *C. asiatica* and *V. amygdalina* extracts.

Plant of study	[A]TPC vs [B]DPPH	TPC vs [C]FRAP	TPC vs [D]Anti-haemolysis	DPPH vs FRAP	DPPH vs Anti-haemolysis	FRAP vs Anti-haemolysis
[E]*C. asiatica*	0.9996***	0.7624*	0.8403**	0.7556*	0.8435**	0.8040**
[E]*V. amygdalina*	0.0665^{ns}	0.4336^{ns}	0.2926^{ns}	0.4311^{ns}	0.6885*	0.2810^{ns}
[F]*C. asiatica* and *V. amygdalina*	0.9968***	0.9099*	0.9933***	0.9109*	0.9878***	0.9211**

Table displays the correlation coefficient, r between assays. Statistical significant between correlation coefficient was determined by Pearson correlation analysis for two-tailed p value. $p < .001$ - highly significant***; $p < .01$ -very significant**; $p < .05$ – significant*; ns - not significant. **[A]Total phenolic content (TPC)** - milligram gallic acid equivalent per gram dry weight (mg GAE/g d.w.). **[B]Antioxidant capacity (DPPH)** - milimole Trolox equivalent per gram dry weight (mmol TE/g d.w.). **[C]Antioxidant capacity (FRAP)** - milimole ferrous sulphate per gram dry weight (mmol Fe^{2+} /g d.w.). **[D]Antioxidant capacity (anti-haemolysis assay)** - milimole ascorbic acid equivalent per gram dry weight (mmol AAE/g d.w.). [E]Correlation between aqueous, ethanolic and methanolic extracts of each plant. [F]Correlation between *C. asiatica* and *V. amygdalina* ethanolic extract.

V. amygdalina extracts displayed weak correlation between TPC and antioxidant capacity and between the antioxidant assays. The highest correlation was between DPPH and anti-haemolysis assay ($r = 0.6885$, $R^2 = 0.4740$, $p < 0.05$). The lowest correlation was observed between TPC and DPPH ($r = 0.0665$, $R^2 = 0.0044$, $p > 0.05$; Table 4-10, Figure 4-17).

Comparison of the correlation of TPC with antioxidant capacity and between antioxidant assays were was made between ethanolic extract of *C. asiatica* and *V. amygdalina*. The results generally showed strong significant correlation ($p < 0.05$) with r values between 0.9968 and 0.9099. The strongest correlation was observed between TPC and DPPH ($r = 0.9968$; $R^2 = 0.9935$; $p < 0.001$) followed by TPC and anti-haemolysis assay ($r = 0.9933$; $R^2 = 0.9867$; $p < 0.001$). The lowest correlation was displayed between TPC and FRAP ($r = 0.9099$; $R^2 = 0.8280$; $p < 0.05$; Table 4-10, Figure 4-18).

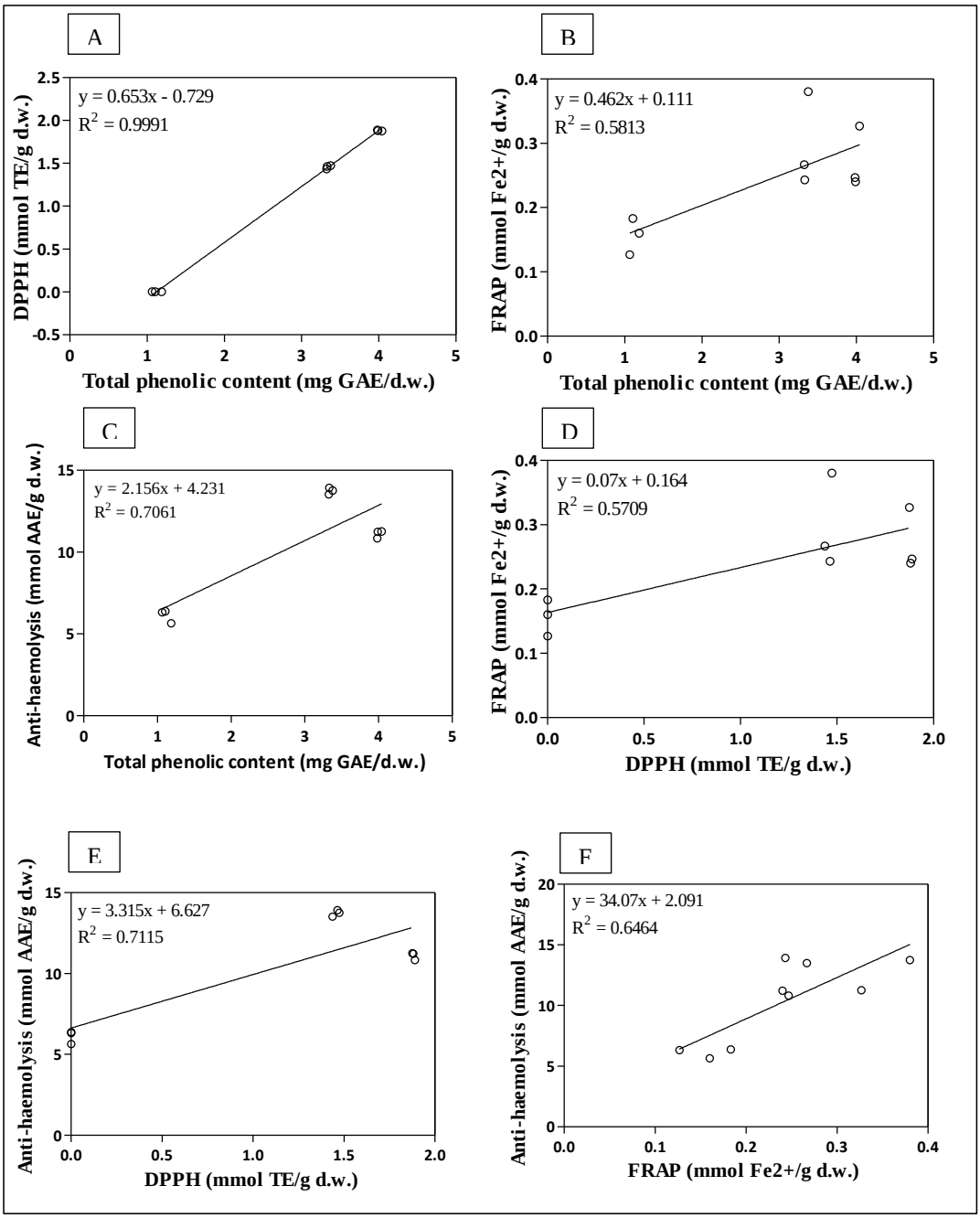

Figure 4- 16: Correlation between total phenolic content and antioxidant capacity and between antioxidant capacities of *C. asiatica* extracts.

Correlation between TPC of *C. asiatica* extracts and their antioxidant capacity determined by DPPH assay (A), FRAP assay (B) and anti-haemolysis assay (C) and between antioxidant capacities DPPH and FRAP (D), DPPH and anti-haemolysis assay (E), and FRAP and anti-haemolysis assay (F). The total phenolic content (TPC) was determined by Folin's Ciocalteu assay, presented as milligram gallic acid equivalent per gram dry weight (mg GAE/g d.w.). The antioxidant activity were DPPH presented as milimole Trolox equivalent per gram dry weight (mmol TE/g d.w.), FRAP assay presented as milimole ferrous sulphate per gram dry weight (mmol Fe^{2+} /g d.w.) and anti-haemolysis assay presented as milimole ascorbic acid equivalent per gram dry weight (mmol AAE/g d.w.). Aqueous, ethanolic and methanolic extracts were used. Results were presented as mean ± SD where n = 3. Coefficient of determinant (R^2) - measures how well the regression line represents the data.

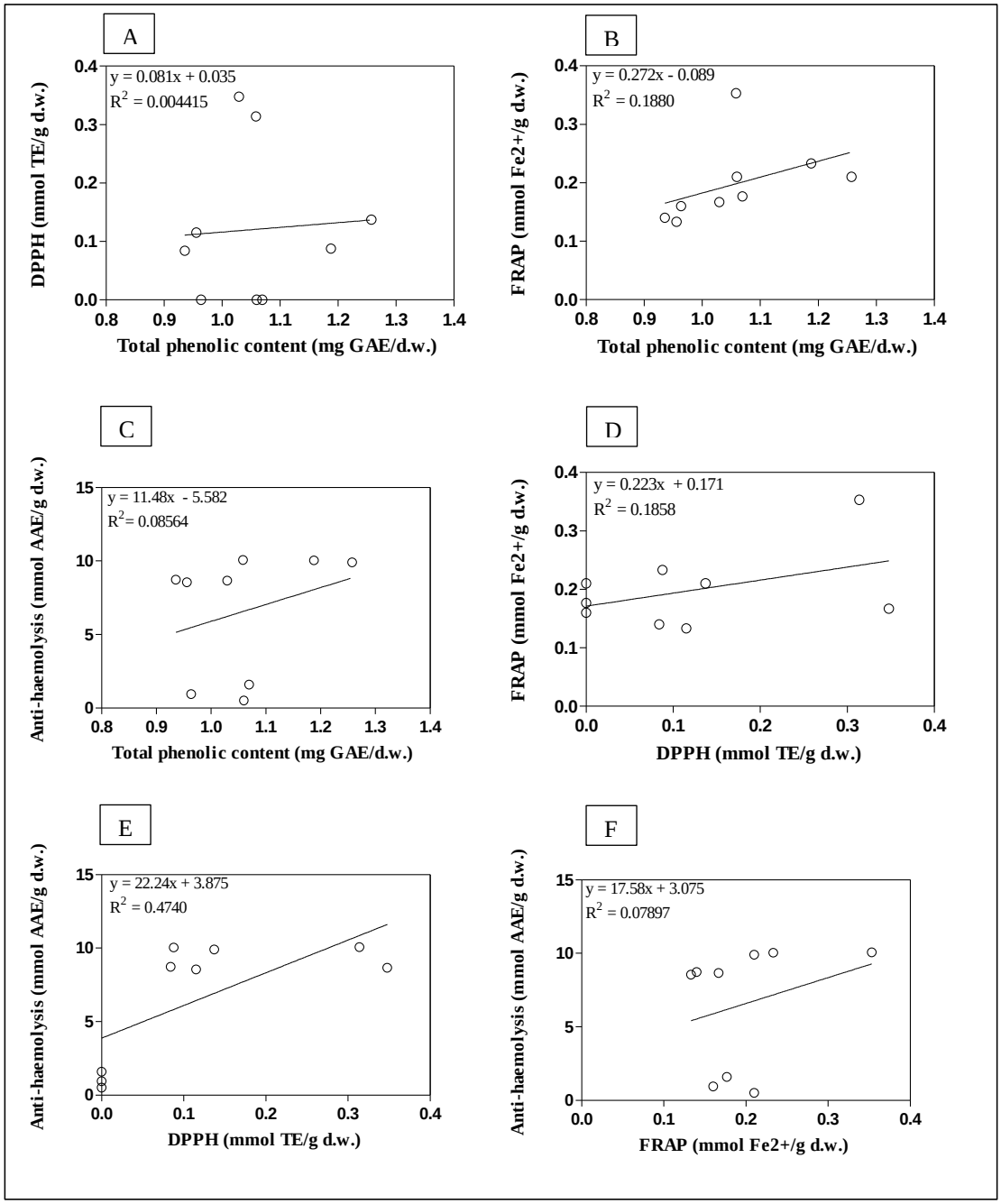

Figure 4- 17: Correlation between total phenolic content and antioxidant capacity and between antioxidant assays of *V. amygdalina* extracts.

Correlation between TPC of *V. amygdalina* extracts and their antioxidant capacity determined by DPPH assay (A), FRAP assay (B) and anti-haemolysis assay (C) and between antioxidant capacities DPPH and FRAP (D), DPPH and anti-haemolysis assay (E), and FRAP and anti-haemolysis assay (F). The total phenolic content (TPC) was determined by Folin's Ciocalteu assay, presented as milligram gallic acid equivalent per gram dry weight (mg GAE/g d.w.). The antioxidant activity were DPPH presented as milimole Trolox equivalent per gram dry weight (mmol TE/g d.w.), FRAP assay presented as milimole ferrous sulphate per gram dry weight (mmol Fe^{2+} /g d.w.) and anti-haemolysis assay presented as milimole ascorbic acid equivalent per gram dry weight (mmol AAE/g d.w.). Aqueous, ethanolic and methanolic extracts were used. Results were presented as mean ± SD where n = 3. Coefficient of determinant (R^2) - measures how well the regression line represents the data.

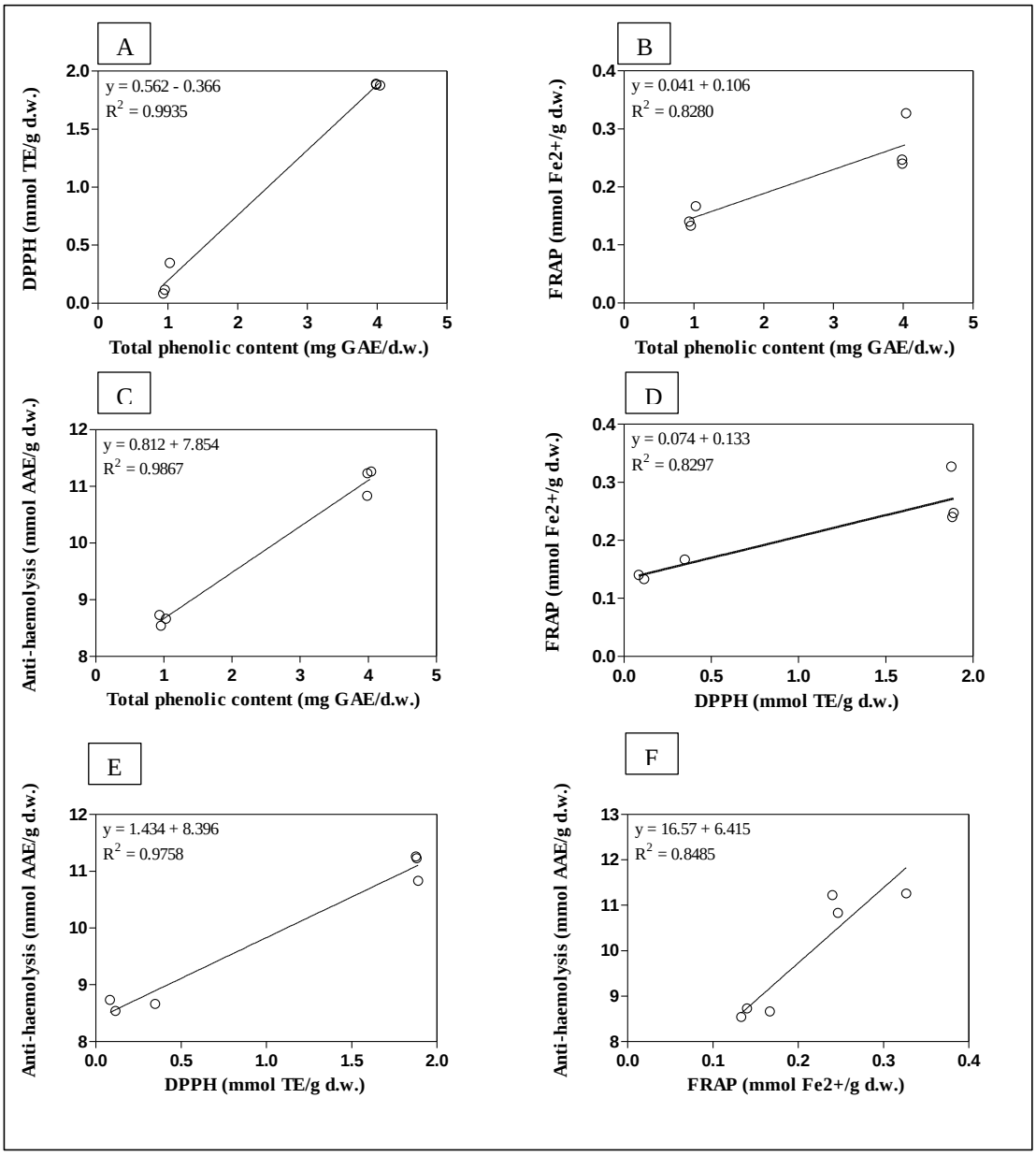

Figure 4- 18: Correlation between total phenolic content and antioxidant capacity and between antioxidant assays of *C. asiatica* and *V. amygdalina* ethanolic extract.

Correlation between TPC of *C. asiatica* and *V. amygdalina* extracts and their antioxidant capacity determined by DPPH assay (A), FRAP assay (B) and anti-haemolysis assay (C) and between antioxidant capacities DPPH and FRAP (D), DPPH and anti-haemolysis assay (E), and FRAP and anti-haemolysis assay (F). The total phenolic content (TPC) was determined by Folin's Ciocalteu assay, presented as milligram gallic acid equivalent per gram dry weight (mg GAE/g d.w.). The antioxidant activity were DPPH presented as milimole Trolox equivalent per gram dry weight (mmol TE/g d.w.), FRAP assay presented as milimole ferrous sulphate per gram dry weight (mmol Fe^{2+} /g d.w.) and anti-haemolysis assay presented as milimole ascorbic acid equivalent per gram dry weight (mmol AAE/g d.w.). Results were presented as mean ± SD where n = 3. Coefficient of determinant (R^2) - measures how well the regression line represents the data.

4.9 Comparison of the Bioactivity in the Aqueous Ethanolic and Methanolic extracts of *C. asiatica* and *V. amygdalina*

The extracts with the highest bioactivity to the lowest bioactivity for antimicrobial activity, total phenolic content and antioxidant capacity by DPPH, FRAP, and anti-haemolysis assays are shown (Table 4-11). It was observed for *C. asiatica*, the aqueous extract displayed strong antimicrobial activity whereas the alcoholic extracts displayed strong phenolic and antioxidant activity. For *V. amygdalina*, the methanolic extract showed highest activity for most of the assays tested and the aqueous extract showed the lowest activity.

Table 4- 11: Bioactivity of *C. asiatica* and *V. amygdalina* aqueous, ethanolic and methanolic extracts.

Bioactivity	*C. asiatica*		*V. amygdalina*	
	Extract with highest activity	**Extract with lowest activity**	**Extract with highest activity**	**Extract with lowest activity**
Antimicrobial activity	aqueous	ethanol and methanol	methanol	aqueous
Total phenolic content	ethanol	aqueous	methanol	ethanol
DPPH antioxidant capacity	ethanol	aqueous	ethanol	aqueous
FRAP antioxidant capacity	methanol	aqueous	methanol	ethanol
Anti-haemolysis assay antioxidant capacity	methanol	aqueous	methanol	aqueous

Table displays the summary of the bioactivity of *C. asiatica* and *V. amygdalina* aqueous, ethanolic and methanolic extracts evaluated by extracts showing the highest and lowest antimicrobial, antioxidant activity and total phenolic content.

4.10 Reverse Phase High Performance Liquid Chromatography (RP-HPLC) Analysis of Phenolic Compounds in Extracts of *C. asiatica* and *V. amygdalina*

Phenolic compounds present in the medicinal plants studied were separated and identified by RP-HPLC using a C_{18} column (280 nm detection wavelength). Methanolic and ethanolic extracts displayed good separation.

For *C. asiatica* good separation of peaks were observed and compared with retention time of eight standards (Table 4-12). The peaks of phenolic compounds were identified as chloramphenicol (peak 6) and benzoic acid (peak 7; Figure 4-19). The presence of the detected phenolic compounds was confirmed by spiking the samples with the appropriate standard (**Appendix J**).

Table 4- 12: Retention time of standards for phenolic compounds of *C. asiatica*

Peak	Compound	RT[a]	Area	Height	Area (%)	Height (%)
1	Gallic acid	4.481	2139145	80255	17.089	10.081
2	Catechin	12.555	540913	29833	4.321	3.748
3	Chlorogenic acid	14.539	1639647	97907	13.098	12.299
4	Caffeic acid	15.990	3013046	206966	24.070	25.999
5	p-coumaric acid	nd	nd	nd	nd	nd
6	Chloramphenicol	19.965	4232124	294366	33.808	36.978
7	Benzoic acid	21.067	745940	63391	5.959	7.963
8	Quercetin	24.071	19033	2858	0.152	0.359

Table displays the retention time of eight commercially available standards by reverse phase high performance liquid chromatography (RP-HPLC). [a]RT - Retention time of standards. nd – not detected.

Table 4- 13: Retention time and concentration of phenolic compounds of major peaks of *C. asiatica* methanolic extract

Peak	Compound identified	RT[a]	Area	Height	Area (%)	Height (%)	Concentration (mg/100g d.w.)[b]	Total phenolic content (mg GAE/100g d.w.)[c]
6	Chloramphenicol	20.561	7903550	835184	73.871	79.114	46.69	738±1.76
7	Benzoic acid	21.751	1288734	104011	12.045	9.853	43.19	94±0.42

Table displays the peak profile of *C. asiatica* methanolic extract by reverse phase high performance liquid chromatography (RP-HPLC). Absorbance was measured at 280 nm. Results were presented as mean ± SD. [a]Retention time of compounds. [b]Concentration determined by RP-HPLC expressed as mg per g dry weight of extracts (mg/g d.w.). [c]Total phenolic content determined by Folin-Ciocalteu's assay expressed as mg gallic acid equivalent per g dry weight (mg GAE/g d.w.).

Quantitation of the major detected peaks of *C. asiatica* was performed as described in Section 3.10.5 based on the peak profiles of standards chloramphenicol and benzoic acid (Table 4-12) and sample (Table 4-13). The concentration of chloramphenicol and benzoic acid obtained were 46.69 and 43.19 mg/ 100g dry weight respectively.

The fractions corresponding to the two major peaks obtained were collected and the total phenolic content was determined with the Folin Coicalteu assay. The chloramphenicol obtained had total phenolic content of 7.38±1.76 mg GAE/g d.w. The benzoic acid obtained had total phenolic content of 0.94±0.42 mg GAE/g d.w.

Retention time of peaks obtained in chromatography of *V. amygdalina* methanolic and ethanolic extract (U1, U2, U3, U4, U5, U6; Figure 4-20B, C) were compared with eight standards (Figure 4-20A). None of the retention time of the standards matched that of the sample. The phenolic compounds present in *V. amygdalina* could not be identified by RP-HPLC (Figure 4-20).

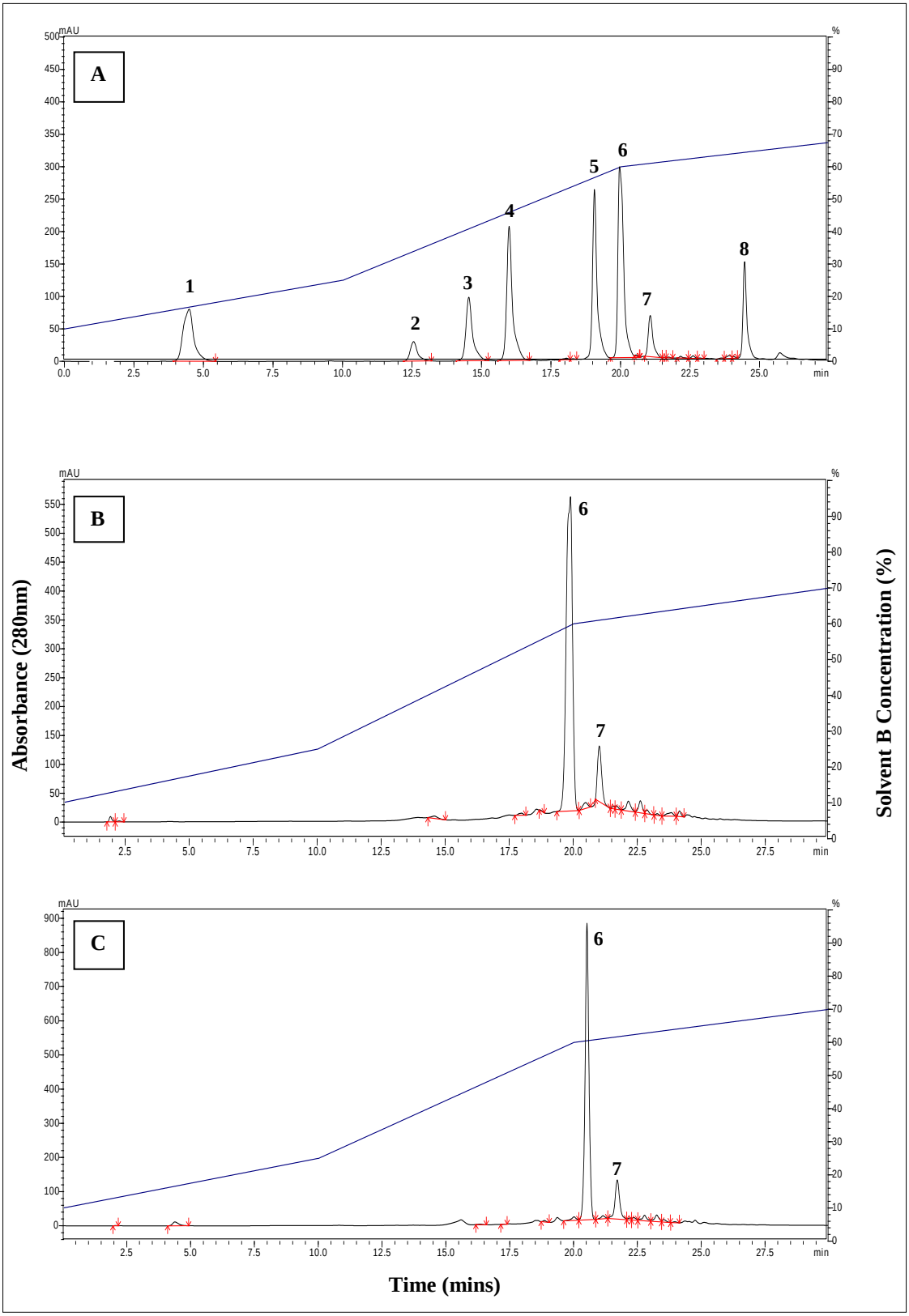

Figure 4- 19: Chromatogram of *C. asiatica*
Chromatogram of external standards (**A**), *C. asiatica* ethanol extract (**B**) and *C. asiatica* methanol extract (**C**) at 280 nm absorbance.1-gallic acid, 2-catechin, 3-chlorogenic acid, 4-caffeic acid, 5-coumaric acid, 6- chloramphenicol, 7-benzoic acid and 8-quercetin.

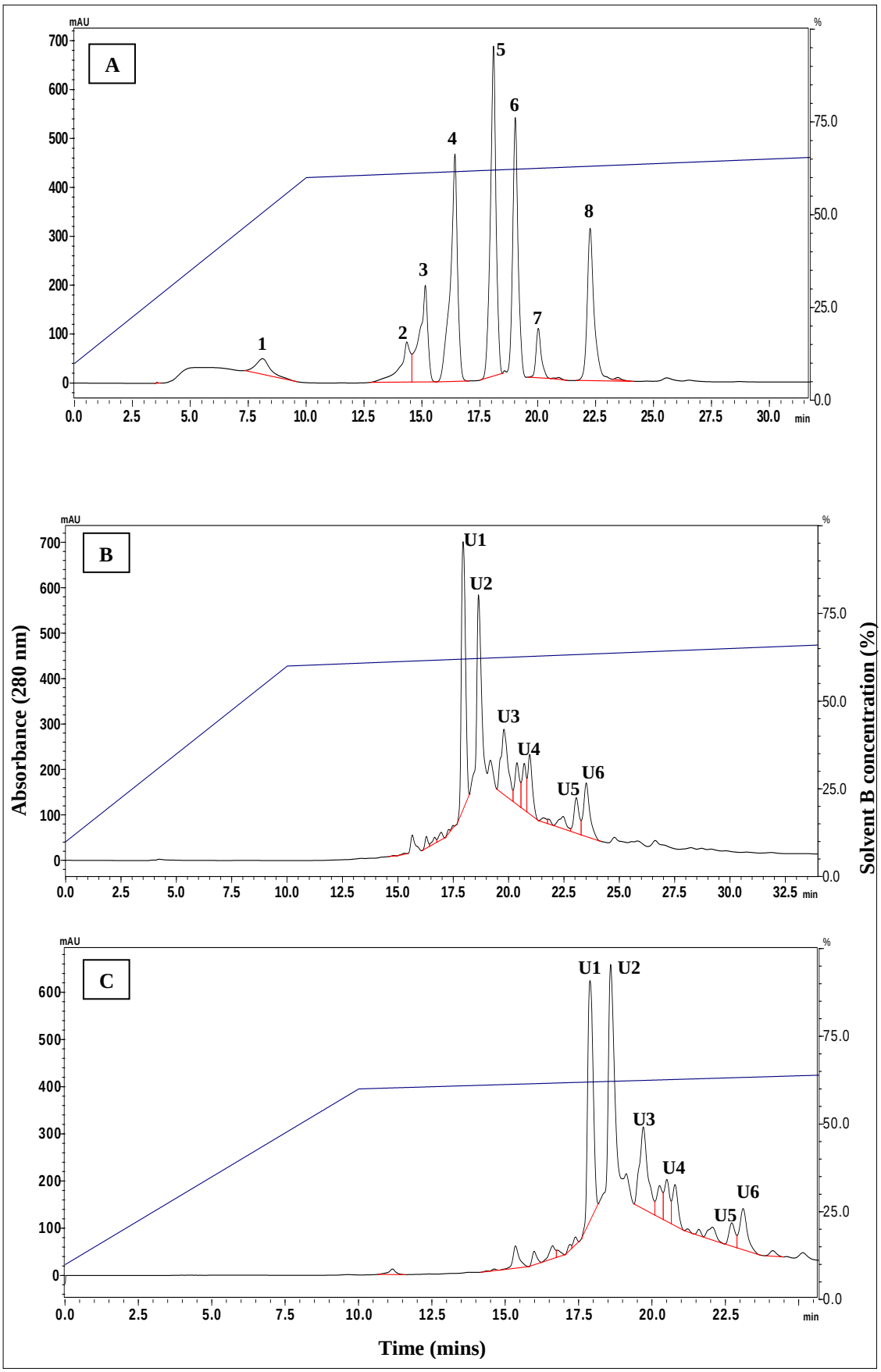

Figure 4- 20: Chromatogram of *V. amygdalina*

Chromatogram of external standards (**A**), *V. amygdalina* ethanol extract (**B**) and *V. amygdalina* methanol extract (**C**) at 280 nm absorbance.1-gallic acid, 2-catechin, 3- chlorogenic acid, 4-caffeic acid, 5-coumaric acid, 6-chloramphenicol, 7-benzoic acid and 8-quercetin. (U1, U2, U3, U4, U5, U6) - unknown compounds.

4.11 Fourier Transform Infrared Spectroscopy (FT-IR) analysis of major peaks from *C. asiatica*.

Fourier Transform Infrared Spectroscopy (FT-IR) was performed to characterize the bonds and functional groups present in peak 6 (retention time – 20.56 mins) and peak 7 (retention time 21.75) collected from RP-HPLC analysis of *C. asiatica* methanol extract. Table 4-14 and 4-15 shows the wave number and assignment of the main bands observed in Figure 4-21.

Table 4- 14: IR spectrum data of peak 6 from *C. asiatica*

Wave number (cm-1)	Bond(s)	Functional group(s)
3304.45	O–H stretch, H–bonded	alcohols, phenols
1672.93	C=C	aromatic
1065.44	C–O	phenols
980.70	=C–H bend	alkenes
858.26	C–H "oop"	aromatics, para-disub benzene

Table depicts the main bands obtained by IR spectrum for peak 6 – chloramphenicol from RP-HPLC

Table 4- 15: IR spectrum data of peak 7 from *C. asiatica*

Wave number (cm-1)	Bond(s)	Functional group(s)
3321.07	O–H stretch, H–bonded	alcohols, phenols
1671.18	C=O	aromatic carboxylic acid
1070.98	C–O	phenols
980.96	=C–H bend	alkenes
859.72	C–H "oop"	aromatics, para-disub benzene

Table depicts the main bands obtained by IR spectrum for peak 7 – benzoic acid from RP-HPLC

The FT-IR of peak 6 consisted of 6 peaks (Figure 4-21A). Of that 5 peaks were characteristics of chloramphenicol (Table 4-14). The peaks were 3304.45, 1672.93, 1065.44, 980.70 and 858.26cm^{-1}. The FT-IR of peak 7 consisted of 7 peaks (Figure 4-21B). Of that 5 peaks were characteristics of benzoic acid (Table 4-15). The peaks were 3321.07, 1671.18, 1070.98, 980.96 and 859.72 cm^{-1}.

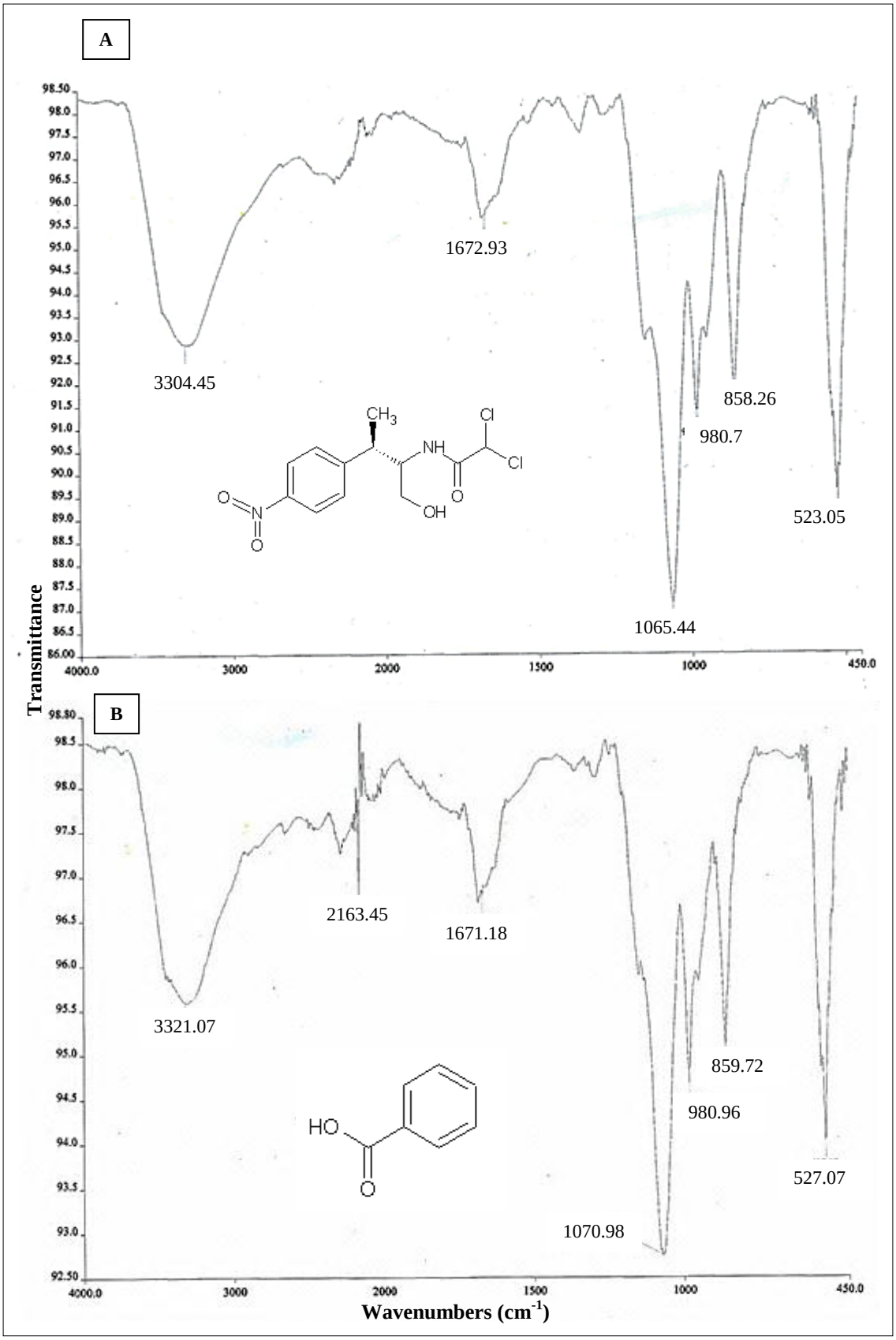

Figure 4- 21: FT-IR Spectrum of *C. asiatica* major peaks
Fourier Transform Infrared Spectroscopy (FT-IR) of *C. asiatica* peak 6 – chloramphenicol (**A**) and peak 7- benzoic acid (**B**) and their respective molecular structures.

4.12 Identification of Phenolic Compounds in Methanolic Extract of *C. asiatica* and *V. amygdalina* by Liquid Chromatography-Mass Spectroscopy (LC-MS)

Liquid chromatography (LC) coupled to negative and positive electrospray ionization (±ESI) mass spectrometry (MS) was used for the identification of phenolic compounds in the methanolic extract of *C. asiatica* and *V. amygdalina*. Table 4-16 and 4-17 summarizes the acquired time, molecular weight, molecular formula and the mass spectra with their respective ionization mode of the compounds identified. The total ion chromatogram (TIC) and the electrospray ionisation-mass spectra in the positive mode (+ESI-MS) for the major peak are displayed in Figure 4-22. The +ESI-MS and –ESI-MS spectra of all the phenolic compounds identified with their respective molecular structure are shown in **Appendix K**.

LC-MS analysis revealed the presence of thirty eight phenolic compounds in the methanolic extract of *C. asiatica*. Among them were theobromine, isorhamnetin-3-*O*-rutinoside, rhamnetin-*O*-rutinoside, syringic acid and naringin were. Chloramphenicol and benzoic acid which had been previously identified by RP-HPLC were also detected by LC-MS (Table 4-16).

In the methanolic extract of *V. amygdalina*, forty phenolic compounds were detected when analysed using LC-MS. Among them were syringic acid, phloridzin, dicaffeoylquinic acid, rhamnetin-*O*-rutinoside, feruloylquinic acid and rutin (Table 4-17).

Table 4- 16: Phenolic compounds identified in methanolic extract of *C. asiatica* by LC-MS

Compound Identified	RT[a] (min)	MW[b]	ESI-MS m/z (+/−)	Abundance[c] (%)	MF[d]
Gallic acid derivatives					
5-galloylquinic acid	0.623	344.3	343.9901 (−)	14.17	$C_{14}H_{16}O_{10}$
Hydroxybenzoic acid					
Syringic acid	0.315	198.17	197.8091 (−)	25.16	$C_9H_{10}O_5$
Ellagic acid pentoside	1.162	434.0	435.2061 (+)	6.63	$C_{19}H_{14}O12$
Hydroxybenzoic acid-*O*-hexoside	30.229	300	301.1415 (+)	20.65	$C_{13}H_{16}O_8$
Protocatechuic acid/ 3,4-Dihydroxybenzoic acid	37.822	154.12	153.8673 (−)	12.73	$C_7H_6O_4$
Methyl gallate	38.579	184.15	183.9348 (−)	9.8	$C_8H_8O_5$
Hydroxycinnamic acid					
Caftaric acid	1.126	312.23	313.1412 (+)	5.56	$C_{13}H_{12}O_9$
Sinapic acid	1.262	224.21	223.8468 (−)	5.63	$C_{11}H_{12}O_5$
Ferulic acid	38.262	194.18	195.1279 (+)	5.04	$C_{10}H_{10}O_4$
Hydroxycinammate quinic esters					
Dicaffeoylquinic acid	1.055	516.45	517.9470 (+)	8.55	$C_{25}H_{24}O_{12}$
Caffeoylquinic acid	1.191	354.31	353.0879 (−)	5.82	$C_{16}H_{18}O_9$
Coumaroylquinic acid	38.155	338.31	339.3445 (+)	18.91	$C_{16}H_{18}O_8$
Feruloylquinic acid	38.191	368.34	369.2952 (+)	9.89	$C_{17}H_{20}O_9$
Flavan-3-ols					
(-)-Gallocatechin	1.333	306.27	305.0695 (−)	8.82	$C_{15}H_{14}O_7$
(-)-Gallocatechin gallate	38.191	458.37	459.2932 (+)	5.73	$C_{22}H_{18}O_{11}$
Flavonol and flavonol glycosides					
Rutin	0.949	610.52	611.3295 (+)	9.27	$C_{27}H_{30}O_{16}$
Isorhamnetin-3-*O*-glucoside	1.126	478.4	479.2369 (+)	8.61	$C_{22}H_{22}O_{12}$
Isorhamnetin-3-*O*-rutinoside	1.162	624.54	625.2829 (+)	38.23	$C_{28}H_{32}O_{16}$
Quercetin-3-*O*-glucuronide	1.162	478.36	479.2386 (+)	8.28	$C_{21}H_{18}O_{13}$
Rhamnetin-*O*-rutinoside	1.162	624.54	625.2829 (+)	38.23	$C_{28}H_{32}O_{16}$
Cirsimaritin	1.162	314.29	315.1560 (+)	6.52	$C_{17}H_{14}O_6$
(±)-Taxifolin	1.162	304.25	305.1554 (+)	7.09	$C_{15}H_{12}O_7$
Phloridzin	1.197	436.41	437.2345 (+)	12.99	$C_{21}H_{24}O_{10}$
Myricetin	1.262	318.24	317.0846 (−)	13.01	$C_{15}H_{10}O_8$
Acacetin / Methyl apigenin	1.446	284.26	285.2887 (+)	7.11	$C_{16}H_{12}O_5$
Chrysin	1.569	254.24	253.8795 (−)	6.28	$C_{15}H_{10}O_4$
Kaempferol	15.034	286.24	285.0749 (−)	5.8	$C_{15}H_{10}O_6$
Apigenin-7-*O*-glucoside	20.181	432.38	431.1807 (−)	12.94	$C_{21}H_{20}O_{10}$
Quercetin 3-*O*-rhamnoside	28.265	448.38	449.2850 (+)	7.26	$C_{21}H_{20}O_{11}$
Quercetin-3-*O*-glucoside	28.159	464.38	465.2626 (+)	9.58	$C_{21}H_{20}O_{12}$
Isorhamnetin	30.300	316.26	317.1147 (+)	10.5	$C_{16}H_{12}O_7$
Flavanone and flavanone glycosides					
Naringin	1.162	580.54	581.2579 (+)	24.9	$C_{27}H_{32}O_{14}$
Luteolin-7-*O*-glucoside	28.123	448.38	449.2856 (+)	6.31	$C_{21}H_{20}O_{11}$
Purine alkaloids					
Theobromine	1.131	180.16	181.0716 (+)	53.99	$C_7H_8N_4O_2$
Phenolic terpenes and lignin					
Rosmadial	0.481	344.40	343.9946 (−)	14.69	$C_{20}H_{24}O_5$
Medioresinol	1.162	388.41	389.0855 (+)	13.59	$C_{21}H_{24}O_7$
Others					
Chloramphenicol	23.387	323.13	322.2112 (−)	9.94	$C_{11}H_{12}Cl_2N_2O_5$
Benzoic acid	35.387	122.12	123.0697 (+)	13.53	$C_7H_6O_2$

Table displays the phenolic compounds identified in the methanolic extract of *C. asiatica* analysed by Liquid Chromatography-Mass Spectroscopy (LC-MS Q-TOF). The analysis was performed with an Electrospray Ionisation (ESI-MS) source in negative and positive mode. [a]RT - Retention time; [b]MW - Molecular weight; Abundance[c] - Relative abundance of the major ion and [d]MF - Molecular formula of the compounds identified.

Table 4- 17: Phenolic compounds identified in methanolic extract of *V. amygdalina* by LC-MS

Compound Identified	RT[a] (min)	MW[b]	ESI-MS m/z (+/−)	Abundance[c] (%)	MF[d]
Gallic acid derivatives					
5-galloylquinic acid	0.584	344.3	343.9918 (−)	11.17	$C_{14}H_{16}O_{10}$
Hydroxybenzoic acid					
Syringic acid	0.039	198.17	197.8067 (−)	100	$C_9H_{10}O_5$
Ellagic acid pentoside	1.116	434.0	433.2060 (−)	9.98	$C_{19}H_{14}O12$
Methyl gallate	1.589	184.15	183.8496 (−)	5.42	$C_8H_8O_5$
Vanillic acid	57.427	168.15	169.0865 (+)	5.30	$C_8H_8O_4$
Protocatechuic acid/ 3,4-Dihydroxybenzoic acid	57.447	154.12	153.8703 (−)	5.93	$C_7H_6O_4$
Hydroxycinnamic acid					
trans-Cinnamic acid	0.393	148.16	149.9509 (+)	5.19	$C_9H_8O_2$
Sinapic acid	0.442	224.21	223.8542 (−)	5.05	$C_{11}H_{12}O_5$
Ferulic acid	0.584	194.18	193.8159 (−)	5.41	$C_{10}H_{10}O_4$
p-Coumaric acid	0.631	164.16	163.8459 (−)	11.23	$C_9H_8O_3$
Caftaric acid	18.828	312.23	311.1666 (−)	11.59	$C_{13}H_{12}O_9$
Caffeic acid	58.019	180.16	181.1657 (+)	5.7	$C_9H_8O_4$
Hydroxycinammate quinic esters					
Dicaffeoylquinic acid	1.069	516.45	515.1231 (−)	35.08	$C_{25}H_{24}O_{12}$
Caffeoylquinic acid	1.234	354.31	353.0885 (−)	20.17	$C_{16}H_{18}O_9$
Feruloylquinic acid	11.528	368.34	367.0509 (−)	28.31	$C_{17}H_{20}O_9$
Coumaroylquinic acid	60.953	338.31	339.3429 (+)	8.63	$C_{16}H_{18}O_8$
Flavan-3-ols					
(+)-Catechin/(−)-Epicatechin	1.115	290.27	291.1301 (+)	5.94	
(-)-Gallocatechin gallate	1.174	458.37	459.2540 (+)	11.29	$C_{22}H_{18}O_{11}$
(-)-Gallocatechin	59.214	306.27	307.1580 (+)	5.32	$C_{15}H_{14}O_7$
Flavonol and flavonol glycosides					
Quercetin 3-*O*-rhamnoside	1.104	448.38	447.0979 (−)	19.15	$C_{21}H_{20}O_{11}$
Rutin	1.115	610.52	611.3310 (+)	27.15	$C_{27}H_{30}O_{16}$
(±)-Taxifolin	1.115	304.25	305.1595 (+)	8.01	$C_{15}H_{12}O_7$
Isorhamnetin-3-*O*-glucoside	1.115	478.40	479.2425 (+)	9.72	$C_{22}H_{22}O_{12}$
Isorhamnetin-3-*O*-rutinoside	1.115	624.54	625.2916 (+)	25.14	$C_{28}H_{32}O_{16}$
Phloridzin	1.127	436.41	437.2339 (+)	52.84	$C_{21}H_{24}O_{10}$
Rhamnetin-*O*-rutinoside	1.174	624.54	625.2845 (+)	34.47	$C_{28}H_{32}O_{16}$
Cirsimaritin	1.174	314.29	315.1573 (+)	5.01	$C_{17}H_{14}O_6$
Apigenin-7-*O*-glucoside	1.175	432.38	431.1784 (−)	13.88	$C_{21}H_{20}O_{10}$
Chrysin	3.932	254.24	253.8765 (−)	6.68	$C_{15}H_{10}O_4$
Flavanone and flavanone glycosides					
Neohesperidin	1.115	610.56	611.8362 (+)	9.89	$C_{28}H_{34}O_{15}$
Naringin	1.115	580.54	581.2676 (+)	15.86	$C_{27}H_{32}O_{14}$
(±)-Naringenin	1.175	272.25	271.1044 (−)	17.48	$C_{15}H_{12}O_5$
Luteolin-7-*O*-glucoside	1.234	448.38	447.0985 (−)	14.45	$C_{21}H_{20}O_{11}$
Luteolin	8.759	286.24	285.0799 (−)	5.22	$C_{15}H_{10}O_6$
Hesperitin	58.681	302.28	303.1537 (+)	5.51	$C_{16}H_{14}O_6$
Resveratrols					
trans-Resveratrol/*cis*-Resveratrol	1.175	228.24	227.0768 (−)	23.56	$C_{14}H_{12}O_3$
Purine alkaloids					
Theobromine	59.545	180.16	181.1695 (+)	7.79	$C_7H_8N_4O_2$
Phenolic terpenes and lignin					
Rosmadial	0.607	344.40	343.9953 (−)	18.7	$C_{20}H_{24}O_5$
Methyl carnosate	61.426	346.46	347.1727 (+)	6.62	$C_{21}H_{30}O_4$
Epirosmanol	62.455	346.42	347.1721 (+)	5.84	$C_{20}H_{26}O_5$

Table displays the phenolic compounds identified in the methanolic extract of *V. amygdalina* analysed by Liquid Chromatography-Mass Spectroscopy (LC-MS Q-TOF). The analysis was performed with an Electrospray Ionisation (ESI-MS) source in negative and positive mode. [a]RT - Retention time; [b]MW - Molecular weight; Abundance[c] - Relative abundance of the major ion; and [d]MF - Molecular formula of the compounds identified.

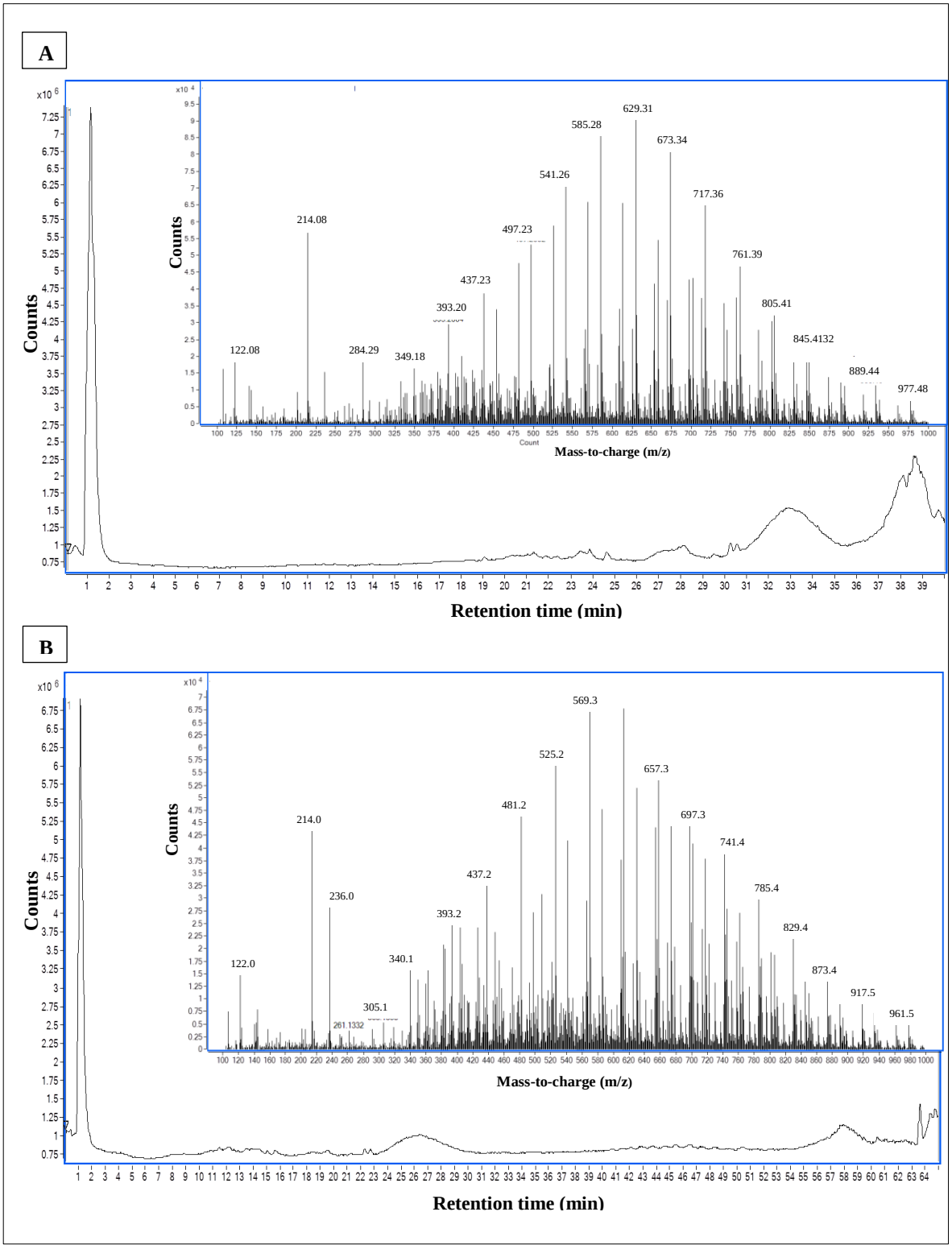

Figure 4- 22: Total Ion Current (TIC) chromatogram of *C. asiatica* (A) and *V. amygdalina* (B) methanolic extract by LC-ESI-MS analysis

Figure displays the TIC chromatogram of the phenolic compounds detected in the methanolic extract of *C. asiatica* and *V. amygdalina* by LC-MS Q-TOF with an Electrospray Ionisation (ESI-MS) source in the positive mode. Inset is the Electrospray ionization–mass spectra (ESI-MS) positive ionization for the major peak of each extract at retention time 1.138 and 1.115 respectively.

5.0 DISCUSSION

5.1 Choice of Plant Used in the Study

Based on traditional folklore medicine (Anbarashan *et al.*, 2011), chemotaxonomic approach and by random selection (Jantan, 2004), six medicinal plants *Aloe vera* (leaf and gel), *Azadirachta indica* (leaf), *Carica papaya* (leaf), *Centella asiatica* (whole plant), *Hymenocallis speciosa* (leaf, tuber and root) and *Vernonia amygdalina* (leaf and stem) were selected.

These plants and their parts were chosen specifically because (i) *A. vera* leaf and gel were reported to possess wound healing properties (Anbarashan *et al.*, 2011; Kumar *et al.*, 2010).

(ii) *A. indica* leaf has antibacterial properties (Almas, 1999) and have been used to cure various skin disorders (Anbarashan *et al.*, 2011).

(iii) The leaf of *Carica papaya* was proven to treat dengue fever in the tropics (Ahmad *et al.*, 2011).

(iv) The whole plant of *C. asiatica* was widely used in folklore medicine and had a wide range of health benefits such as improving memory, curing skin disorders and in the wound healing process (Duke, 2010; Hargono *et al.*, 1999). It belongs to the Apiaceae family which comprises of important medicinal plants such as coriander and fennel (Gurib-Fakim, 2006), thus the same active biological compounds may be present in *C. asiatica* (Eloff & McGaw, 2006).

(v) *H. speciosa* was selected because it had been used to cure jaundice in Malaysian folklore medicine (Azliza *et al.*, 2012). Furthermore it belongs to the Amaryllidaceae family which possess bioactive compounds such as amaryllidaceae alkaloids that display

antimalarial activity (Şener *et al.*, 2003). The tuber and root were chosen because in general, underground plant parts were found to possess bioactive compounds with antimicrobial properties compared to other aerial parts of plants (Das *et al.*, 2010). Furthermore, these plant parts have not been studied for *H. speciosa* to the best of our knowledge and the antimicrobial properties have not been evaluated.

(vi) The leaf and stem of *V. amygdalina* were selected because it had been reported to possess high antidiabetic and antimicrobial properties (Akinpelu, 1999; Ekpenyong *et al.*, 1999). The main purpose for selecting these plants was because of their antimicrobial trait due to the secondary metabolites in these plants which may be significant in therapeutic treatments.

5.2 Extraction Yield and Antimicrobial Activity of the Medicinal Plants Screened

The percentage yield was highest for ethanolic extract *H. speciosa* root because it was homogenized to a fine powder with the absence of fibre. This was followed by ethanolic extract of *A. vera* gel because it has high water content of 99.5% (Eshun & He, 2004). The difference in extraction yield ascribes to the difference in extractable compounds based on the different bioactive compounds found in the plants and different types of solvent possess varying extraction capabilities.

The antimicrobial susceptibility test is important to determine efficiency of novel antimicrobial agent isolated from biological samples against various microorganisms (Das *et al.*, 2010). In this study, the screening of the selected plants was based on their antimicrobial property which was determined by well diffusion assay. Both Gram-negative

bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Bacillus cereus, Staphylococcus aureus,* and *Streptococcus mutans*) were selected due to the following reasons:

(i) Gram-negative bacteria were more resistant to antimicrobials due to the presence of an additional outer membrane layer (Alberts *et al.*, 2002) and they make up most plant pathogens. In general, purified plant bioactive compounds display low antimicrobial activity towards Gram-negative bacteria (González-Lamothe *et al.*, 2009).

(ii) *E. coli* was selected because it is easily cultured in the laboratory, well characterized, found in a wide range of hosts and acquires resistance to antimicrobial agents easily (Erb *et al.*, 2007; Welch, 2006).

(iii) *P. aeruginosa* is resistant to antibiotics due to the lipopolysaccharide layer and forms biofilm to colonize surfaces. It is a notorious pathogen that may be potentially inhibited by antimicrobial agents of plant origin (Kim *et al.*, 1995; Iinuma *et al.*, 1994).

(iv) *B. cereus* is a soil bacterium and can be easily transmitted into food products, hence infecting the human intestine (Vilain *et al.*, 2006). It is resistant to a wide array of antimicrobials including clindamycin, cefazolin, cefotaxime, cephalosporin and penicillin (Bottone, 2010).

(v) *S. aureus* was chosen because it is a commensal in humans and develops resistance to antibiotics easily (Rasigade & Vandenesch, 2013).

(vi) The oral bacterium *S. mutans* was chosen because it invades the oral cavity and is known to cause dental caries (Loesche, 1986). Some of the medicinal plants in this study

such as *A. vera* and *A. indica*, were known to inhibit oral bacteria that cause gum diseases (Kumar *et al.*, 2010; Ajik, 1990).

Well diffusion assay with the aqueous extract revealed that *C. asiatica* significantly inhibited all the five microbial strains while the other medicinal plants did not inhibit any of the test microorganisms. However ethanolic extracts displayed stronger antimicrobial activities. The ethanolic extracts of *C. asiatica* inhibited all the test microorganism and ethanolic extract of *V. amygdalina* leaf significantly inhibited *B. cereus*. Slight inhibition was recorded for the ethanolic extracts of *A.vera* gel against *B. cereus* and *S. aureus*; *A.vera* leaf against *B. cereus* and *E. coli*; and *H. speciosa* leaf against *B. cereus* and *P. aeruginosa* but the inhibition was insignificant.

Previous studies have reported the inhibition of *A. vera* gel and leaf extracts against *S. aureus, P. aeruginosa* (Agarry *et al.*, 2005), and *E.coli* (Kaithwas *et al.*, 2008). However Alemdar and Agaoglu (2009) reported similar results to this study in *A. vera* juice which failed to inhibit these microorganism. *A. indica* extract was reported to inhibit *S. mutans* (Biswas *et al.*, 2002), *P. aeruginosa, S. aureus* and *E. coli* (Abdullah Al *et al.*, 2011). Ethanolic extract of *Carica papaya* leaf was reported to inhibit *E. coli, B. cereus, P. aeruginosa* and *S. aureus* (Baskaran *et al.*, 2012). No antimicrobial activity has been reported for extracts of *H. speciosa*.

Dash *et al.* (2011) reported higher inhibition of *C. asiatica* ethanolic extract towards *E. coli* and *S. aureus* compared to aqueous extract which was in contrary with the results in this study and Kannabiran *et al.* (2009) reported slight inhibition of aqueous extract and no inhibition with ethanolic extract against *P. aeruginosa*. The leaf sap of *V. amygdalina*, was reported to inhibit *S. aureus, E. coli,* and *P. aeroginosa* (Ijeh *et al.*, 1996), while the

methanolic leaf extract inhibited *P. aeruginosa,* and *S. aureus* (Akinpelu, 1999) Vernolide and vernodalol isolated from the plant was reported to inhibit *Bacillus cereus* and *S. aureus* (Erasto *et al.*, 2006).

The difference in findings between this study and previous studies are difficult to be compared to because different methodologies such as extract, extraction technique, test microorganism and choice of antimicrobial test were employed (Weerakkody *et al.*, 2010; Nostro *et al.*, 2000; Hammer *et al.*, 1999). However, the antimicrobial susceptibility test in this study followed all the standards of the Clinical and Laboratory Standards Institute 2007). Screening of various plant species confirmed the therapeutic potency of *C. asiatica* and *V. amygdalina* as used in traditional medicine. These findings were the basis for choosing *C. asiatica* and *V. amygdalina* as candidate plant species for the determination of the bioactive constituents present.

The type of solvent used could greatly affect the antimicrobial activity in the plants. Most plant antimicrobial are aromatic or saturated organic compounds, therefore the most effective solvents used for preliminary screening of plant antimicrobial activity are methanol, ethanol and water (Parekh *et al.*, 2006; Rojas *et al.*, 2006; Lourens *et al.*, 2004; Bisignano *et al.*, 1996; Salie *et al.*, 1996). For *C. asiatica* and *V. amygdalina*, methanol extraction was also included and the results between extracts were compared. Aqueous extracts of *C. asiatica* possess greater zone of inhibition compared to ethanolic and methanolic extracts. Ethanolic and methanolic extracts exhibited similar inhibition at corresponding plant extract concentration. Aqueous extraction was chosen because most traditional medicines used water as solvent to dissolve the traditional preparations (Das *et al.*, 2010) and this study revealed that aqueous extract of *C. asiatica* have strong

antimicrobial activity. This result proved the presence of water soluble bioactive compounds active against the tested microorganisms in *C. asiatica*.

C. asiatica plant extracts displayed antimicrobial activity towards both Gram-positive and Gram-negative bacteria that possess an outer layer making it a strong antibacterial plant. The most susceptible bacteria amongst the bacterial strains investigated in this study was *S. mutans*. Hence this plant may be useful as an oral antibacterial agent and can be incorporated into mouthwashes and toothpastes. *C. asiatica* extracts also had significantly greater levels of inhibition on *P. aeruginosa* compared to the positive control tetracycline. *P. aeruginosa* is a nosocomial pathogen that easily acquires resistance towards antibacterial agents (Morita *et al.*, 2014). Thus *C. asiatica* may be an effective agent to control this nosocomial pathogen. The usage of this plant in wound healing and healing skin disorders in folklore was shown to be correct since this plant extract possess antimicrobial compounds which can inhibit pathogens.

The aqueous extracts of *V. amygdalina* exhibited no antimicrobial activity, however strong inhibition was observed in ethanolic and methanolic leaf extracts. This finding was in agreement with previous study by Parekh *et al.* (2005), whereby organic solvent extracts provide better antimicrobial activity compared to water extracts. The antimicrobial compounds from *V. amygdalina* dissolved in polar solvents. Most plant antimicrobial compounds are aromatic or saturated organic compounds that are extracted by ethanol or methanol (Serkedjieva & Manolova, 1992). The methanolic and ethanolic extracts were more active against Gram-positive microorganisms compared to Gram-negative microorganisms in agreement with previous studies (González-Lamothe *et al.*, 2009; Alberts *et al.*, 2002). *B. cereus*, a foodborne pathogen was found to be most susceptible

towards methanolic leaf extract proving the folklore usage of this plant in treating gastrointestinal disorders (Aregheore *et al.*, 1998).

The yield of *C. asiatica* was slightly higher in aqueous extracts and almost similar between ethanolic and methanolic extracts in agreement with the antimicrobial results that aqueous extracts showed better inhibitory activity between the three extracts ethanolic and methanolic extracts display similar inhibition. Similar results were obtained in *V. amygdalina*, whereby the methanolic and ethanolic extracts displayed higher yield and aqueous extract displayed lowest yield in agreement with the antimicrobial results obtained. This indicated that the solvent used played a major role in extracting the bioactive compounds such as antimicrobial compounds present in the plant.

5.3 Authentication of the Medicinal Plants in the Study

When utilizing medicinal benefits from plants, it is important that the medicinal compound is prepared from the correct and authenticated plant species. A rapid and reliable method used to authenticate plant samples is by DNA barcoding method through sequence comparison of the ITS2 region (Chen *et al.*, 2010; Shiba *et al.*, 2006; Lau *et al.*, 2001; Zhao *et al.*, 2001). Variation between species can be distinguished with nucleotide sequencing of the ITS2 region whereby sequence identities between the unknown plant and the sequence from the Genbank is capable of identifying species.

BLAST search of the unidentified medicinal plant CA and VA was determined as *Centella asiatica* and *Vernonia amygdalina* respectively when compared with sequences from the Genbank, each with at least 99% identity.

5.4 Antimicrobial Activity after Ammonium Sulphate Precipitation of Ethanolic Extracts

Various studies have shown that plant defence mechanism against pathogen is by secondary metabolites or antimicrobial peptides (Feng *et al.,* 2003). The antimicrobial activity reported in the whole plant extract of *C. asiatica* and leaf extract of *V. amygdalina* was tested to determine if it was due to the presence of secondary metabolites or antimicrobial peptides in the plant by performing 100% ammonium sulphate precipitation of the plant ethanolic extract.

Based on the result, the supernatant obtained after ammonium sulphate precipitation of *C. asiatica* ethanolic extract displayed high antimicrobial activity against *B. cereus, E. coli, P. aeruginosa* and *S. aureus* while no activity was displayed against *S. mutans*. The pellet of ammonium sulphate precipitate showed relative insignificant activity against *E. coli* and *P. aeruginosa*, while no activity was observed against *B. cereus, S. aureus* and *S. mutans*.

Similarly, the supernatant of *V. amygdalina* leaf ethanolic extract after ammonium sulphate precipitation exhibited antimicrobial activity against *B. cereus* while the pellet exhibited insignificant activity against *B. cereus*. No inhibitory activity was observed for both the pellet and supernatant against the other test microorganism. Thus this study found that the antimicrobial activity was prominent in the supernatant for both *C. asiatica* and *V. amygdalina* extracts. Hence, antimicrobial compounds present in the plant were due to secondary metabolites and not peptides.

5.5 Total phenolic content (TPC)

Phenolic compounds are secondary metabolites present in all plants. Structurally phenolic compound comprises of an aromatic ring with one or more hydroxyl groups (Michalak, 2006). Phenolic compounds contribute towards many biological effects such as antioxidant, antimicrobial and anti-haemolysis activity (Middleton *et al.*, 2000; Osawa, 1994). In this study, the antimicrobial activities of *C. asiatica* and *V. amygdalina* extracts were attributed to the phenolic compounds present in the plant as reported by Gurjar *et al.* (2012) when the supernatant of ammonium sulphate precipitation showed antimicrobial activity.

Antioxidant activity of phenolic compounds is based on the redox properties that scavenge free radicals, decompose peroxides and quench singlet and triplet oxygen (Osawa, 1994). The number and configuration of the hydroxyl functional group are the main features that determine the antioxidant capacity of phenolic compound in medicinal plants (Pannala *et al.*, 2001; Cao *et al.*, 1997). Therefore, it is crucial to study the TPC in plants as phenolics with natural antioxidant are potential therapeutic agents of various ailments such as cancer, neurodegenerative diseases, diabetes, cardiovascular dysfunctions, inflammatory diseases and aging.

The Folin-Ciocalteu method was used to determine the TPC in the plants. TPC was expressed as milligram gallic acid equivalent per g dry weight (mg GAE/g d.w). The Folin reagent produced a blue coloured complex when reacted with phenols which can be quantified spectrophotometrically (Slinkard & Singleton, 1977). In this study, the highest amount of polyphenols (4.006 mg GAE/g d.w.) were found in the ethanolic extract of the whole plant of *C. asiatica* which was slightly lower than the amount earlier reported by Andarwulan *et al.* (2010) (5.82 mg GAE/g d.w.) and much lower than the amount reported

by Wongsa *et al.* (2012) (52.5 mg GAE/g d.w.). Previously, the TPC in the whole plant extracted using methanol reported by Guleria *et al.* (2013) (27.43 mg GAE/g d.w.) was much higher than found in the methanolic extracts in this study. However in another study, Wongsa *et al.* (2012) reported a much lower TPC (1.4 mg GAE/g d.w.) comparable to this study. A low amount of phenolic content had been recorded in the aqueous extract which was consistent with this study (Wongsa *et al.*, 2012).

The three extracts of the leaf of *V. amygdalina* displayed similar TPC with the highest of them being the methanolic extract. Previous studies showed similarly low TPC in ethanolic and aqueous extracts (3.97 and 2.71 mg GAE/g d.w.) respectively (Audu *et al.*, 2012). However higher TPC value was recorded by Fasakin *et al.* (2011) in ethanolic (8.2 mg GAE/g d.w.) and methanolic (14.8 mg GAE/g d.w.) extracts contrasting with the results of this study.

Generally the TPC of *V. amygdalina* was found to be lower than *C. asiatica* which may explain better antimicrobial activity displayed by *C. asiatica*. *C. asiatica* had higher phenolic content compared to methanolic extracts of medicinal plants such as *Alpinia oxyphylla*, *Asparagus cochinchinensis*, *Astragalus membranaceus*, *Atractylodes macrocephala*, *Dendrobium nobile*, *Dioscorea opposite*, *Lilium brownii*, *Morinda officinalis*, *Ophiopogon japonicas*, *Polygonatum odoratum* and *Tremella fuciformis* (Wong, Li, *et al.*, 2006) and ethanolic extracts of vegetables such as *Apium graveolens* (leaf celery), *Justica gendarussa* (willow-leaved justice), *Kaempferia galanga* (chinese ginger), *Micromelum minutum* (lime berry), *Morinda elliptica* (indian mulberry), *Ocimum basilicum* (basil), *Vigna sinensis* (long beans) and *Vigna radiate* (mung bean sprout) (Sulaiman *et al.*, 2011).

5.6 Antioxidant Activity in the Plant of Study

Antioxidant activity present in the plant must be determined to evaluate if it is related to the phenolic compounds present in the plant. More than one antioxidant assay was employed to measure the antioxidant activity present to take into account the different modes of actions of antioxidants present in the plant extracts related to difference in chemical structure and complexity of the antioxidants (Huang *et al.*, 2005; Prior & Cao, 1999). In this study DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay, Ferric Reducing Antioxidant Power (FRAP) Assay and hydrogen peroxide (H_2O_2) induced haemolysis assay were performed to determine the antioxidant activity in *C. asiatica* and *V. amygdalina*.

5.6.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity Assay

DPPH radical scavenging assay is the most common method used to evaluate antioxidant activity because it is simple and rapid. The mechanism of radical scavenging activity employed in this assay is via the hydrogen atom donated by phenolic compounds with antioxidant activity which binds to the purple coloured DPPH thus reducing DPPH which turns yellow in colour (Gulcin *et al.*, 2007). Antioxidant activity was measured by comparing the inhibition of phenols to Trolox and expressed as milimole Trolox equivalent per gram dry weight sample (mmol TE/g d.w.). The greater scavenging activity of the plant extracts, higher antioxidant capacity was displayed.

From the experimental data, all extracts of *C. asiatica* showed scavenging activity towards DPPH. The highest scavenging activity and antioxidant capacity was observed with the ethanolic extract which was comparable to ascorbic acid but was much higher than

with BHT. Ethanolic extract showed the lowest IC_{50}. Antioxidant capacity of methanolic extract was comparable to ethanolic extract. Aqueous extract displayed the lowest scavenging activity with little antioxidant capacity. These results were in agreement with those of Hamid *et al.* (2002).

However, ethanolic and methanolic extracts of *V. amygdalina* leaf displayed radical scavenging activity which was lower compared to that of *C. asiatica*. The aqueous extract showed apparently no antioxidant capacity. This result was in agreement with the findings of Anyasor *et al.* (2010). The IC_{50} was very high for all the extracts of *V. amygdalina*, displaying the weak antioxidant activity of this plant.

Based on this assay, phenolic compounds with antioxidant activity was better extracted by ethanol/methanol compared to water for both the plants of study. This property was also observed by Hamid *et al.* (2002). A common pattern of inhibition towards DPPH was observed amongst all the extracts for both the plants where a steady increase in inhibition was followed by a much slower increase with a levelling off. This general pattern was found to be common in plant extracts (Razali *et al.*, 2008; Kumaran & Karunakaran, 2007). However, aqueous extracts of *V. amygdalina* did not follow the same pattern because it possessed weak radical scavenging activity and higher concentration of this extract is required to display significant results.

5.6.2 Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay involves the reduction of the almost colourless ferric tripyridyl triazine (Fe(III)-TPTZ) complex to a blue coloured ferrous (Fe(II)-TPTZ) complex by antioxidants present in the plant extracts. The assay is by monitoring the change in absorbance at 593 nm. This assay is used to evaluate the reducing power of samples where the greater the antioxidants present in the plant extract, the greater the intensity of the blue colour formation and hence higher the absorbance.

The reaction of the samples and standards were measured at an interval of 15 seconds for 4 minutes. The antioxidant capacity of the extracts and standards were measured by comparison with a ferrous sulphate ($FeSO_4.7H_2O$) standard curve and expressed as milimole ferrous sulphate per gram of dry weight (mmole Fe^{2+}/g dry weight).

A linear pattern was observed for absorbance of the controls and extracts with regards to time. The absorbance of all the extracts of *C. asiatica*, *V. amygdalina* and standards reached a plateau by the fourth minute of incubation indicating there was no further increase in the reduction of ferric ions.

At 1.0 mg/ml concentration, methanolic and ethanolic extracts of *C. asiatica* showed rapid reaction with high absorbance within the 4 minute reaction time (0.466±0.012 AU at 0 seconds to 0.555±0.012 AU at 4 minutes) and (0.421±0.011 AU at 0 seconds to 0.502±0.014 AU at 4 minutes) respectively. Antioxidant capacity was highest in the methanolic extract. The antioxidant capacity was lower than previous reports by Malinowska (2013) who showed high antioxidant capacity of *C. asiatica* extracted from commercial cosmetic (1.02 mg Fe^{2+}/g d.w) and Guleria *et al.* (2013) who showed high antioxidant capacity in 80% methanolic extract (22.5 mg Fe^{2+}/g d.w.). The aqueous extract in this study displayed low antioxidant activity in agreement with the findings of Wong *et*

al. (2006) and Reihani and Azhar (2012). The antioxidant capacities of all extracts were higher than ascorbic acid, but lower than BHT (Table 4-7).

The methanolic extract of *V. amygdalina* possesses the highest absorbance compared to other extracts (0.142±0.020 AU at 0 seconds to 0.222±0.012 AU at 4 minutes) at 1.0 mg/ml concentration. The highest antioxidant capacity was recorded for the methanolic extract while the lowest was for the aqueous extract. All extracts had higher antioxidant capacity than ascorbic acid but lower than BHT (Table 4-8). Oriakhi *et al.* (2014) reported FRAP antioxidant capacity value in the ethanolic extract of *V. amygdalina* to be two times higher than in this study. The difference in result could be due to the extraction technique used or the plant growth conditions met.

Overall the extracts of *C. asiatica* showed higher FRAP antioxidant capacity compared to extracts of *V. amygdalina* suggesting that *C. asiatica* is a more potent antioxidant. This study shows methanol was a better extractant of phenolic compounds possessing antioxidant activity.

The methanolic extract of *C. asiatica* was shown to possess good reducing capabilities such that it was able to donate electrons to free radicals. Hence in actual biological systems, this extract may be useful as a natural remedy in the treatment of free radical related diseases such as cancer, diabetes, arthritis, and acceleration of the ageing process (Shahidi, 1997).

5.6.3 Protective Effects of the Plant of Study against Hydrogen Peroxide Induced Red Blood Cell Lysis

Hydrogen peroxide (H_2O_2) a reactive compound formed from superoxide, can inactivate enzymes in the body. It easily diffuses across the cell membrane and reacts with Fe^{2+} or Cu^{2+} forming the hydroxyl radicals which reacts with macromolecules in the body resulting in cellular stress which is the root cause of many diseases (Bhatia *et al.*, 2011; Sebastia *et al.*, 2006). Therefore, it is important to evaluate compounds that can scavenge the H_2O_2 and control their accumulation in the cell for the benefit of health and wellbeing.

In this study the erythrocytes cell membranes were used to determine oxidative damage because they are susceptible to oxidation and are targets of oxidizing agents. Erythrocytes generate reactive oxygen species because its membrane has a high concentration of polyunsaturated fatty acids. Furthermore, it contains redox active haemoglobin molecules involved in the transport of oxygen (Shalini *et al.*, 2009). Haemoglobin exposed to H_2O_2 causes degradation of haem, which results in the release of iron ions forming light yellow colour that can be measured at 540 nm. The greater the protective effect of the plant extracts, the lower the absorbance measured. Antioxidant capacity was determined in comparison with ascorbic acid and expressed as milimole ascorbic acid equivalent per gram dry weight plant sample (mmol AAE/g d.w.).

Firstly it was discovered that extracts of both *C. asiatica* and *V. amygdalina* pre-incubated with erythrocytes did not exhibit any lytic effect on the red blood cells. In fact, it was discovered that both the plants showed protective effect against H_2O_2 induced haemolysis. Maximal protective effect and antioxidant capacity in both *C. asiatica* and *V. amygdalina* was found in methanolic extract which was higher than that of ascorbic acid indicating that the compounds extracted with methanolic extract are potent anti-haemolytic agent(s). The protective effect against haemolysis was in a dose dependent pattern for

methanolic and ethanolic extracts. Aqueous extracts did not follow a dose dependent pattern and displayed varying protective effects against extract concentration showing it to be a weak extractant of anti-haemolytic agents in both the plants.

In this study, different concentrations of H_2O_2 (10 mM and 50 mM) were tested and in both *C. asiatica* and *V. amygdalina*, aqueous extracts against 50 mM H_2O_2 displayed lower protective effects than against 10 mM H_2O_2 and ethanolic extracts showed similar protective effect against both the concentration of H_2O_2. However, methanolic extract displayed higher protective effect against 50 mM H_2O_2 compared to 10 mM H_2O_2. Aqueous extracts were weak antioxidants and displayed lower protective effect. The strongest antioxidant activity was in the methanolic extract that displayed stronger protective effect against higher concentration of H_2O_2.

Previous studies such as on red wine and mango peel showed protective properties against H_2O_2 induced erythrocyte haemolysis (Ajila & Rao, 2008; Tedesco *et al.*, 2001). However some plants such as *Allium stracheyi* promote haemolysis of erythrocyte due to the oxidative reaction of phenols (Mukherjee & Rajasekaran, 2010; Bukowska & Kowalska, 2004). Thus measuring haemolytic activity in plant extracts is important in toxicological evaluation (Gandhi & Cherian, 2000). From this study, phenolic compound in both the plants were shown not to cause harmful effects on haemoglobin. *C. asiatica* and *V. amygdalina* possessed compound to effectively scavenge H_2O_2 before it could affect the erythrocytes and thus are safe to be utilized as pharmacological applicants. This study is also the first to report protective effect of *C. asiatica* whole plant extracts and *V. amygdalina* leaf extracts against H_2O_2 induced haemolysis.

5.7 Relationship between Phenolic Compounds and Bioactivity

5.7.1 Relationship between Phenolic Compounds and Antimicrobial Activity

Antimicrobial activity in medicinal plants has been attributed to the phenolic compounds present (Das *et al.*, 2010; Urs & Dunleavy, 1975). Interestingly, this study revealed that the relationship between the antimicrobial activity and phenolic content of *C. asiatica* was generally low for each test microorganisms when compared using the aqueous, ethanolic and methanolic extracts for each plant. The high negative correlation in *C. asiatica* towards *B. cereus* and *S. mutans* indicated that the aqueous extract displayed high antimicrobial activity but low phenolic content and the ethanolic extract possessed high phenolic content but low antimicrobial activity. This result indicated that water extracted certain bioactive compounds believed to inhibit *B. cereus* and *S. aureus* effectively. This suggests the presence of other compounds such as organic acids or aldehydes in addition to the phenolic compounds that may be responsible for the antimicrobial activity. The results also indicated that phenolic compounds were extracted better in ethanol.

Extracts of *V. amygdalina* displayed significantly relationship between the antimicrobial activity and phenolic content towards *B. cereus* and *S. mutans* with the later showing about 0.60 coefficient of determination (R^2). This result indicated that phenolic compounds present in *V. amygdalina* were responsible for the antimicrobial activity displayed by the plant extracts.

When comparison was made between both plants using one of the extract (ethanol) the relationship between antimicrobial activity and total phenolic content was significantly high in agreement with the findings by Shan *et al.* (2007) who discovered high correlation between the antimicrobial activity and total phenolic content of 46 spice and herb extracts.

This finding suggests that the phenolic compounds significantly contributed to the antimicrobial activity of many plant extracts including those of *C. asiatica* and *V. amygdalina*.

5.7.2 Relationship between Phenolic Compounds and Antioxidant Activity

Correlation between total phenolic content (TPC) and antioxidant capacity was determined to evaluate if antioxidant capacity may be attributed to the phenolic compounds present in *C. asiatica* and *V. amygdalina* as reported by previous studies on other plants (Jahan, 2011; Ćetković *et al.*, 2007; Pietta, Simonetti, Gardana, *et al.*, 1998; Shahidi *et al.*, 1992). Phenolic compounds in plants are composed of one or more aromatic rings which are bound to hydroxyl group(s) that forms resonance-stabilized phenoxyl radicals by quenching free radicals giving them their antioxidant properties (Bors & Michel, 2002; Rice-Evans *et al.*, 1997).

The correlation between TPC with DPPH, FRAP and anti-haemolysis activity of *C. asiatica* extracts were significantly high indicating that the phenolic hydroxyl groups were the major contributors of the free radical scavenging activity, ferric reducing capacities and protective effect against hydrogen peroxide shown by the plant extracts. Strong correlation was also observed between the antioxidant capacities determined by DPPH, FRAP and anti-haemolysis assays indicating that the antioxidants present in the plant extracts possess strong antioxidant capacities, although they might act through different modes of action (Huang *et al.*, 2005; Prior & Cao, 1999). Based on the phenolic compounds present in the plant *C. asiatica* is a good source of antioxidant.

The correlation between TPC and antioxidant capacity for *V. amygdalina* extracts was generally low for all the antioxidant assays suggesting that the phenolic compounds in the plant did not display antioxidant potential. Interestingly, the results revealed significantly high correlation between DPPH and anti-haemolysis assay while the other antioxidant assays showed non-significant correlations. This is probably because the phenolic compounds with strong radical scavenging potential also showed strong protective effect towards hydrogen peroxide, but did not efficiently reduce ferric ions. In general, the difference in antioxidant capacities between assays may be due to (i) the difference in stoichiometry between the antioxidants and free radicals; (ii) the different solubility of the antioxidant compounds in aqueous, ethanol or methanol solvents and (iii) stereo selectivity of the free radical ions (Khan *et al.*, 2012). *V. amygdalina* was found to be a weak source of antioxidant.

Correlation between TPC and antioxidant capacities were significantly higher between *C. asiatica* and *V. amygdalina* (ethanolic extract) compared to the correlation between the aqueous, ethanolic and methanolic extracts of each plant. These findings revealed that the presence of phenolic compounds in each plant contributes significantly to their antioxidant capacity as previously reported (Wong, Leong, *et al.*, 2006; Cai *et al.*, 2004). A clear correlation was established in *C. asiatica* but not in *V. amygdalina*. Thus the phenolic compounds of *C. asiatica* possessed stronger antioxidant capacities compared to *V. amygdalina*.

5.8 Bioactivity of Aqueous, Ethanolic and Methanolic extracts of *C. asiatica* and *V. amygdalina.*

When the overall comparison of the bioactivity present in the aqueous, ethanolic and methanolic extracts of *C. asiatica* and *V. amygdalina* were made, it was revealed that aqueous extract of *C. asiatica* showed strongest antimicrobial activity. However methanolic extraction was effective in the isolation of bioactive compounds and the methanolic extract of *V. amygdalina* showed higher antimicrobial activity in agreement with Parekh *et al.* (2005). The ethanolic and methanolic extracts showed highest TPC compared to aqueous extract in both plants. This indicates that phenolic compounds are extracted effectively by ethanol or methanol.

Amongst all the extracts tested, methanolic extract displayed the highest ferric reducing capabilities and anti-haemolysis activity while ethanolic extract displayed highest DPPH radical scavenging capacity in both *C. asiatica* and *V. amygdalina*. It may be assumed that methanol extracted most phenolic compounds possessing ferric reducing capacities and protective effect against hydrogen peroxide and ethanol effectively extracted phenolic compounds possessing DPPH radical scavenging activity.

Ethanolic extract of *C. asiatica* displayed significantly higher DPPH radical scavenging capacity compared to the standard BHT. Synthetic antioxidants such as BHT have been added in to food product to prolong shelf life by controlling lipid oxidation in food. However synthetic antioxidants may be hazardous to health (Kahl & Kappus, 1993).

Therefore natural antioxidants especially from medicinal plants are suitable replacement for the synthetic ones in the food industry. In this study, ethanolic extract of *C. asiatica* may be the best replacement for BHT in the food industry as it possessed high phenolic content and strong antioxidant activity.

The methanolic extract of both *C. asiatica* and *V. amygdalina* displayed significantly higher ferric reducing capacity and anti-haemolysis capacity compared to ascorbic acid. Therefore antioxidant agent from methanolic extracts of these plants may be developed for the management of disorders linked to free radicals such as ageing, cancer, cardiovascular disease, diabetes, rheumatoid arthritis, epilepsy & degradation of essential fatty acids (Subedi *et al.*, 2012; Barros *et al.*, 2007; Singh *et al.*, 2007).

5.9 Identification of Phenolic Compounds through Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and Fourier Transform Infrared Spectroscopy (FT-IR)

Based on the findings of the current study, it was concluded that phenolic compounds were present in both the medicinal plants and the antioxidant and antimicrobial properties displayed in both the plants were attributed to phenolic compounds. RP-HPLC used for the separation, identification and quantification of phenolic compounds present in *C. asiatica* and *V. amygdalina* same as others, has shown to be a reliable method (Molnar-Perl & Fuzfai, 2005).

The ethanolic and methanolic extracts of *C. asiatica* and *V. amygdalina* showed high TPC as determined by the Folin-Coicalteu method. Purification of plant extracts was required prior to injection into HPLC to remove compounds such as chlorophyll, waxes sterols, etc. which may damage the HPLC column and interfere with the elution of compounds (Głowniak *et al.*, 1996). SPE (solid phase extraction) method was chosen as the pretreatment method over the hydrolysis method because pretreatment with SPE columns is known to give higher recovery of phenolic compounds whereas, pretreatment by hydrolysis caused decomposition of polyphenols which often led to the loss in recovery of phenolic

compounds (Kermasha *et al.*, 1995; Hertog *et al.*, 1992). Furthermore the SPE pretreatment method is highly reproducible and a low volume of sample is required (Głowniak *et al.*, 1996).

The identity of the phenolic compounds were determined by comparing with the retention time of standards as established by Bartolomé *et al.* (1993). In this study, the RP-HPLC analysis of *C. asiatica* extracts revealed the presence of chloramphenicol and benzoic acid whereby both ethanolic and methanolic extracts displayed similar chromatographic patterns. The peaks for chloramphenicol and benzoic acid obtained were confirmed firstly by spiking with authentic chloramphenicol and benzoic acid standards and then by FT-IR. Interestingly, this study is the first to report the presence of chloramphenicol and benzoic acid in *C. asiatica* which may contribute to the plant's antioxidant and antimicrobial activities.

In this study, chloramphenicol is the major compound in extracts of *C. asiatica* whole plant. It is a broad spectrum antibiotic which was first isolated from *Streptomyces venezuelae* in 1947. Chloramphenicol inhibits both gram positive and negative bacteria and is effectively used in the treatment of meningitis, typhoid fever, and cystic fibrosis (Baselt & Cravey, 1995). This may explain the antimicrobial activity displayed by the plant extracts. Chloramphenicol was discovered to exist naturally in herbs, grass and in plants from the Artemisia family (Berendsen *et al.*, 2013; Berendsen *et al.*, 2010).

The other major compound present, benzoic acid is the most widely used preservative in the food, drug and cosmetic industries (Davidson, 2001). It exists as a white powder in pure form and is slightly soluble in water. It was found to occur naturally in blackberries, mushrooms and tomatoes (Abdullah *et al.*, 1994; Marlatt *et al.*, 1992; Humpf & Schreier, 1991).

Previous studies reported the presence of triterpenes such as asiatic acid, madecassic acid, asiaticosside, madecassoside, terminolic acid, 11,12-dehydroursolic acid lactone, ursolic acid, pomolic acid, 2a ,3a -dihydroxyurs-12-en-28-oic acid, 3-epimaslinic acid, corosolic acid, 8-acetoxy-1,9-pentadecadiene-4,6-diyn-3-ol, β-sitosterol 3-*O*-β-glucopyranoside and asiaticoside-B; and rosmaric acid in the plant (Bonfill *et al.*, 2006; Yoshida *et al.*, 2005; Schaneberg *et al.*, 2003; Verma *et al.*, 1999; Inamdar *et al.*, 1996).

Quantification of the total phenolic content (TPC) of the two major peaks obtained was done by (i) Folin-Coicalteu assay; and (ii) comparison with the peak profiles of commercial standards chloramphenicol and benzoic acid. The TPC of the peaks obtained by Folin-Coicalteu method was much higher than by comparison of peak profiles to standards. The difference in the phenolic content obtained by both methods may be due to the lack of accuracy of the Folin-Coicalteu method, which may be affected by interference such as sulphur dioxide, ascorbic acid, sugar, aromatic amines, organic acids and other non-phenolic substances that may react with the assay (Singleton *et al.*, 1999).

The RP-HPLC analysis of *V. amygdalina* detected six major unknown peaks. Further analysis by LC-MS and GC-MS was done to identify the phenolic compounds present. Previous studies of *V. amygdalina* extracts revealed the presence of caffeoylquinic acid (Johnson *et al.*, 2011) flavonoids (Igile *et al.*, 1994), steroidal alcohol (Arene, 1972), steroid glucosides (Igile *et al.*, 1994; Jisaka *et al.*, 1993), fatty acids (Erasto *et al.*, 2007) and sesquiterpene lactones (vernodalin and vernoamygdalin) (Luo *et al.*, 2011; Erasto *et al.*, 2006).

5.10 Liquid Chromatography-Mass Spectroscopy Analysis of the Plant extracts

Currently LC-MS is considered as the best analytical method for identifying phenolic compounds present in plant extracts (Motilva *et al.*, 2013; Savarese *et al.*, 2007). Previous studies indicated that the negative ionization mode of the ESI-MS provides better identification of phenolic compounds with low molecular weight and low concentration (Abad-García *et al.*, 2012; Sun *et al.*, 2007; Charrouf *et al.*, 2007). However in this study, both the ionization modes (positive and negative) were used to provide certainty of the molecular mass determination. Identification of phenolic compounds was based on the accurate molecular mass by search of the literature.

In this study, thirty eight phenolic compounds were identified in the methanolic extract of *C. asiatica*. The phenolic compounds were classified into the following groups: gallic acid derivatives, hydroxybenzoic acid, hydroxycinnamic acid, flavonoids (flavan-3-ols, flavonol and flavonol glycosides, flavanone and flavanone glycosides), purine alkaloids and phenolic terpenes and lignin. Other compounds such as chloramphenicol and benzoic acid which were previously identified by RP-HPLC and FT-IR were also detected by LC-MS confirming the presence of these compounds in *C. asiatica* extracts.

The LC-MS analysis showed that the methanolic extract of *V. amygdalina* contained forty phenolic compounds ranging from gallic acid derivatives, hydroxybenzoic acid, hydroxycinnamic acid, flavonoids (flavan-3-ols, flavonol and flavonol glycosides, flavanone and flavanone glycosides), resveratrols, purine alkaloids and phenolic terpenes and lignin.

Interestingly, this study is the first to report the presence of the phenolic compound phloridzin. Phloridzin is characterized as a dihydrochalcone, which is present as a major phenolic compound in *Malus domestica* (Gosch *et al.*, 2009). In a recent study conducted

by Najafian *et al.* (2012), this compound significantly reduced the blood glucose levels and improved dyslipidemia in streptozotocin-induced diabetic rats. Phloridzin was discovered to help reduce diabetes, obesity, stress and hyperglycaemia and is a strong antioxidant agent (Kobori *et al.*, 2012; Gosch *et al.*, 2010). The leaf extracts of *V. amygdalina* is known to possess antidiabetic activity (Osinubi, 2008; Ekpenyong *et al.*, 1999) and this may be related to the presence of phloridzin.

The combination of phenolic compounds detected in both *C. asiatica* and *V. amygdalina* may work in synergy to exhibit the antimicrobial, antioxidant and anti-haemolyis properties. Synergy between different constituents of plant extracts has been shown in *Cinchona* bark, whereby thirty alkaloids have been detected and the mixtures of alkaloids have a greater inhibition against *Plasmodium falciparumin* than any of the alkaloids used separately (Druilhe *et al.*, 1988). This study is also the first to identify various phenolic compounds present in *C. asiatica* and *V. amygdalina*.

6.0 CONCLUSION

Six medicinal plants: *Aloe vera* (leaf and gel), *Azadirachta indica* (leaf), *Carica papaya* (leaf), *Centella asiatica* (whole plant), *Hymenocallis speciosa* (leaf, tuber and root) and *Vernonia amygdalina* (leaf and stem) were screened for antimicrobial activity. On the basis of antimicrobial activity, *C. asiatica* (whole plant) and *V. amygdalina* (leaf) were chosen as candidate plant species for the determination of bioactive constituents present. The aqueous, ethanolic and methanolic extracts of *C. asiatica* inhibited *B. cereus*, *E. coli*, *P. aeruginosa*, *S. mutans* and *S. aureus*. Ethanolic extracts of *V. amygdalina* inhibited *B. cereus* while the methanolic extract inhibited *B. cereus* and *S. mutans*.

The supernatant obtained from the ammonium sulphate precipitation of the ethanolic extracts of *C. asiatica* displayed high antimicrobial activity against *B. cereus*, *E. coli*, *P. aeruginosa* and *S. aureus*. The supernatant of the ethanolic extract of *V. amygdalina* exhibited antimicrobial activity against *B. cereus*. Insignificant inhibition was observed for the ammonium sulphate pellet against the test microorganisms. Hence the antimicrobial activity of the plant extracts was not attributed to peptides but may be due to phenolic compounds.

Total phenolic content (TPC) of the extracts were determined. The ethanolic extract of *C. asiatica* showed high TPC whereas the TPC in *V. amygdalina* was similar in the aqueous, ethanolic and methanolic extracts but lower in amount compared to *C. asiatica*. Phenolic compounds have been known to be major contributors of antioxidant activity in plants. The antioxidant potential of both the plants were evaluated based on DPPH free radical scavenging activity, FRAP assay and anti-haemolysis activity.

The ethanolic extract of *C. asiatica* showed high scavenging activity towards DPPH, which was stronger than that of BHT. The methanolic extract displayed high FRAP antioxidant capacity, which was stronger than that of ascorbic acid. Hence the extracts of this plant may be useful as a natural antioxidant agent for the management of health disorders associated with free radicals.

The methanolic and ethanolic extracts of *V. amygdalina* displayed high scavenging activity towards the DPPH radical, and the methanolic extract displayed high FRAP antioxidant capacity. However it was lower than *C. asiatica* and higher concentrations of the extract was required to display significant antioxidant activity.

This study also demonstrated for the first time the protective effect of *C. asiatica* and *V. amygdalina* extracts against hydrogen peroxide induced red blood cell lysis. The protective effect against haemolysis was higher than that of ascorbic acid.

Biologically active phenolic compounds in *C. asiatica* and *V. amygdalina* were identified by RP-HPLC, LC-MS, and FT-IR. RP-HPLC analysis of the methanolic extract of *C. asiatica* identified the presence of chloramphenicol and benzoic acid as the major phenolic compounds in the plant. The identification of these compounds was confirmed by FT-IR analysis. However RP-HPLC analysis of *V. amygdalina* extracts detected six major phenolic compounds which were not identified. Analysis by LC-MS was done to identify the phenolic compounds present in the plant extracts.

Analysis by LC-MS identified thirty eight and forty different phenolic compounds in *C. asiatica* and *V. amygdalina* respectively including gallic acid derivatives, hydroxybenzoic acid, hydroxycinnamic acid, flavonoids, purine alkaloids and phenolic terpenes and lignins. Amongst them, the presence of phloridzin was detected in the methanolic extract of *V. amygdalina*. Phloridzin is known to have antidiabetic properties.

The results from this study suggests that extracts of the selected medicinal plants *C. asiatica* and *V. amygdalina* possesses phenolic compounds with antimicrobial and antioxidant properties that can be used as antimicrobial and antioxidant agents in the search for new drugs. Furthermore the natural plant pheolics could serve as a natural preservative in the food industry to replace synthetic antioxidant agents.

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APPENDICES

Appendix A. Plants Selected in the Study

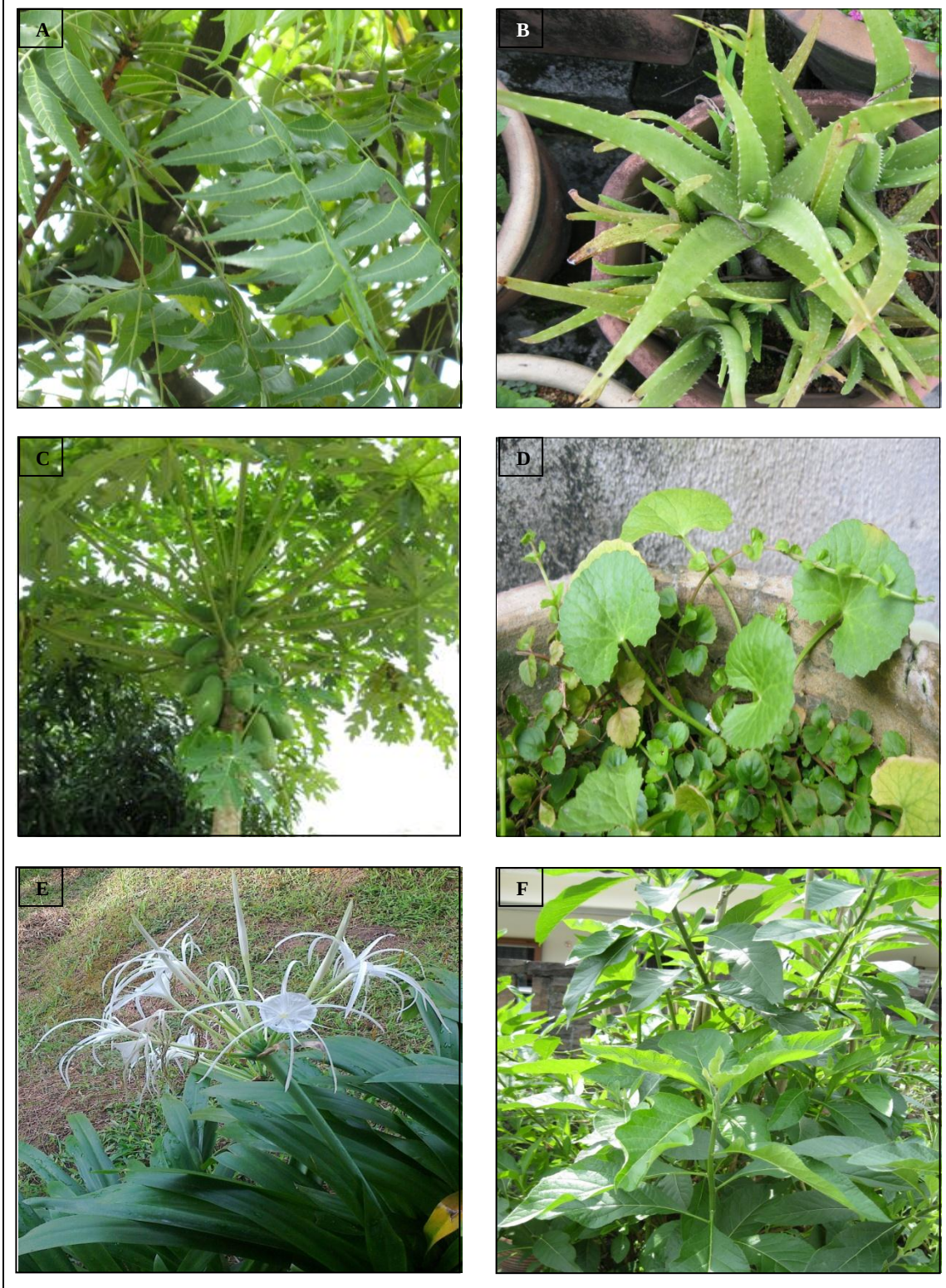

Figure 1: Medicinal plants collected for the study
The medicinal plants *Azadirachta indica* (**A**), *Aloe vera* (**B**), *Carica papaya* (**C**), *Centella asiatica* (**D**), *Hymenocallis speciosa* (E) and *Vernonia amygdalina* (**F**) were collected from Seksyen 4, Petaling Jaya, Selangor

Appendix B. Voucher Deposit of Selected Plants

Figure 2a: Voucher deposited at the Herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.
The voucher was deposited for the six medicinal plants as follows *Aloe vera* (**A**), *Azadirachta indica* (**B**), *Carica papaya* (**C**), and *Centella asiatica* (**D**).

Appendix B continued.

Figure 2b: Voucher deposited at the Herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.
Hymenocallis speciosa (**E**) and *Vernonia amygdalina (***F**).

Appendix C. Well Diffusion Assay

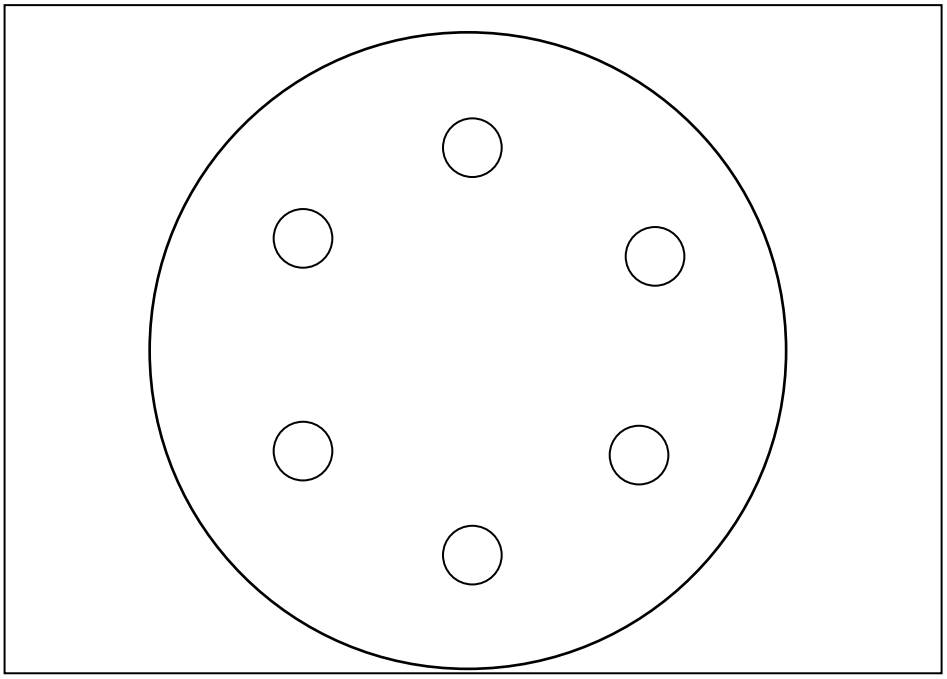

Figure 3a: Template for six wells on a 90 x 15mm petri plate
Template was used as a guide to bore six wells onto agar medium of petri plate in a well diffusion assay.

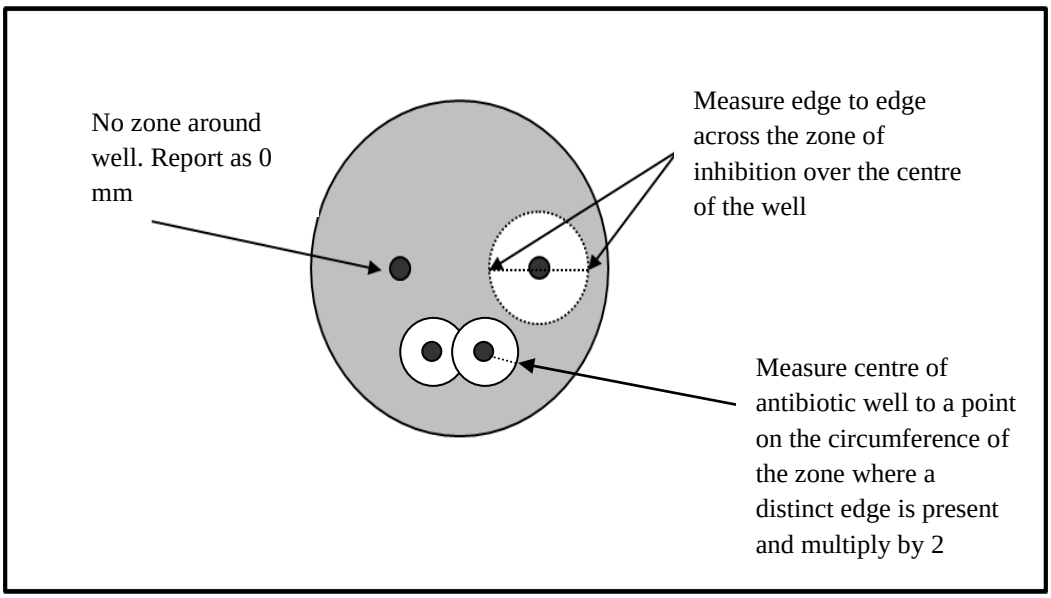

Figure 3b: Measuring zones of inhibition.
Grey shading represents a confluent lawn of bacterial growth. Black circle represents the well bored on the agar. The white circle represents no growth of the test organism (zone of inhibition).

Appendix D. Antimicrobial Activity of Plant Extracts by Well Diffusion Assay

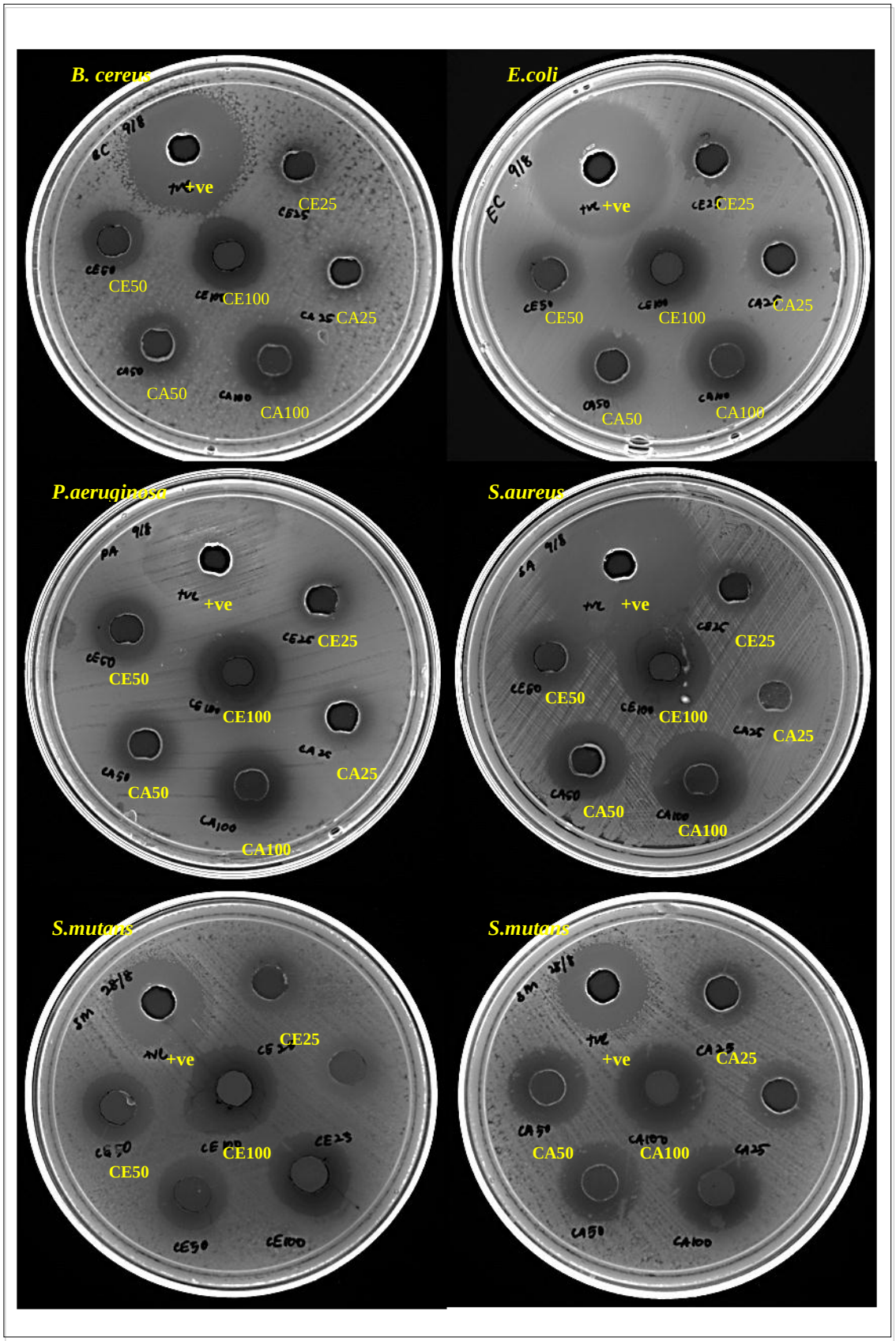

Figure 4a: Well diffusion assay for *C. asiatica* ethanolic and aqueous extracts against test microorganisms
+ve – Positive control; CE – *C. asiatica* ethanolic extract; CA – *C. asiatica* aqueous extract; at 25, 50 and 100 mg/ml concentration respectively

Appendix D continued

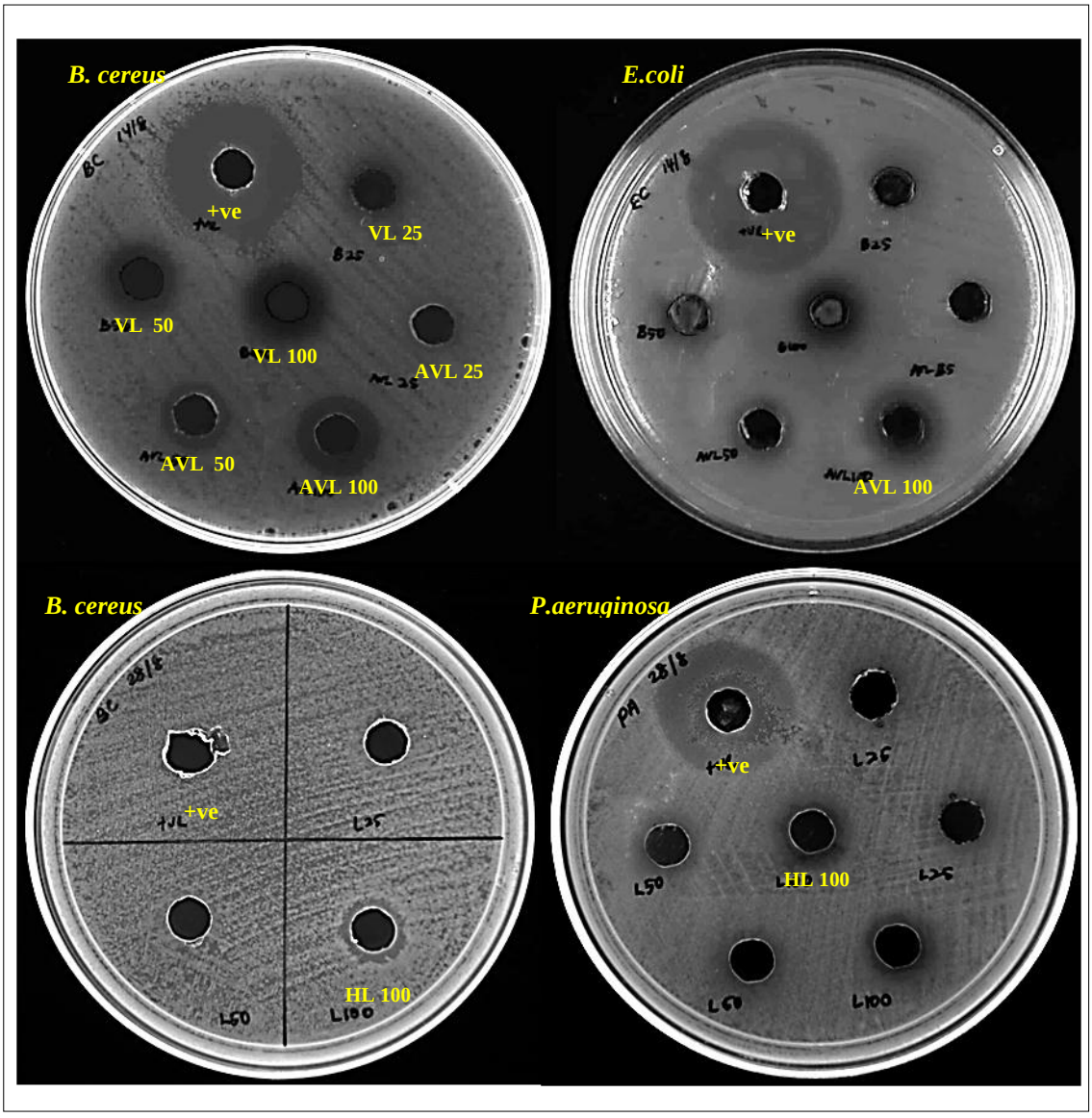

Figure 4b: Well diffusion assay for *A. vera* leaf, *V. amaygdalina* leaf and *H. speciosa* leaf ethanolic extract against test microorganisms

+ve – Positive control; AVL – *A. vera* leaf; VL – *V. amygdalina* leaf and HL – *H. speciosa* leaf extract; at 25, 50 and 100 mg/ml concentration respectively

Appendix D continued

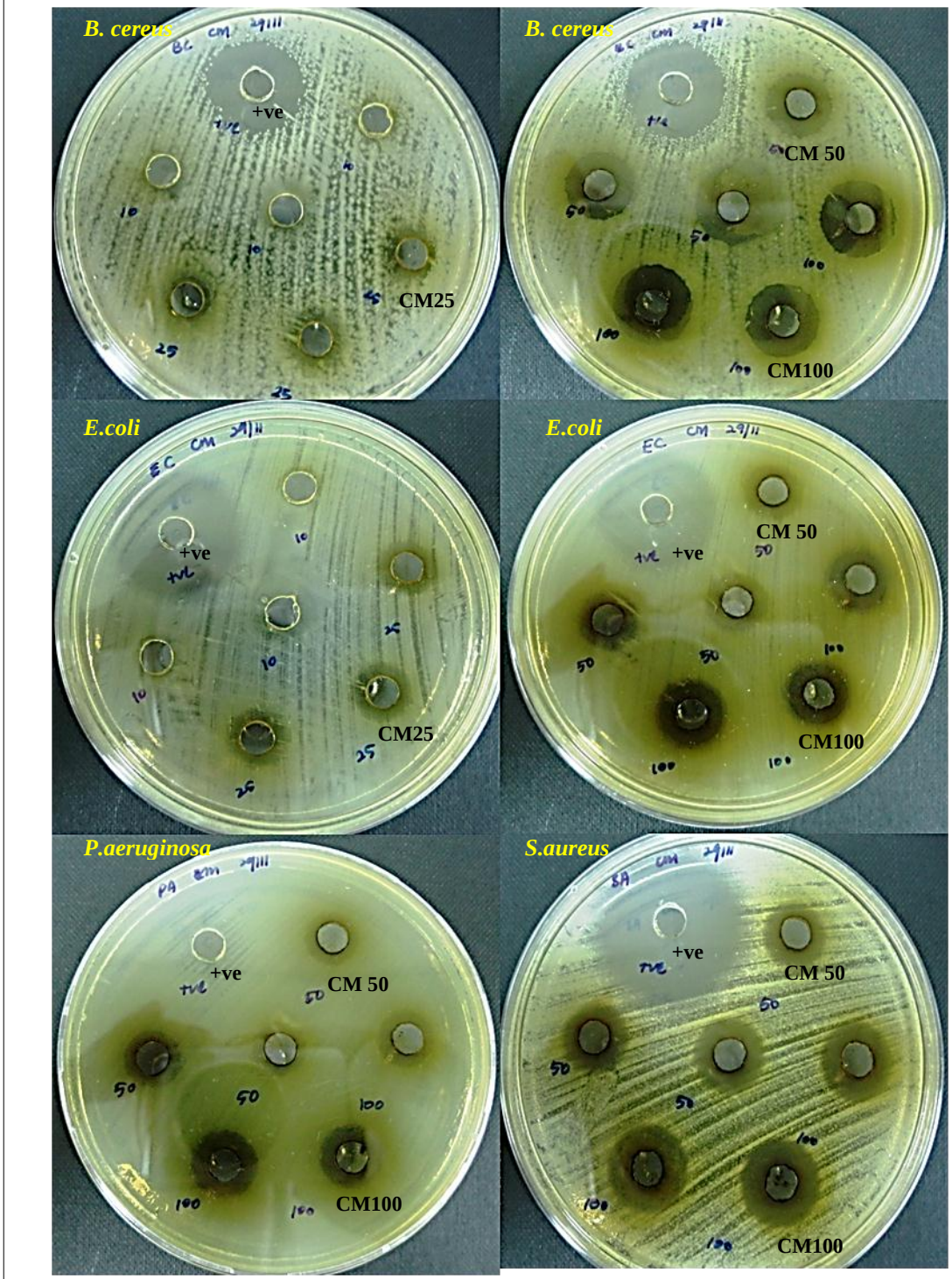

Figure 4c: Well diffusion assay for *C. asiatica* methanolic extract against test microorganisms
+ve – Positive control; CM 25 - *C. asiatica* 25 mg/ml; CM 50 - *C. asiatica* 50 mg/ml and CM 100 - *C. asiatica* 100 mg/ml of methanol extract.

Appendix D continued

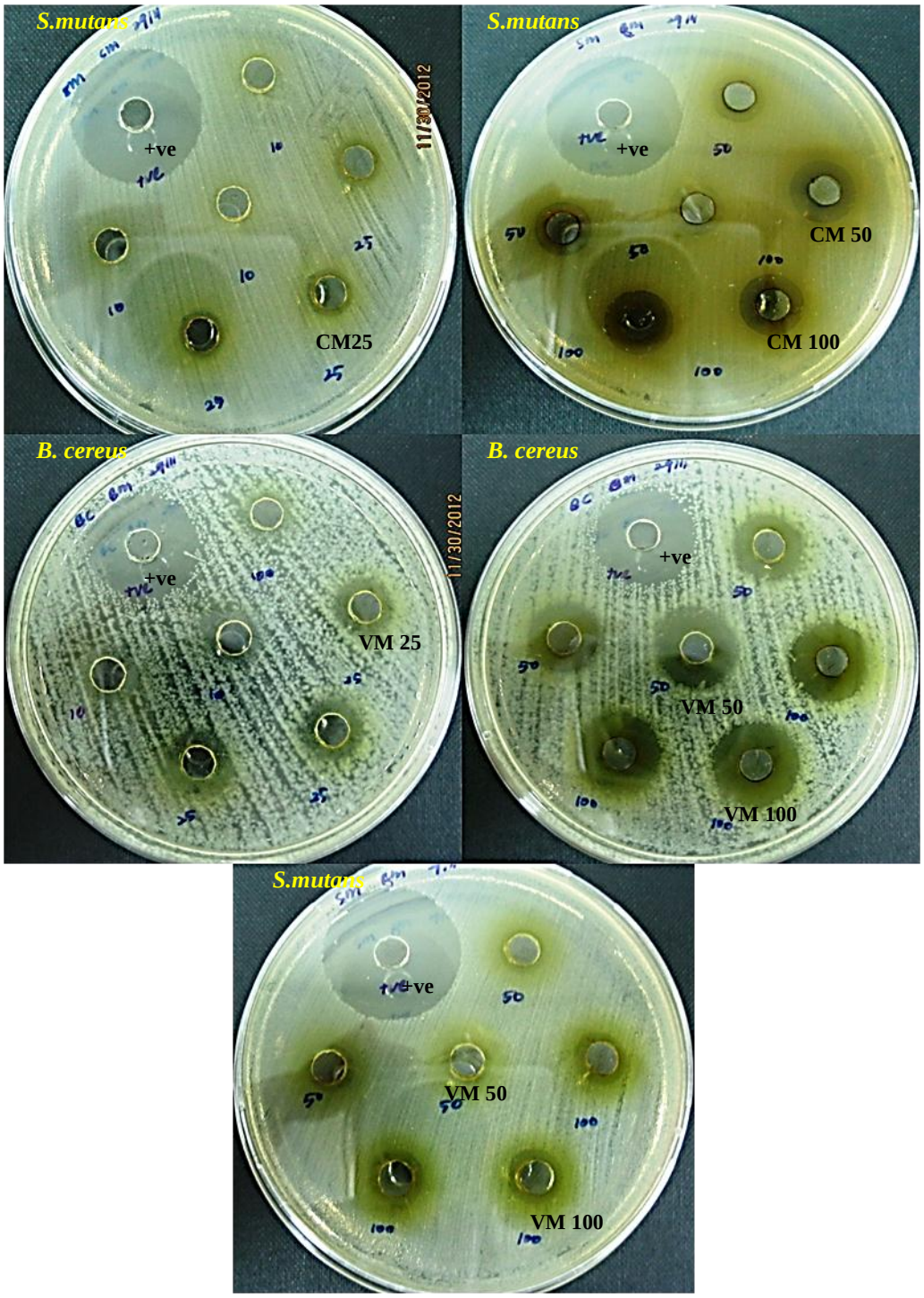

Figure 4d: Well diffusion assay for *C. asiatica* and *V. amaygdalina* methanolic extract against test microorganisms

+ve – Positive control; CM 25 - *C. asiatica* 25 mg/ml; CM 50 - *C. asiatica* 50 mg/ml; CM 100 - *C. asiatica* 100 mg/ml VM 25 – *V. amygdalina* 25 mg/ml; VM 50 - *V. amygdalina* 50 mg/ml and VM 100 - *V. amygdalina* 100 mg/ml of methanol extract.

Appendix E. BLAST Query Search from the NCBI Database for the DNA Sequence of the Selected Medicinal Plants

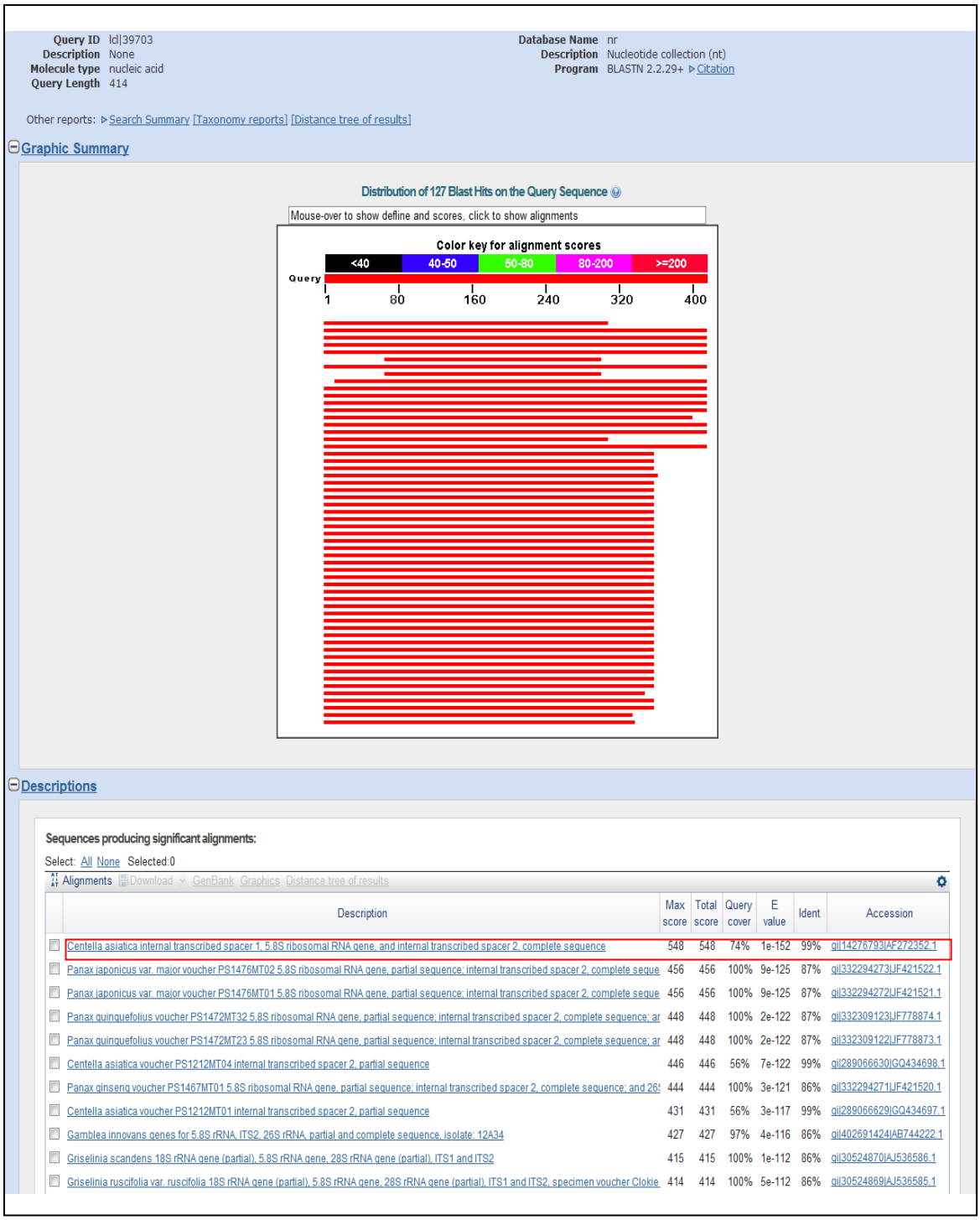

Figure 5a: BLAST query search from NCBI database of the 414 nucleotide of the PCR product. The query results list different sequences. Sequence homology of 99% was obtained for *Centella asiatica* ( ).

Appendix E continued

Centella asiatica internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence

GenBank: AF272352.1

FASTA Graphics PopSet

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  ORGANISM  Centella asiatica
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REFERENCE   1  (bases 1 to 626)
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  TITLE     Relationships among 'ancient araliads' and their significance for
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  JOURNAL   Mol. Phylogenet. Evol. 19 (2), 259-276 (2001)
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  TITLE     Direct Submission
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Figure 5b: Detailed information of *Centella asiatica* (AF272352).
The BLAST query of the ITS2 region with the 99% homology showed *Centella asiatica*.

Appendix E continued

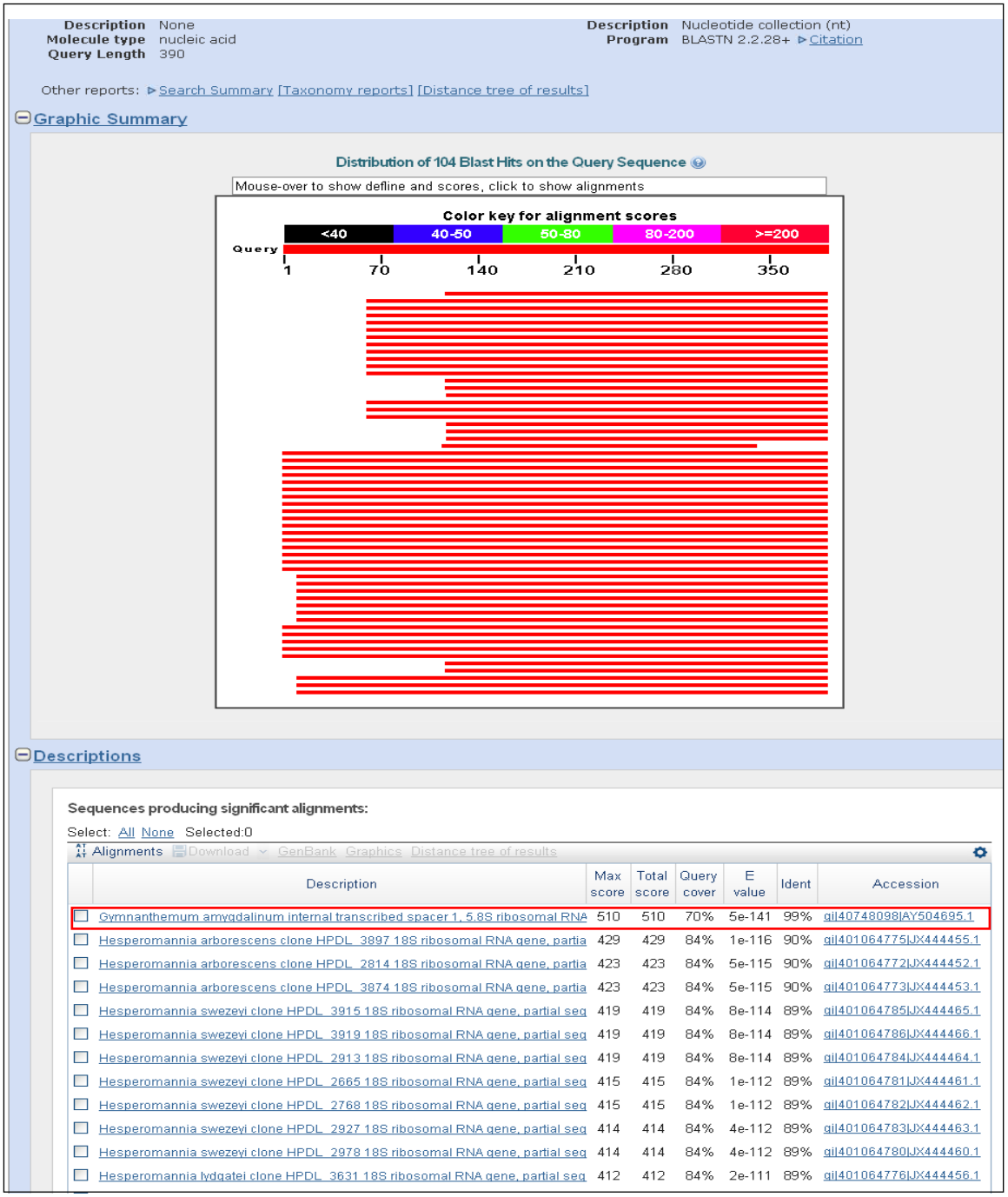

Figure 5c: BLAST query search from NCBI database of the 390 nucleotide of the PCR product. The query results list different sequences. Sequence homology of 99% was obtained for *Gymnanthemum amygdalinnum*/*Vernonia amygdalina* ( ).

Appendix E continued

Gymnanthemum amygdalinum internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence

GenBank: AY504695.1

FASTA Graphics PopSet

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  AUTHORS   Funk,V.A., Chan,R. and Keeley,S.C.
  TITLE     Insights into the evolution of the tribe Arctoteae (Compositae:
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  JOURNAL   Taxon 53 (3), 637-655 (2004)
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  AUTHORS   Funk,V.A., Chan,R. and Keeley,S.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-DEC-2003) Botany, National Museum of Natural History,
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Figure 5d: Detailed information of *Gymnanthemum amygdalinum* (AY504695).
The BLAST query of the ITS2 region with the 99% homology showed *Gymnanthemum amygdalinum*.

Appendix F. Total Phenolic Content (TPC) Determination by Folin-Ciocalteu Assay

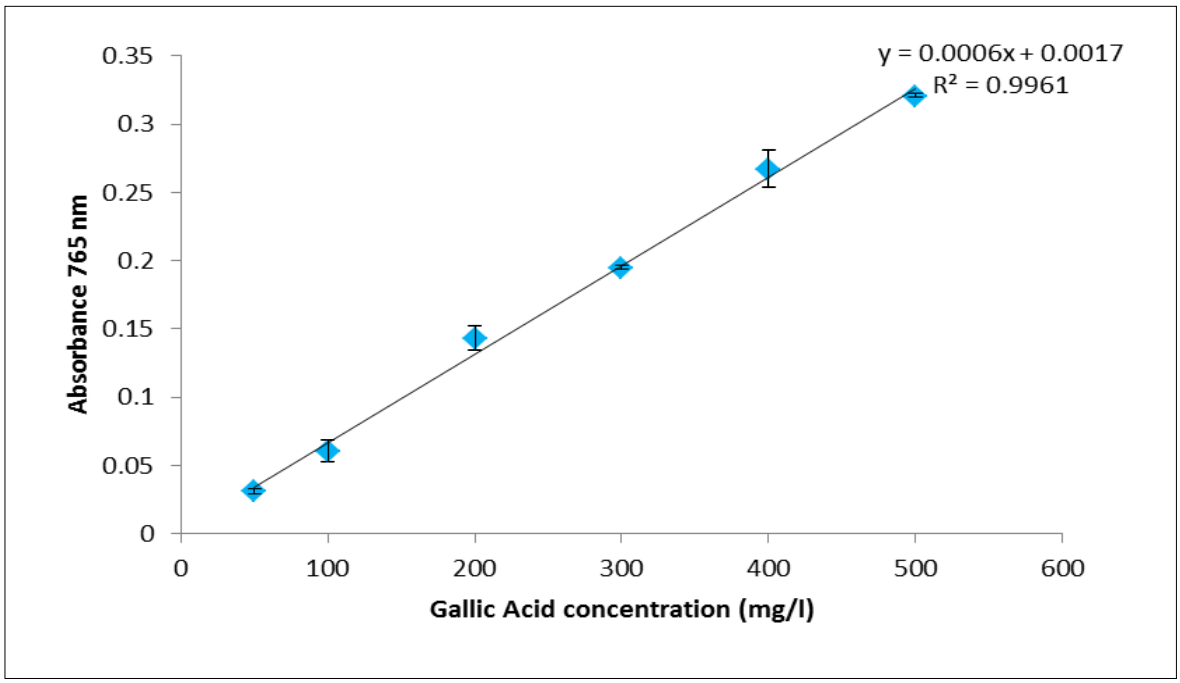

Figure 6: Gallic acid standard curve in Folin-Ciocalteu Assay
Graph shows the gallic acid standard curve in Folin-Ciocalteu assay whereby absorbance at 765nm was plot against concentration of gallic acid in mg/litre for (50-500 mg/l) concentration of gallic acid. Gallic acid standard was used to estimate the total phenolic content in plant extracts by the Folin-Ciocalteu colorimetric method. The assay was performed in trplicate and results were presented as mean ± SD.

Table 1: Total phenolic content of *Centella asiatica* and *Vernonia amygdalina*

Medicinal plant of study	Total phenolic content (mg GAE/g d.w.)		
	Aqueous	Ethanol	Methanol
Centella asiatica	1.120±0.063[a]	4.006±0.032[b]	3.346±0.029[c]
Vernonia amygdalina	1.031±0.058[de]	0.974±0.049[d]	1.168±0.101[e]

Total phenolic content was determined based on Folin-Ciocalteu's method for *C. asiatica* and *V. amygdalina* expressed as milligram gallic acid equivalent per gram dry weight (mg GAE/g d.w.). Results were presented as mean ± SD; n = 3. The mean changes between the samples were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test. Samples represented by different alphabets are significantly different ($p < .05$) from each other.

Appendix G. Results of DPPH Radical Scavenging Activity

Table 2: Percentage inhibition of DPPH radicals by sample/positive control/standard at varying concentrations (μg/ml).

Sample[a]	Concentration (μg/ml)[b]						
	25	50	75	100	200	300	400
C. asiatica aqueous	-0.30±1.89	3.52±0.50	1.85±1.61	3.49±0.27	4.64±2.17	7.15±1.26	8.28±0.74
C. asiatica ethanol	11.39±0.94	25.28±1.62	27.50±1.08	36.72±0.43	64.58±0.28	86.44±0.13	88.83±0.23
C. asiatica methanol	7.33±3.63	16.35±3.19	21.82±2.03	26.56±0.87	46.49±0.63	65.59±0.56	73.17±0.71
V.amygdalina aqueous	4.93±7.56	6.56±5.43	2.21±1.19	1.48±0.27	8.17±8.76	7.72±4.74	5.95±1.37
V.amygdalina ethanol	5.36±6.85	8.30±6.28	9.74±6.10	10.72±5.12	15.62±5.37	21.03±6.58	26.07±5.32
V.amygdalina methanol	7.60±10.81	8.53±8.29	8.58±8.18	10.76±7.38	15.94±7.69	20.88±6.09	25.97±4.39
Ascorbic acid	13.90±1.97	29.29±1.02	46.41±0.71	90.73±0.30	90.56±0.29	90.57±0.32	90.49±0.69
BHT	-7.38±1.40	-3.58±1.05	0.19±0.72	5.43±2.95	13.10±1.44	21.38±0.92	26.34±1.47
Trolox	23.28±3.25	39.38±1.43	49.31±0.85	60.13±1.51	90.51±0.21	90.68±0.19	90.80±0.28

Table presents the percentage inhibition of DPPH radicals by samples *C. asiatica* and *V. amygdalina* aqueous, ethanolic and methanolic extracts; positive controls ascorbic acid and BHT; and standard Trolox.

[a]Results were presented as mean ± SD, where n = 3.

[b]Concentration of sample/positive control and standard was (25-400 μg/ml)

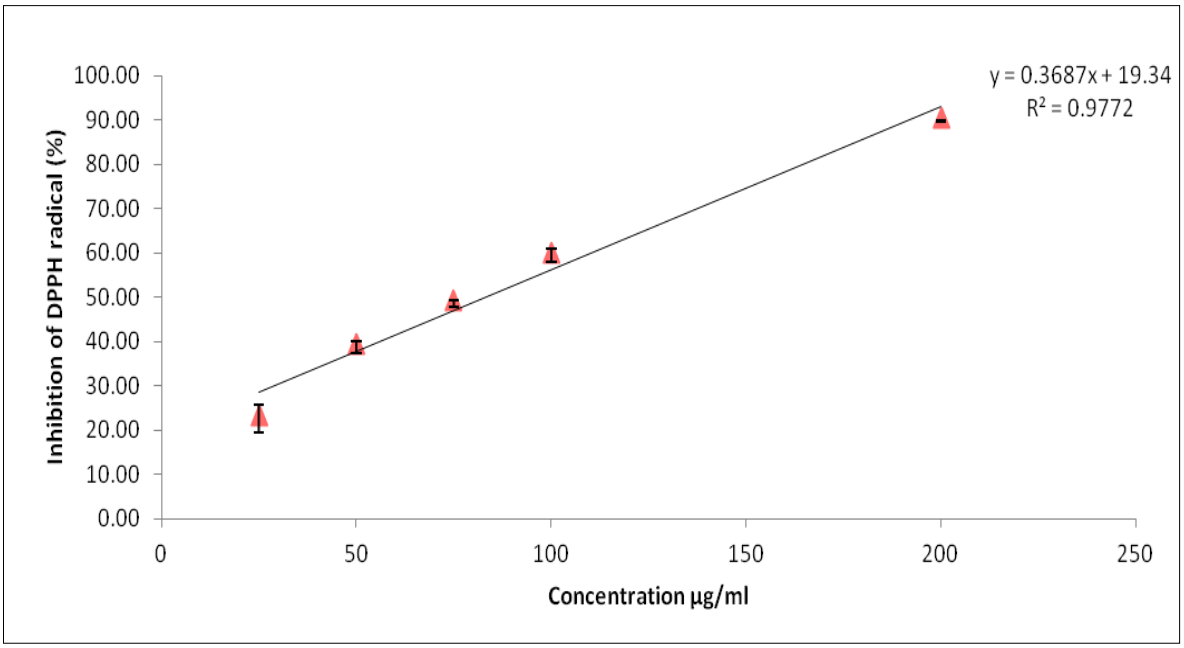

Figure 7a: Trolox standard curve with DPPH assay

Graph depicts the trolox standard curve in DPPH assay whereby the percentage inhibition of DPPH radical was plot against concentration (25-200μg/ml) of trolox. Trolox standard curve was used to determine the antioxidant activity of samples, expressed as milimole Trolox equivalent antioxidant capacity per gram dried weight of plant sample (mmol TE/g d.w.). Results were presented as mean ± SD, where n = 3.

Appendix G continued

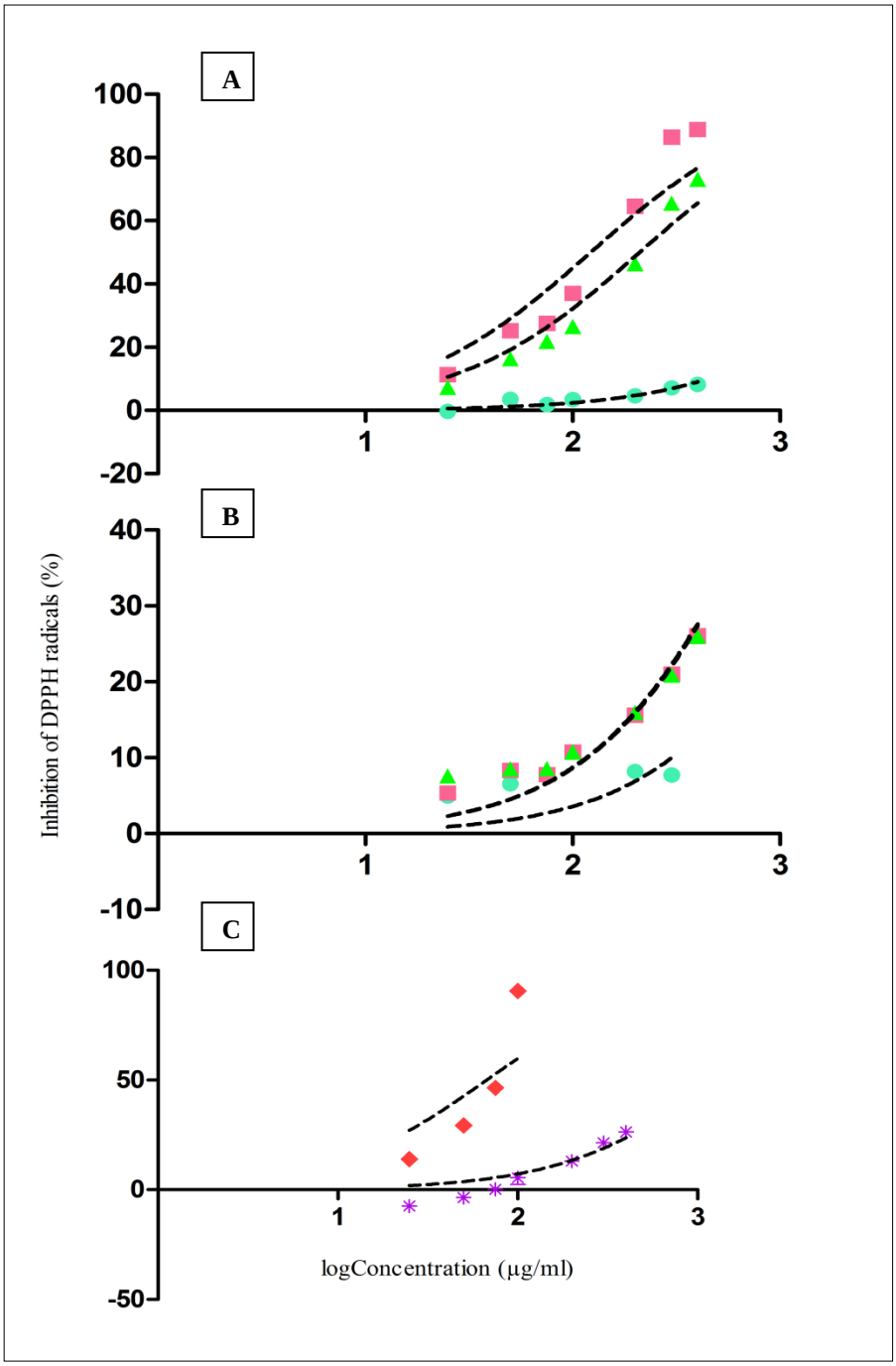

Figure 7b: Non-linear regression analysis of *C. asiatica* (A), *V. amygdalina* (B) extracts and positive control (C) used to determine IC_{50} of DPPH assay

Graph depicts the non-linear regression curve of *C. asiatica* and *V. amygdalina* aqueous (●), ethanolic (■) and methanolic (▲) extracts and positive control ascorbic acid (◆) and BHT (✳) used to determine IC_{50}. The percentage inhibition of DPPH radical was plot against log concentration (1.3 – 2.6 µg/ml). Results were presented as mean ± SD, where n = 3.

Appendix H. Results of FRAP Assay for the Extracts of *C. asiatica* and *V. amygdalina*

Table 3a: Ferric Reducing Antioxidant Power (FRAP) activity in 1 mg/ml of sample and positive control

Time (s)	Absorbance 595nm[a]							
	Centella asiatica			*Vernonia amygdalina*			Ascorbic acid	BHT
	aqueous	ethanolic	methanolic	aqueous	ethanolic	methanolic		
0	0.037±0.004	0.421±0.011	0.466±0.012	0.041±0.003	0.093±0.006	0.142±0.020	3.224±0.103	0.048±0.017
15	0.050±0.003	0.447±0.014	0.496±0.009	0.058±0.001	0.104±0.006	0.170±0.007	3.290±0.036	0.051±0.014
30	0.057±0.005	0.460±0.014	0.512±0.009	0.067±0.001	0.111±0.006	0.183±0.007	3.310±0.037	0.064±0.011
45	0.062±0.005	0.468±0.013	0.521±0.009	0.073±0.002	0.116±0.006	0.192±0.008	3.301±0.021	0.079±0.011
60	0.066±0.005	0.477±0.013	0.527±0.010	0.079±0.004	0.121±0.009	0.198±0.009	3.315±0.011	0.095±0.012
90	0.068±0.005	0.482±0.012	0.532±0.010	0.082±0.005	0.124±0.010	0.202±0.011	3.316±0.014	0.109±0.013
120	0.071±0.006	0.487±0.011	0.538±0.012	0.086±0.005	0.127±0.010	0.207±0.012	3.326±0.009	0.124±0.016
150	0.074±0.006	0.490±0.013	0.542±0.013	0.089±0.006	0.130±0.011	0.211±0.012	3.334±0.014	0.138±0.017
180	0.079±0.005	0.494±0.012	0.547±0.013	0.092±0.275	0.132±0.011	0.215±0.012	3.302±0.033	0.152±0.017
210	0.081±0.005	0.498±0.013	0.550±0.013	0.094±0.006	0.134±0.011	0.218±0.012	3.304±0.011	0.166±0.018
240	0.084±0.005	0.502±0.014	0.555±0.012	0.096±0.006	0.137±0.011	0.222±0.012	3.318±0.021	0.179±0.017

Table depicts Ferric Reducing Antioxidant Power (FRAP) assay of *C. asiatica* and *V. amygdalina* aqueous, ethanolic and methanolic extracts and positive controls ascorbic acid and BHT at 1 mg/ml concentration. Absorbance was recorded at 595 nm.

[a]Assay was performed in trplicate and results presented as mean ± SD.

Table 3b: Ferric Reducing Antioxidant Power (FRAP) activity in 0.5 mg/ml of sample and positive control

Time (s)	Absorbance 595nm[a]							
	Centella asiatica			*Vernonia amygdalina*			Ascorbic acid	BHT
	aqueous	ethanolic	methanolic	aqueous	ethanolic	methanolic		
0	0.025±0.003	0.211±0.003	0.258±0.012	0.025±0.002	0.045±0.003	0.084±0.008	2.192±0.090	0.007±0.007
15	0.034±0.001	0.220±0.002	0.265±0.006	0.035±0.004	0.051±0.001	0.087±0.003	2.242±0.086	0.006±0.004
30	0.037±0.003	0.225±0.002	0.272±0.004	0.039±0.005	0.055±0.003	0.092±0.002	2.246±0.086	0.010±0.005
45	0.039±0.004	0.228±0.003	0.275±0.005	0.043±0.007	0.056±0.003	0.095±0.001	2.253±0.087	0.016±0.008
60	0.041±0.004	0.231±0.003	0.277±0.006	0.044±0.006	0.057±0.003	0.098±0.002	2.251±0.087	0.025±0.010
90	0.044±0.003	0.238±0.006	0.284±0.008	0.047±0.001	0.060±0.004	0.103±0.009	2.261±0.095	0.053±0.038
120	0.045±0.004	0.240±0.006	0.286±0.007	0.048±0.001	0.061±0.005	0.105±0.009	2.268±0.104	0.062±0.039
150	0.046±0.003	0.242±0.006	0.287±0.008	0.049±0.001	0.061±0.005	0.106±0.009	2.274±0.102	0.070±0.039
180	0.046±0.003	0.244±0.005	0.288±0.007	0.049±0.002	0.061±0.005	0.108±0.009	2.284±0.099	0.077±0.039
210	0.048±0.003	0.246±0.005	0.291±0.007	0.051±0.001	0.062±0.005	0.109±0.009	2.287±0.104	0.084±0.040
240	0.049±0.004	0.250±0.005	0.295±0.007	0.053±0.001	0.064±0.006	0.112±0.010	2.295±0.104	0.094±0.044

Table depicts Ferric Reducing Antioxidant Power (FRAP) assay of *C. asiatica* and *V. amygdalina* aqueous, ethanolic and methanolic extracts and positive controls ascorbic acid and BHT at 0.5mg/ml concentration. Absorbance was recorded at 595 nm.

[a]Assay was performed in trplicate and results presented as mean ± SD.

Appendix H continued

Table 3c: Ferric Reducing Antioxidant Power (FRAP) activity of standard ferrous sulphate ($FeSO_4.7H_2O$) at varying concentrations

Concentration (µmol/L)	100	200	400	600	800	1000
Absorbance 595nm	0.015±0.023	0.055±0.012	0.119±0.036	0.208±0.012	0.089±0.049	0.044±0.025

Table depicts Ferric Reducing Antioxidant Power (FRAP) activity of standard ferrous sulphate (100-1000 μmol/L) at absorbance 595 nm which was used to plot the $FeSO_4.7H_2O$ standard curve. Results were presented as mean ± SD, where n = 3.

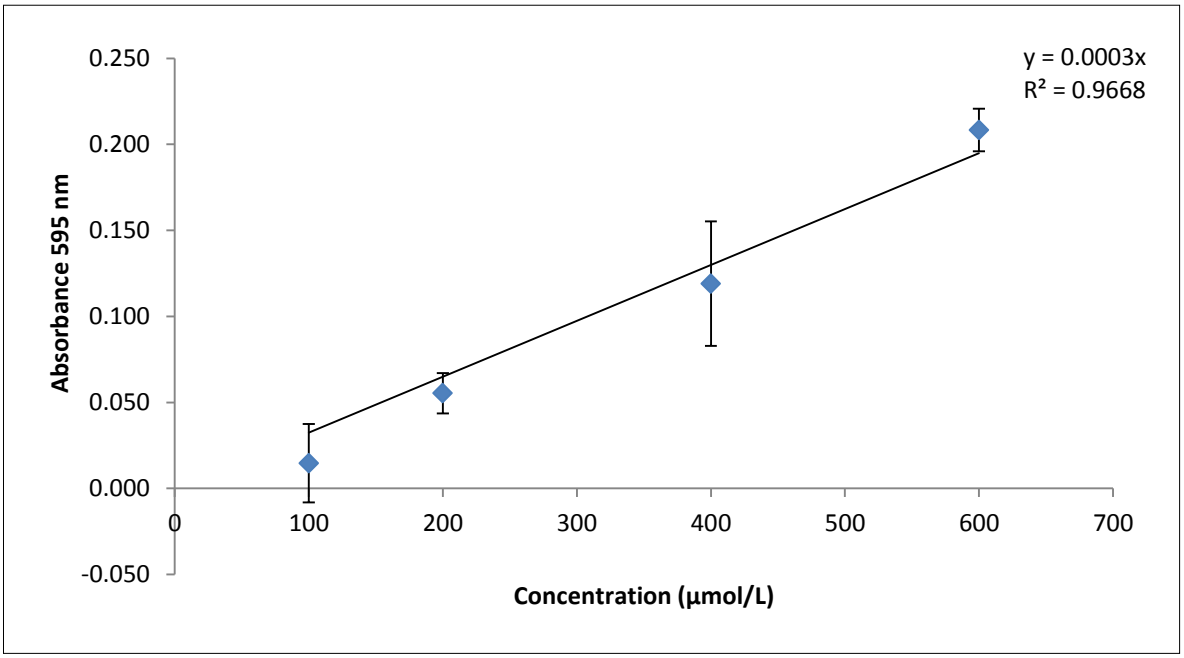

Figure 8: Ferrous sulphate ($FeSO_4.7H_2O$) standard curve with FRAP assay

Graph depicts the ferrous sulphate ($FeSO_4.7H_2O$) standard curve in FRAP assay whereby the absorbance at 595 nm was plot against concentration (100-600 μmol/L) of $FeSO_4.7H_2O$. Standard curve was used to determine the antioxidant activity of samples, expressed as milimole ferrous sulphate per gram dried weight (mmol Fe^{2+} /g d.w.). Results were presented as mean ± SD, where n = 3.

Appendix I. Results for the Protective Effect of *C. asiatica* and *V. amygdalina* Extracts against Hydrogen Peroxide Induced Haemolysis of Rabbit Erythrocytes

Table 4a: Percentages of the *in vitro* protective effect of *C. asiatica* and *V. amygdalina* against 10 mM H_2O_2 induced haemolysis of rabbit erythrocytes.

Sample[a]	Concentration (mg/ml)				
	0.1	0.25	0.5	0.75	1.0
C.asiatica aqueous	77.44±2.28	74.16±2.01	75.83±6.76	74.16±2.01	76.47±0.79
C.asiatica ethanol	73.22±2.37	74.30±1.64	81.03±1.00	81.19±1.94	85.12±1.69
C.asiatica methanol	73.60±1.27	74.27±1.36	80.21±2.05	82.95±1.14	88.14±0.83
V. amygdalina aqueous	75.73±1.23	72.29±0.54	70.38±1.06	71.02±0.52	72.92±0.77
V. amygdalina ethanol	73.74±1.82	74.46±0.61	75.88±0.52	78.75±0.41	78.46±0.35
V. amygdalina methanol	70.23±0.71	73.30±0.20	73.39±1.45	74.27±0.06	79.25±0.64
Ascorbic acid	71.40±0.51	76.69±0.20	79.69±0.75	82.69±0.21	86.05±0.66
Phosphate buffer			72.44±1.04		
Distilled water			0.00±0.00		

Table depicts *in vitro* anti-haemolysis assay of *C. asiatica* and *V. amygdalina* aqueous, ethanolic and methanolic extracts and positive control ascorbic acid at (0.1 – 1.0 mg/ml) concentrations whereby10 mM H_2O_2 was used to induce haemolysis. Isotonic phosphate buffer was the standard used. Distilled water showed 100% haemolysis.

[a]Assay was performed in trplicate and results were presented as mean ± SD.

Table 4b: Percentages of the *in vitro* protective effect of *C. asiatica* and *V. amygdalina* against 50 mM H_2O_2 induced haemolysis of rabbit erythrocytes.

Sample[a]	Concentration (mg/ml)				
	0.1	0.25	0.5	0.75	1.0
C.asiatica aqueous	67.79±1.11	72.54±0.88	69.35±2.03	69.85±0.40	71.61±0.53
C.asiatica ethanol	54.93±1.34	74.53±1.64	75.69±0.42	83.27±0.54	84.73±0.65
C.asiatica methanol	60.34±0.98	66.32±0.62	78.06±2.23	85.87±0.40	91.81±0.55
V. amygdalina aqueous	55.73±0.34	51.76±0.80	59.69±1.62	63.73±0.51	57.55±1.46
V. amygdalina ethanol	62.59±1.16	68.21±0.39	71.84±1.90	75.41±0.63	78.10±0.26
V. amygdalina methanol	60.85±0.86	61.28±0.31	67.22±0.83	70.51±0.37	81.77±0.23
Ascorbic acid	52.70±1.32	64.63±0.65	61.71±1.83	62.74±0.15	71.97±0.76
Phosphate buffer			57.13±0.80		
Distilled water			0.00±0.00		

Table depicts *in vitro* anti-haemolysis assay of *C. asiatica* and *V. amygdalina* aqueous, ethanolic and methanolic extracts and positive control ascorbic acid at (0.1 – 1.0 mg/ml) concentrations whereby 50 mM H_2O_2 was used to induce haemolysis. Isotonic phosphate buffer was the standard used. Distilled water showed 100% haemolysis.

[a]Assay was performed in trplicate and results were presented as mean ± SD.

Appendix I continued

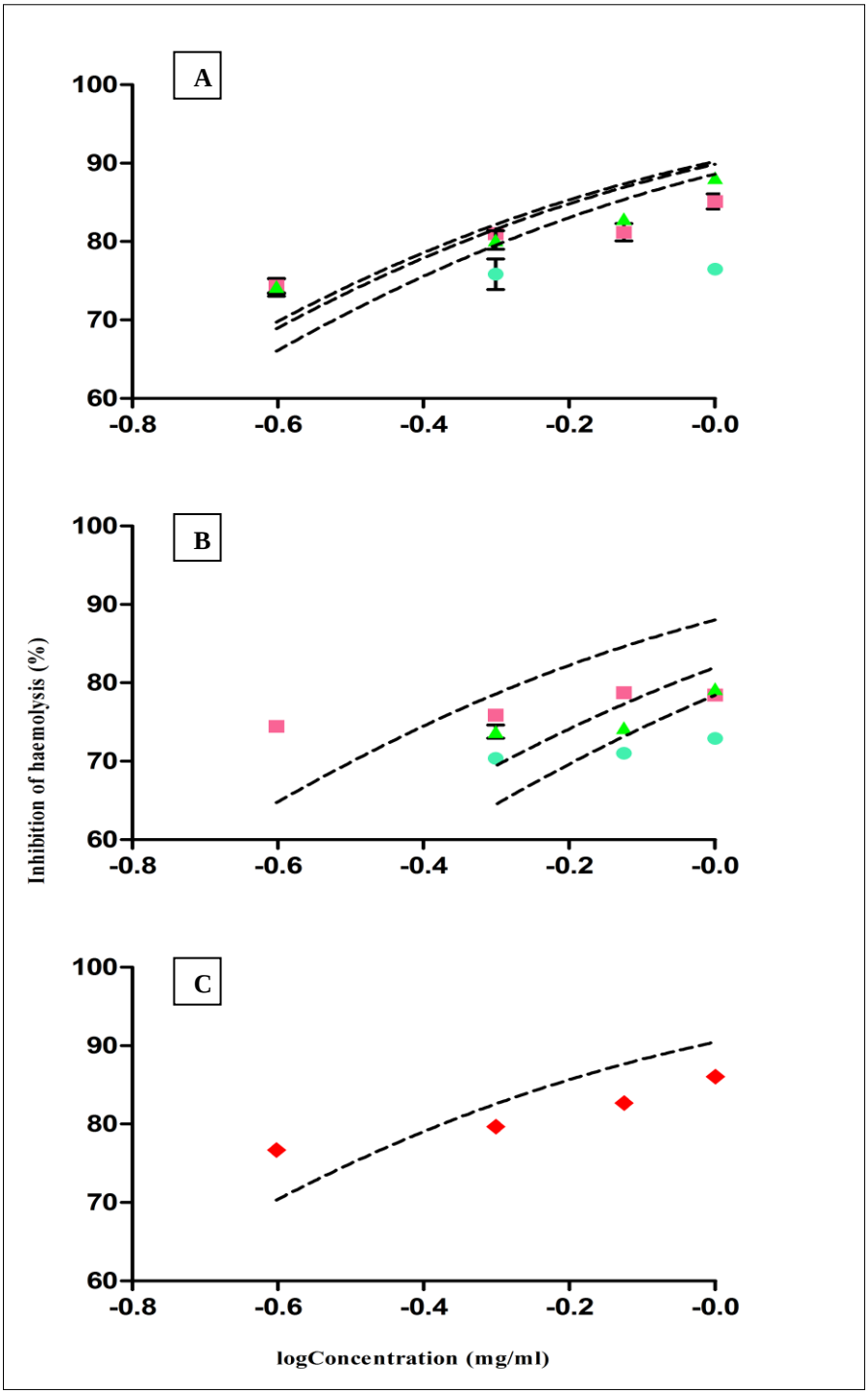

Figure 9a: Non-linear regression analysis of *C. asiatica* (A) *V. amygdalina* (B) extracts and positive control (C) against 10 mM H_2O_2 used to determine IC_{50} of *in vitro* haemolysis assay

Graph depicts the non-linear regression curve of *C. asiatica* and *V. amygdalina* aqueous ( ), ethanolic ( ) and methanolic ( ) extracts and positive control ascorbic acid ( ) used to determine IC_{50}. The percentage inhibition of haemolysis was plot against log concentration (-1 - 0 mg/ml). Results were presented as mean ± SD, where n = 3.

Appendix I continued

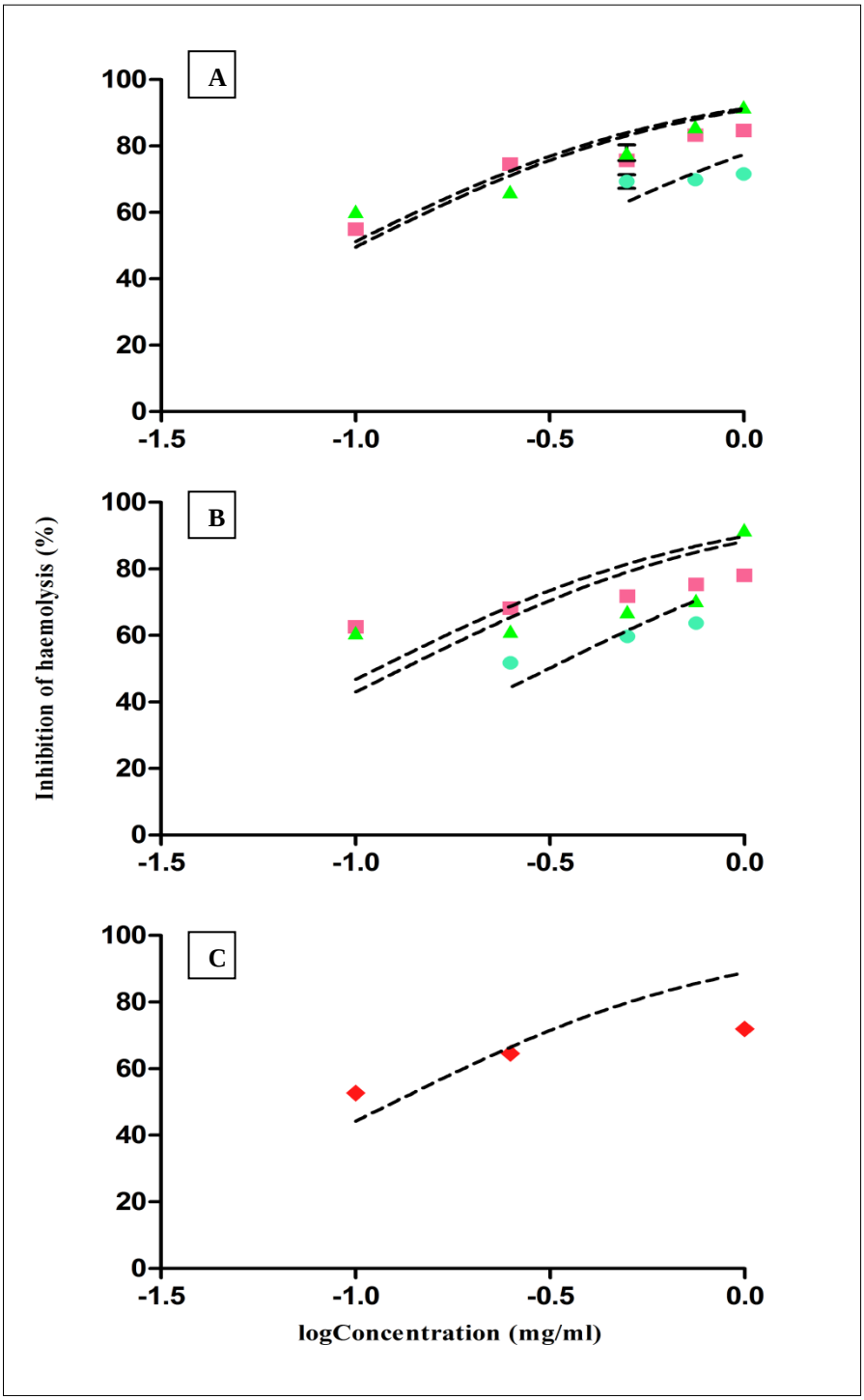

Figure 9b: Non-linear regression analysis of *C. asiatica* (A) and *V. amygdalina* (B) extracts and positive control (C) against 50 mM H_2O_2 used to determine IC_{50} of *in vitro* haemolysis assay

Graph depicts the non-linear regression curve of *C. asiatica* and *V. amygdalina* aqueous (●), ethanolic (■) and methanolic (▲) extracts and positive control ascorbic acid (◆) used to determine IC_{50}. The percentage inhibition of haemolysis was plot against log concentration (-1-0 mg/ml). Results were presented as mean ± SD, where n = 3.

Appendix I continued

Table 4c: *In vitro* anti-haemolysis effect of standard ascorbic acid at varying concentrations

Concentration (mg/ml)	0.1	0.25	0.5	0.75	1.0
Anti-haemolysis (%)	52.70±1.32	64.63±0.65	61.71±1.83	62.74±0.15	71.97±0.76

Table depicts anti-haemolysis effect of standard ascorbic acid (0.1-1.0 mg/ml) at absorbance 540 nm which was used to plot the standard curve. Results were presented as mean ± SD, where n = 3.

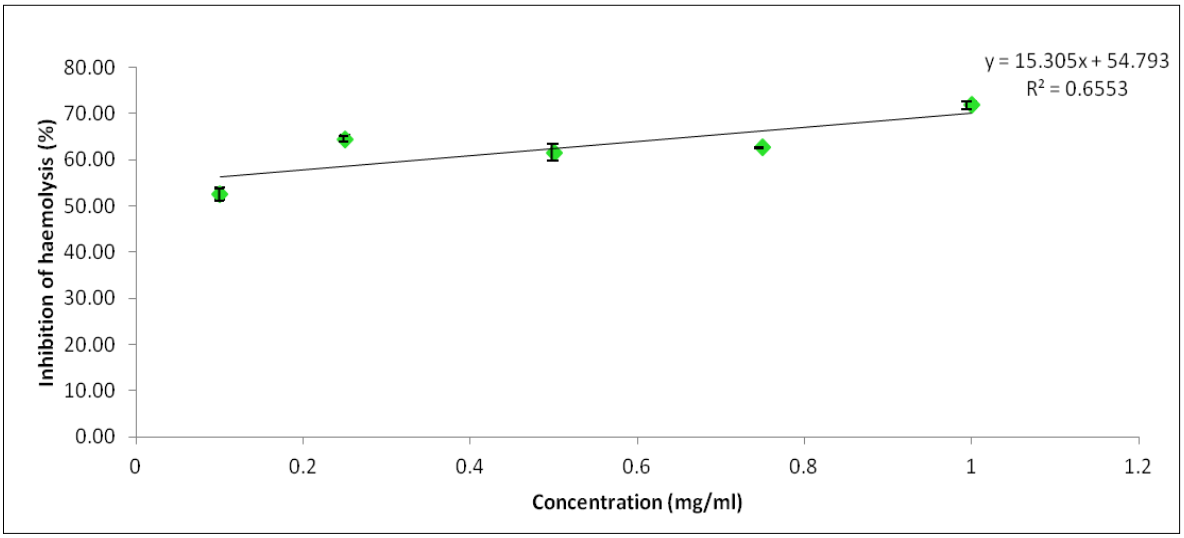

Figure 9c: Ascorbic acid standard curve with anti-haemolysis assay
Graph depicts the ascorbic acid standard curve in anti-haemolysis assay whereby the percentage inhibition of haemolysis was plot against concentration (0.1-1.0 mg/ml) of ascorbic acid. Standard curve was used to determine the antioxidant activity of samples, expressed as milimole ascorbic acid equivalent per gram of dried weight (mmole AAE/g dried weight). Results were presented as mean ± SD, where n = 3.

Appendix J. Homogenous Peak Test of the Methanolic Extract of *C. asiatica*

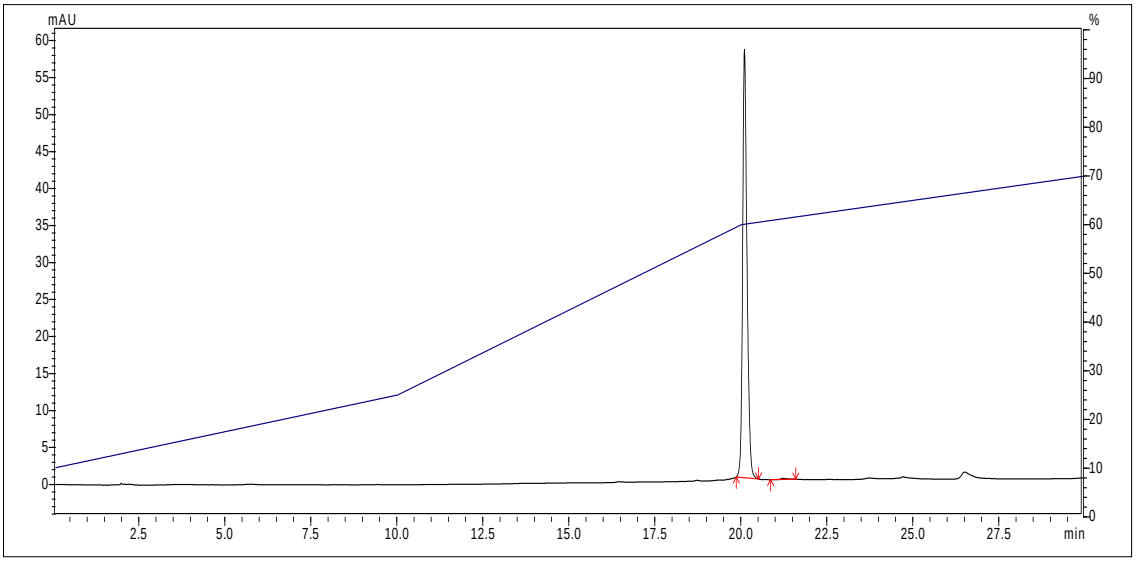

Figure 10a: Homogenous peak test of *C. asiatica* methanolic extract with chloramphenicol standard.
Chromatogram displays peak obtained by spiking the plant sample with chloramphenicol standard at 280 nm wavelength.

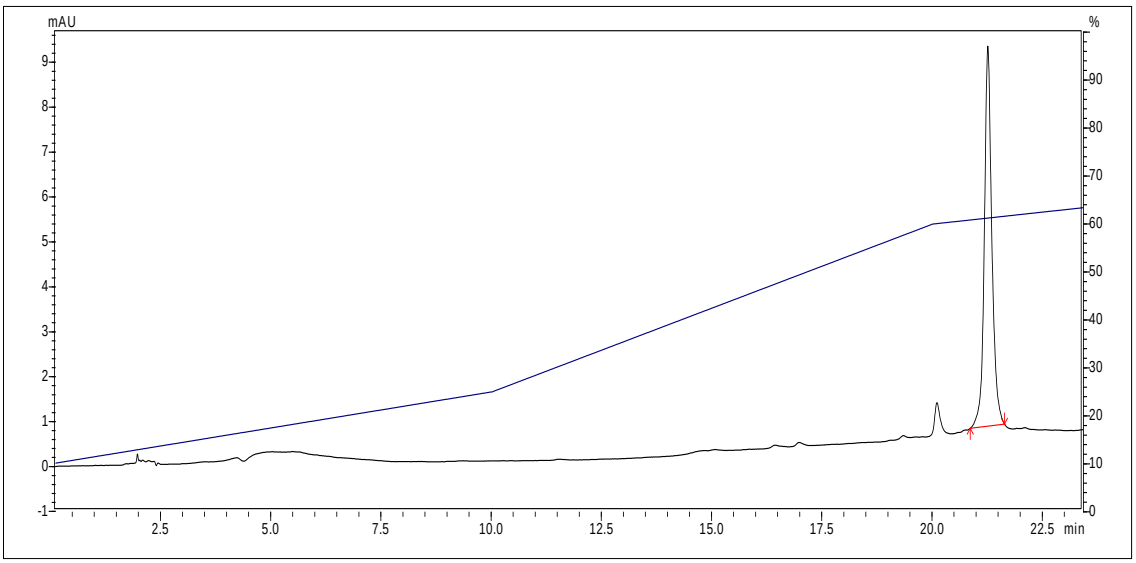

Figure 10b: Homogenous peak test of *C.asiatica* methanolic extract with chloramphenicol standard.
Chromatogram displays peak obtained by spiking the plant sample with benzoic acid standard at 280 nm wavelength.

Appendix K. LC-MS Mass Spectrum of the Extracts of *C. asiatica V. amygdalina*

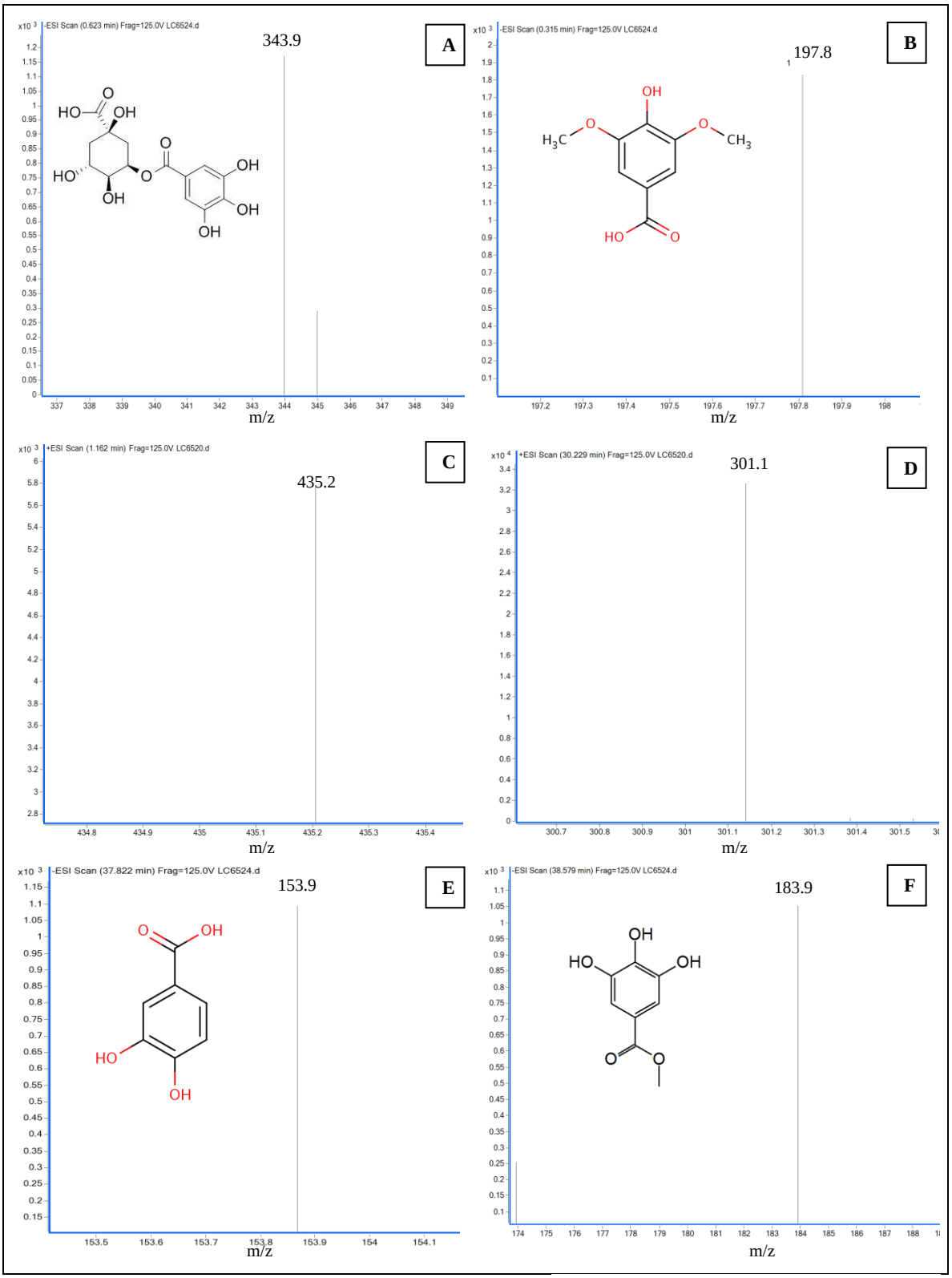

Figure 11a: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *C.asiatica* methanolic extract. Mass spectra from full scan analysis by LC-MS and their molecular structure of 5-galloylquinic acid (**A**); syringic acid (**B**); ellagic acid pentoside (**C**); hydroxybenzoic acid-*O*-hexoside (**D**); protocatechuic acid (**E**); and methyl gallate (**F**)

Appendix K continued

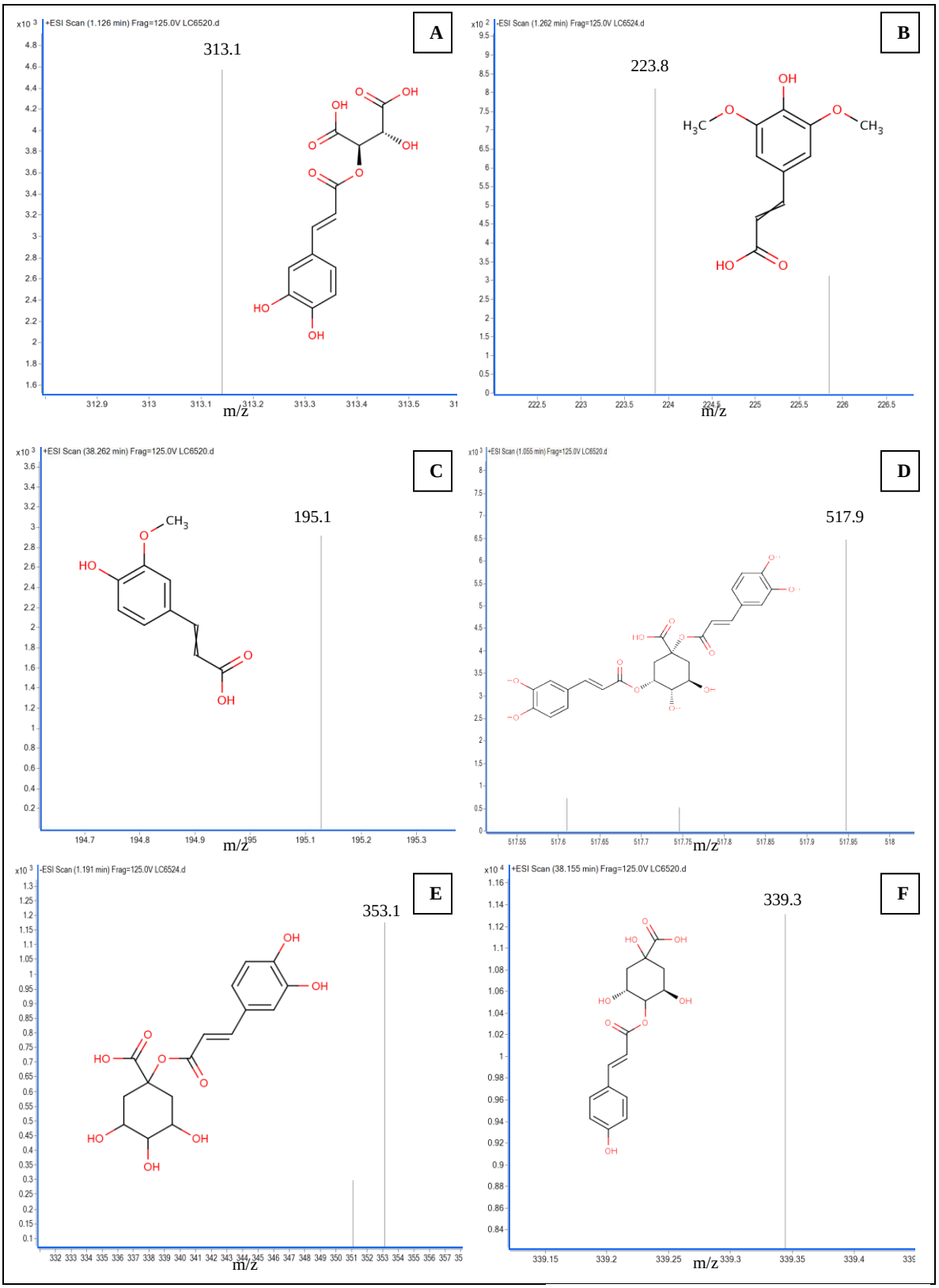

Figure 11b: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *C.asiatica* methanolic extract. Mass spectra from full scan analysis by LC-MS and their molecular structure of caftaric acid (**A**); sinapic acid (**B**); ferulic acid (**C**); dicaffeoylquinic acid (**D**); caffeoylquinic acid (**E**); and coumaroylquinic acid (**F**)

Appendix K continued

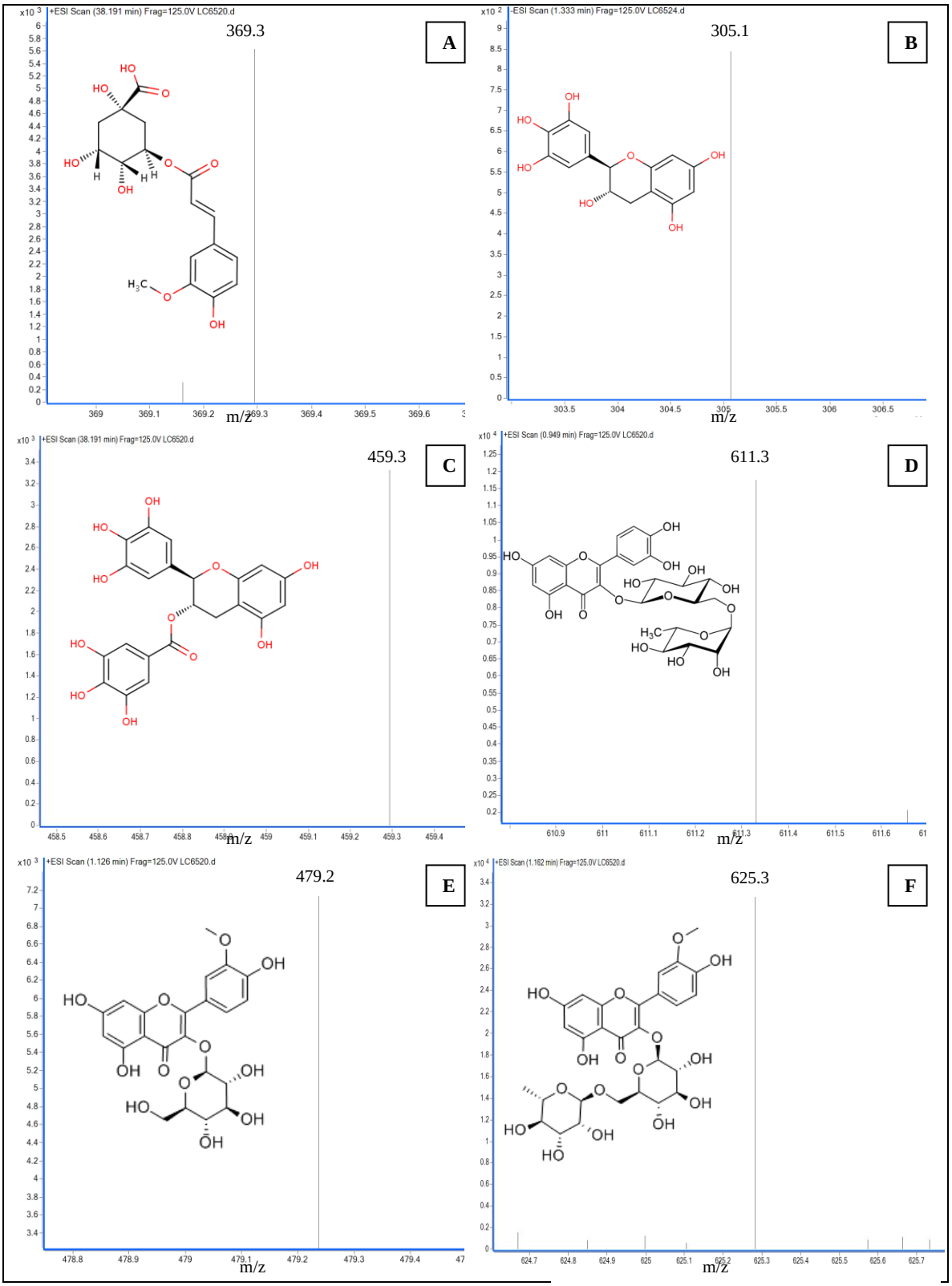

Figure 11c: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *C.asiatica* methanolic extract. Mass spectra from full scan analysis by LC-MS and their molecular structure of feruloylquinic acid (**A**); (-)-gallocatechin (**B**); (-)-gallocatechin gallate (**C**); rutin (**D**); isorhamnetin-3-*O*-glucoside (**E**); and isorhamnetin-3-*O*-rutinoside (**F**)

Appendix K continued

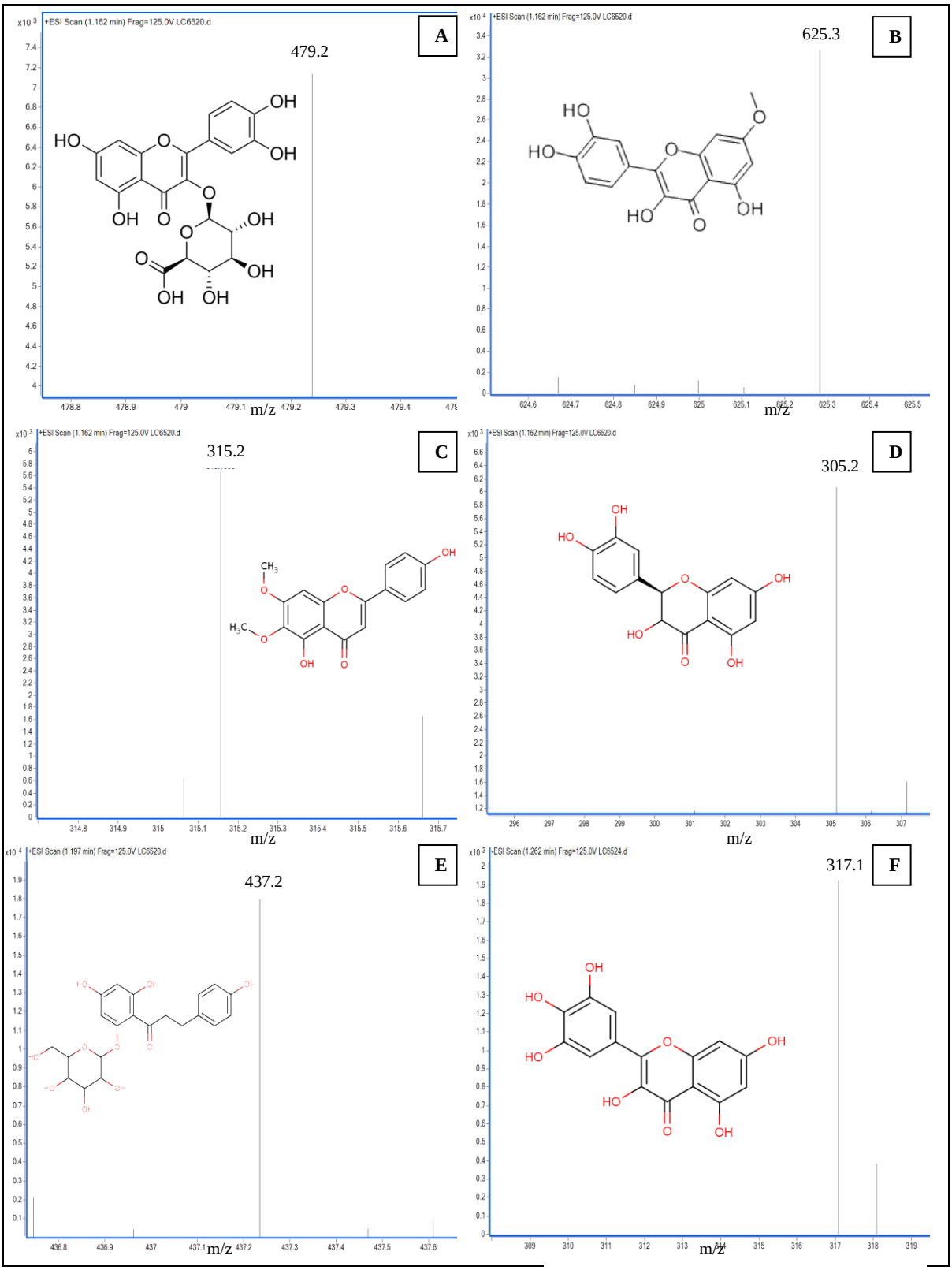

Figure 11d: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *C.asiatica* methanolic extract. Mass spectra from full scan analysis by LC-MS and their molecular structure of quercetin-3-*O*-glucuronide (**A**); rhamnetin-*O*-rutinoside (**B**); cirsimaritin (**C**); (±)-taxifolin (**D**); phloridzin (**E**); and myricetin (**F**)

Appendix L continued

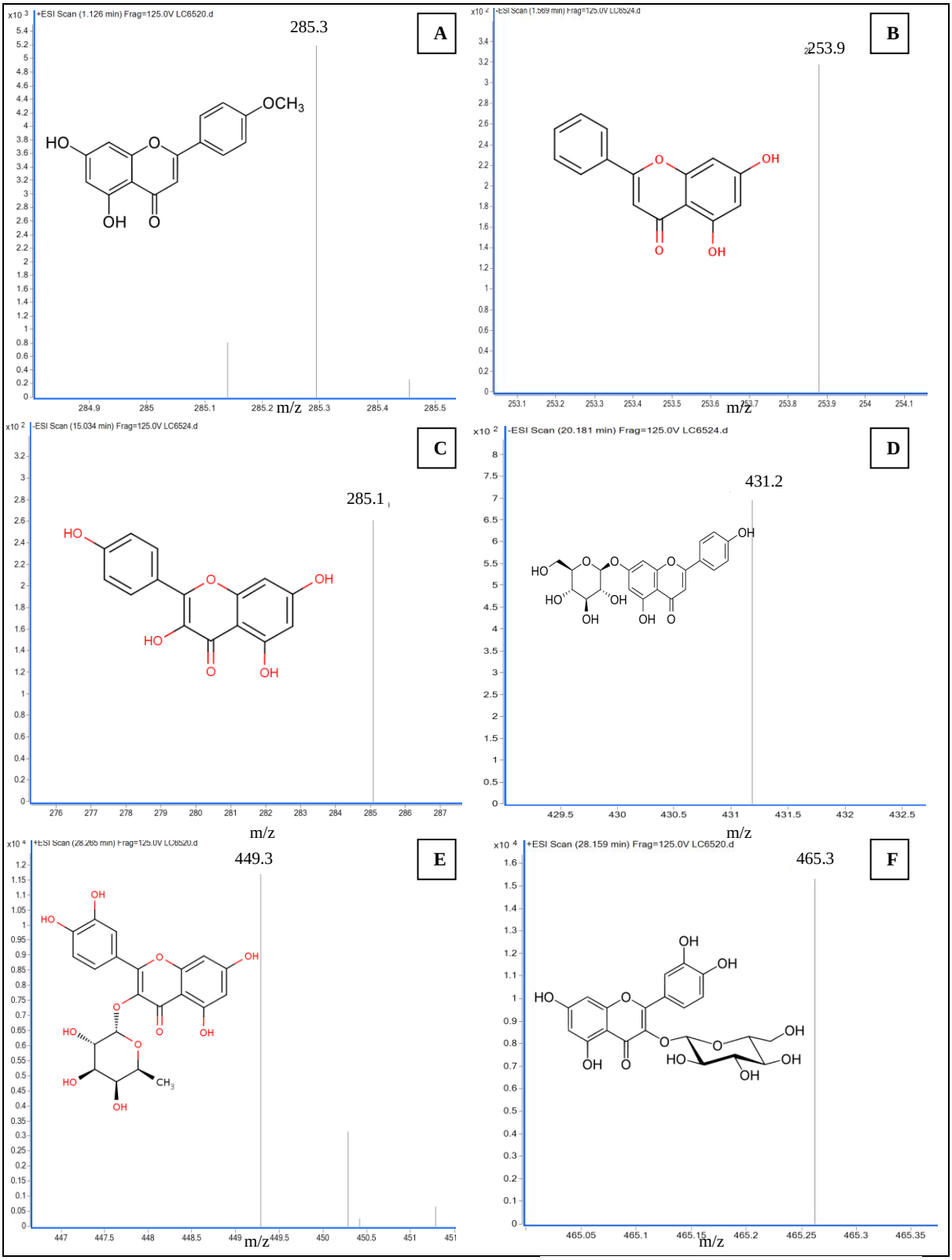

Figure 11e: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *C.asiatica* methanolic extract. Mass spectra from full scan analysis by LC-MS and their molecular structure of acacetin (**A**); chrysin (**B**); kaempferol (**C**); apigenin-7-*O*-glucoside (**D**)**;** quercetin 3-*O*-rhamnoside (**E**); and quercetin-3-*O*-glucoside (**F**)

Appendix k continued

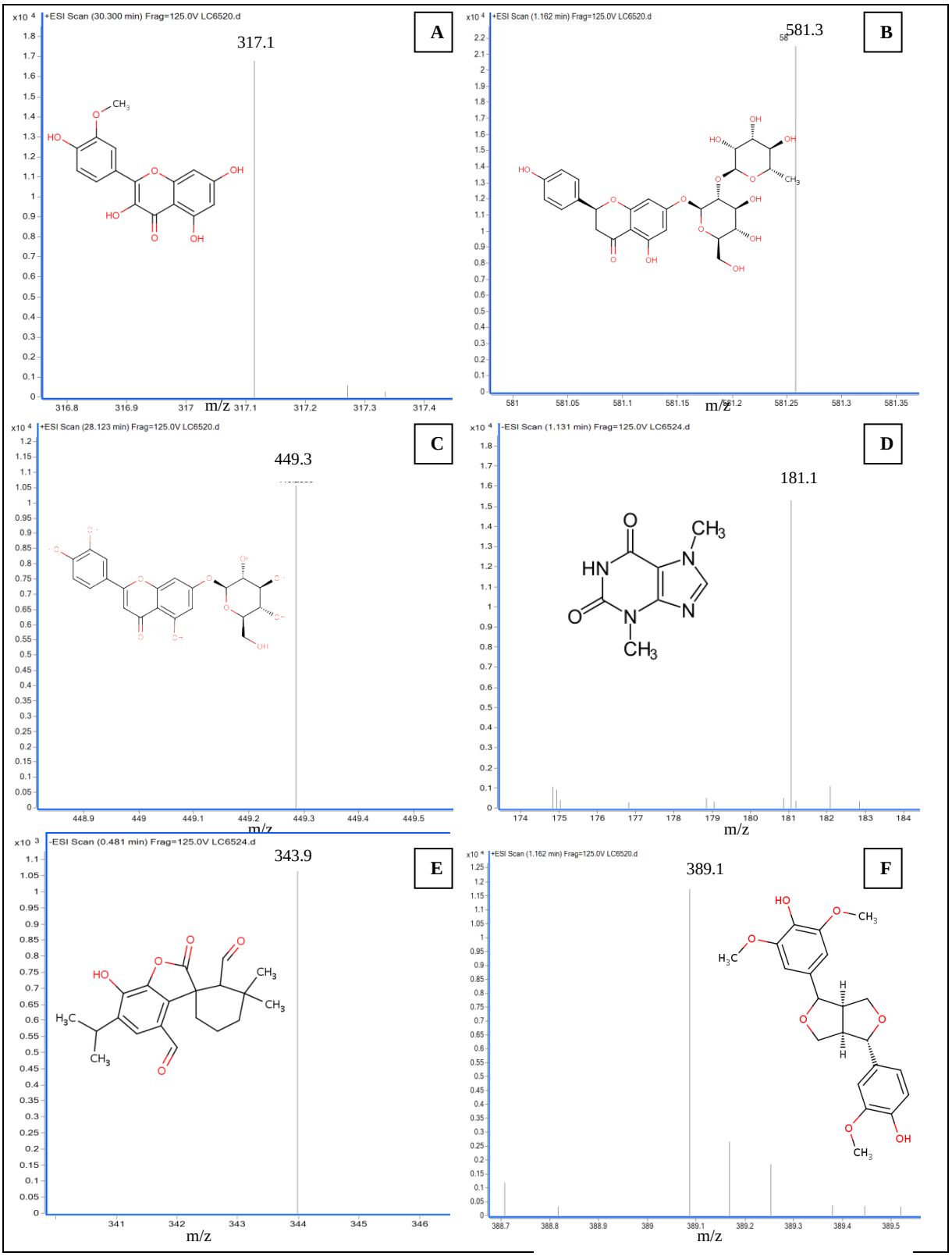

Figure 11f: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *C.asiatica* methanolic extract. Mass spectra from full scan analysis by LC-MS and their molecular structure of isorhamnetin (**A**); naringin (**B**); luteolin-7-*O*-glucoside (**C**); theobromine (**D**) rosmadial (**E**); and medioresinol (**F**)

Appendix k continued

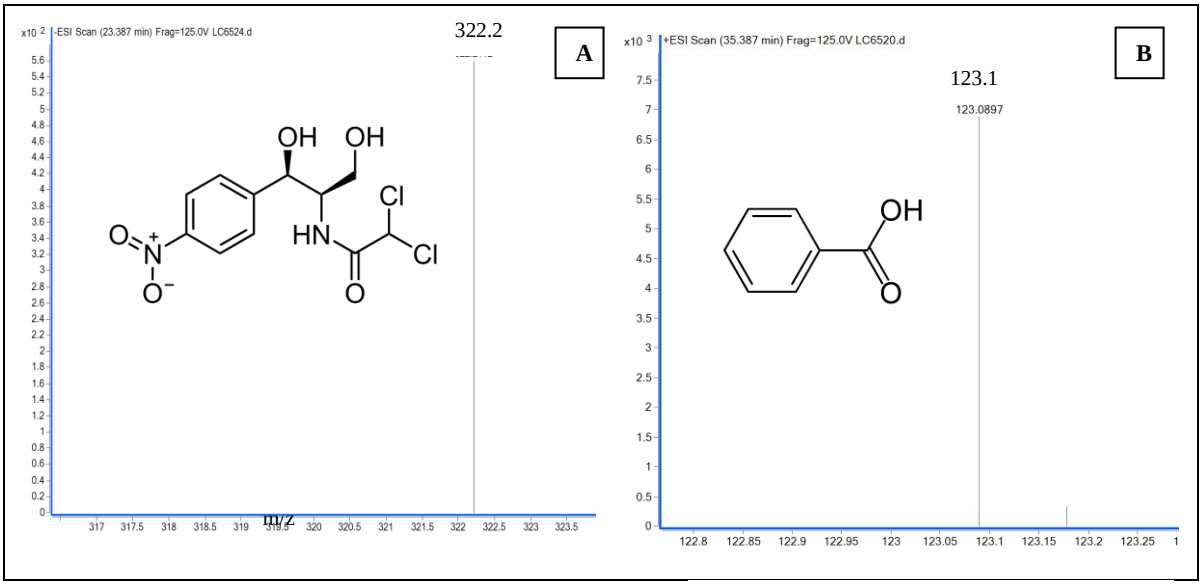

Figure 11g: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *C.asiatica* methanolic extract. Mass spectra from full scan analysis by LC-MS and their molecular structure of chloramphenicol (**A**); and benzoic acid (**B**)

Appendix k continued

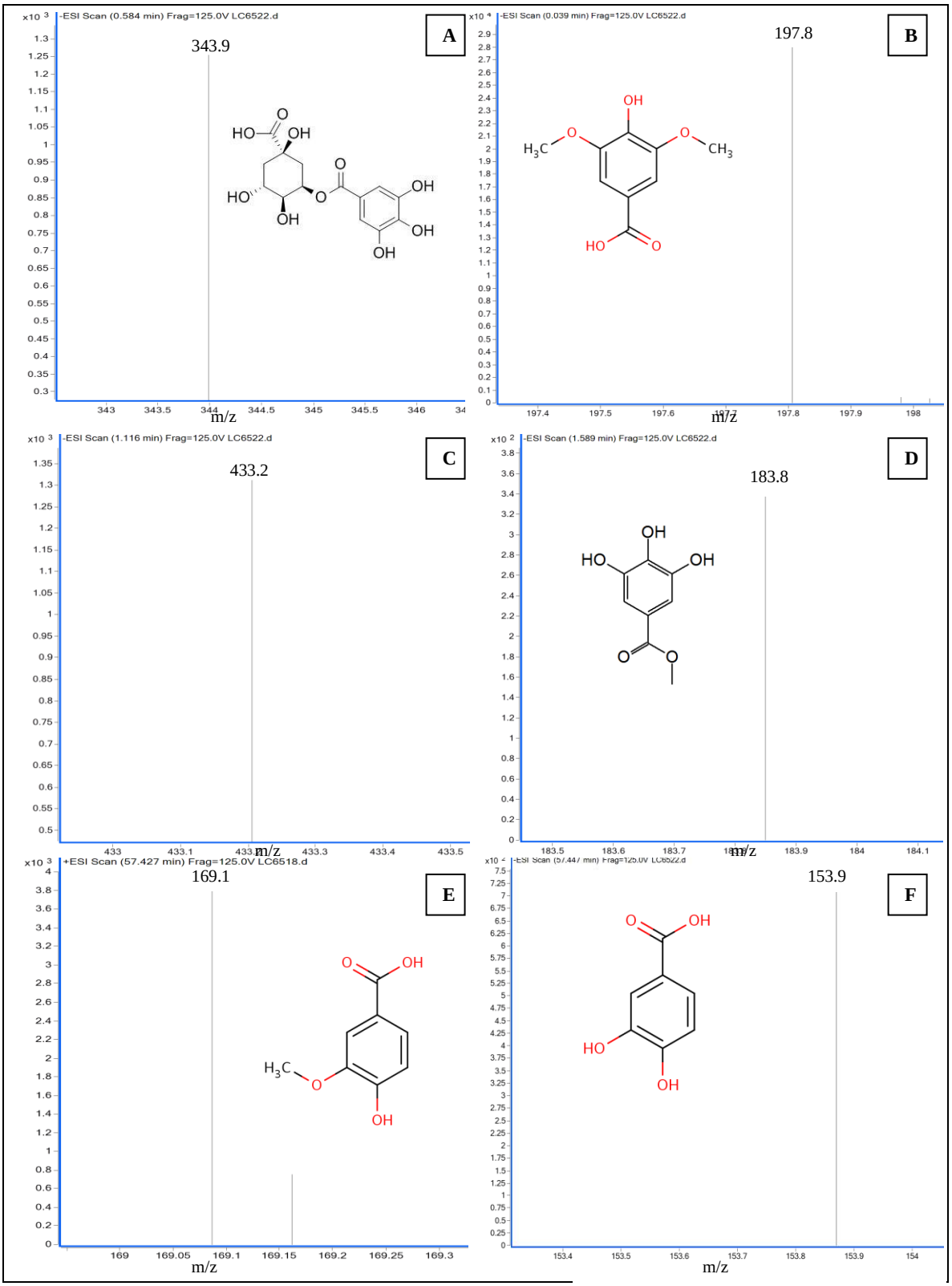

Figure 12a: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *V. amygdalina* methanolic extract.

Mass spectra from full scan analysis by LC-MS and their molecular structure of 5-galloylquinic acid (**A**); syringic acid (**B**); ellagic acid pentoside (**C**); methyl gallate (**D**); vanillic acid (**E**); and protocatechuic acid (**F**)

Appendix k continued

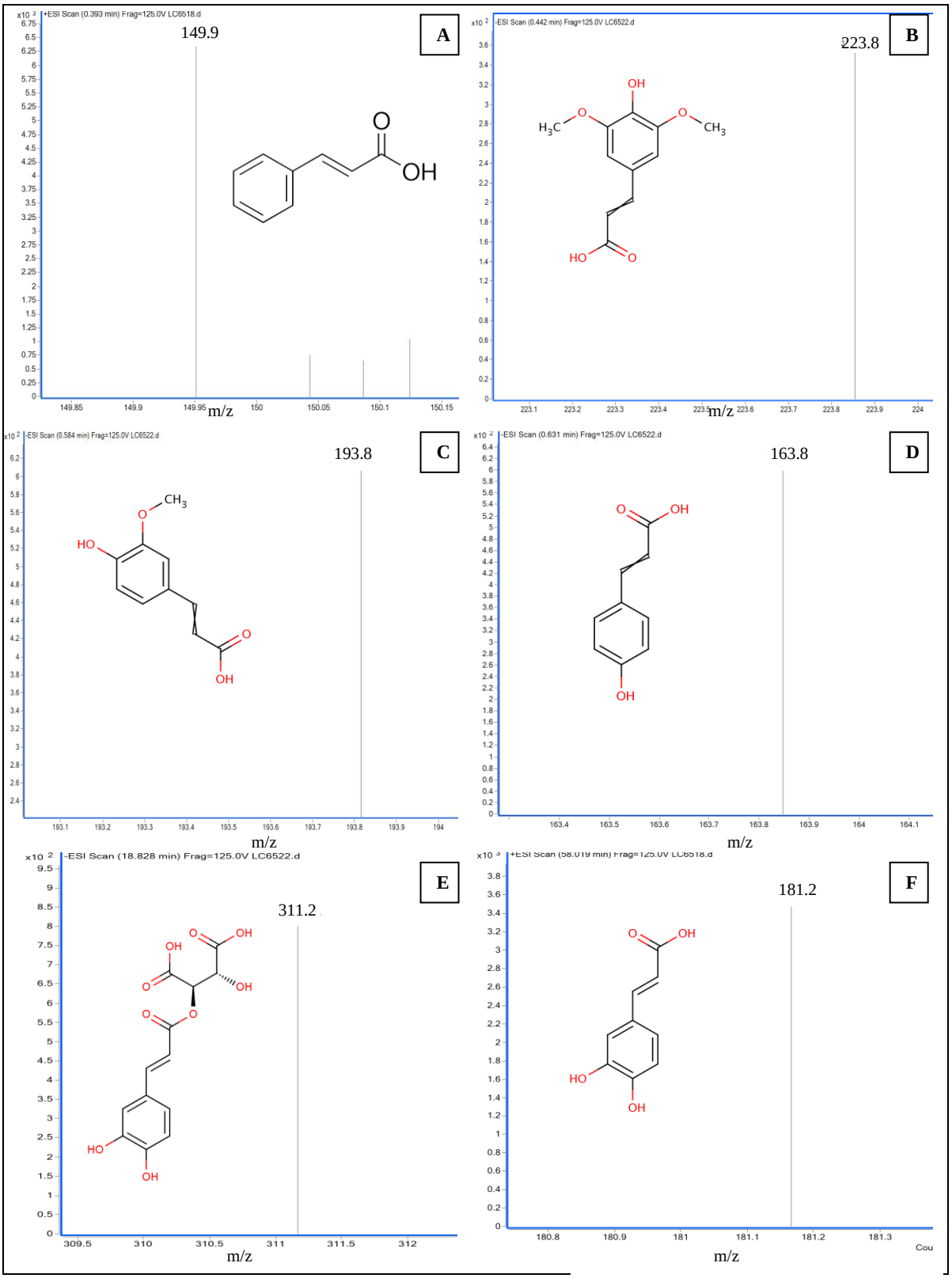

Figure 12b: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *V. amygdalina* methanolic extract.

Mass spectra from full scan analysis by LC-MS and their molecular structure of *trans*-cinnamic acid (**A**); sinapic acid (**B**); ferulic acid (**C**); p-coumaric acid (**D**); caftaric acid (**E**); and caffeic acid (**F**)

Appendix k continued

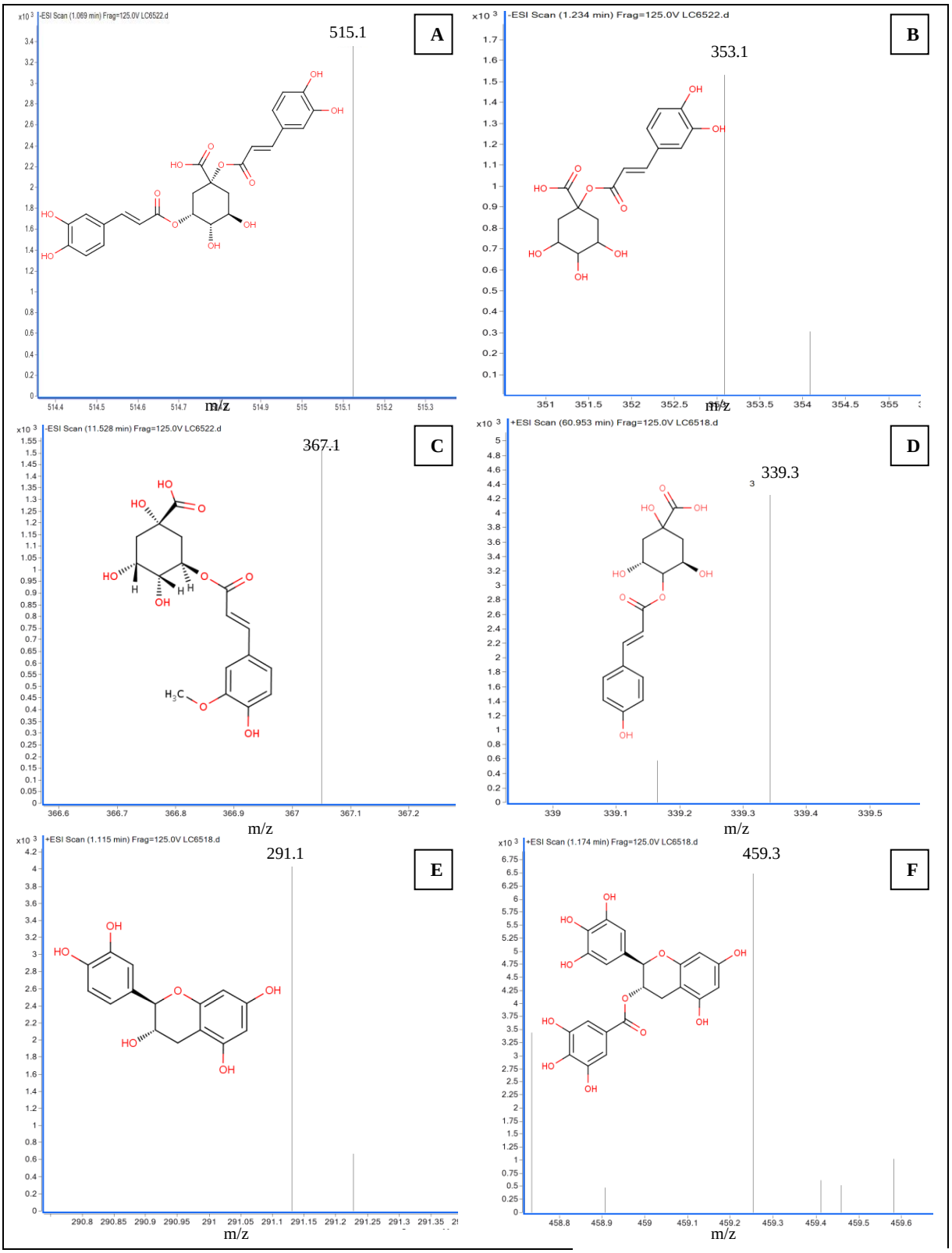

Figure 12c: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *V. amygdalina* methanolic extract.

Mass spectra from full scan analysis by LC-MS and their molecular structure of dicaffeoylquinic acid (**A**); caffeoylquinic acid (**B**); feruloylquinic acid (**C**); coumaroylquinic acid (**D**); (+)-catechin/(−)-epicatechin (**E**); and (-)-gallocatechin gallate (**F**)

Appendix k continued

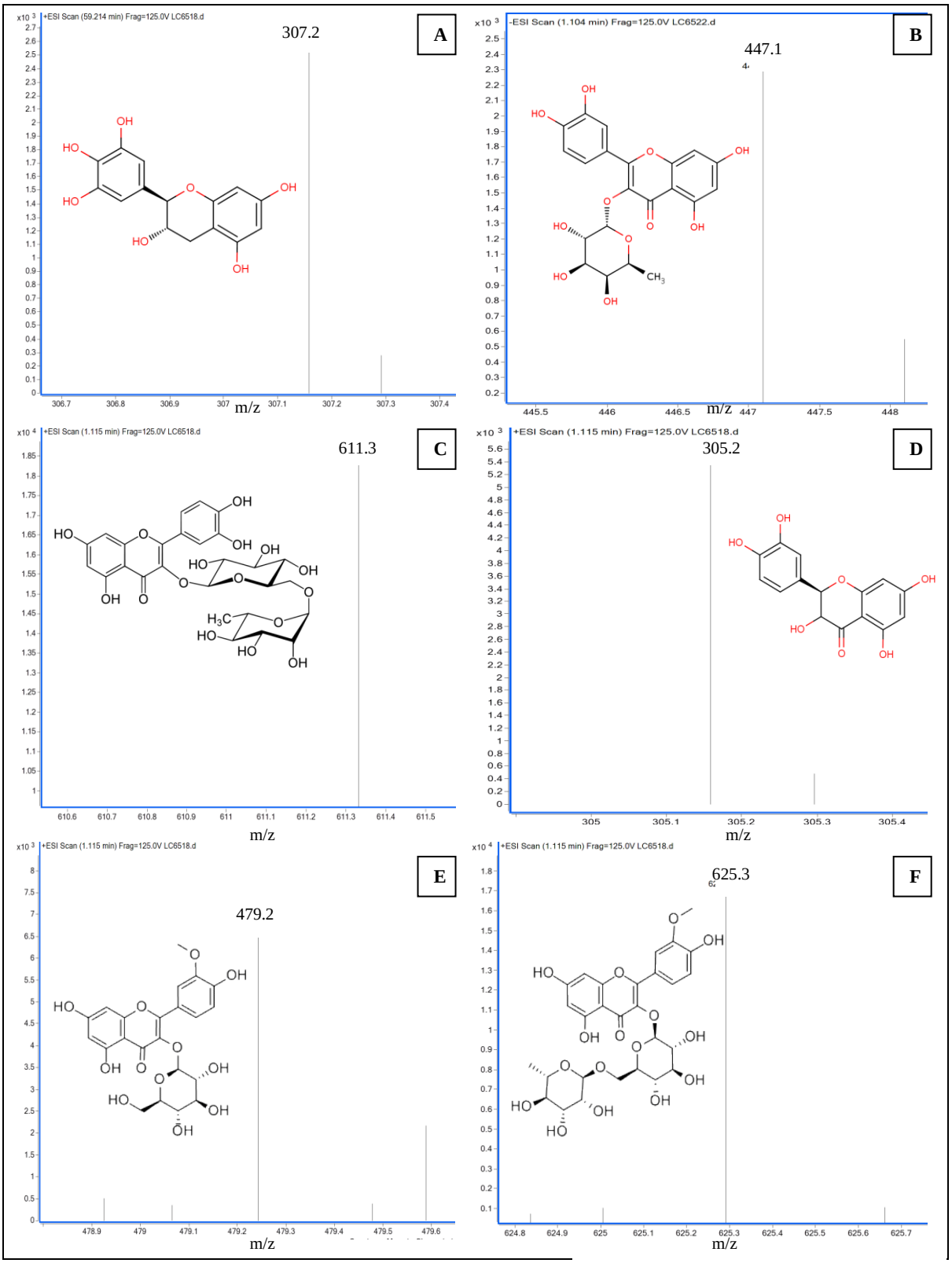

Figure 12d: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *V. amygdalina* methanolic extract.

Mass spectra from full scan analysis by LC-MS and their molecular structure of (-)-gallocatechin (**A**); quercetin 3-*O*-rhamnoside (**B**); rutin (**C**); taxifolin (**D**); isorhamnetin-3-*O*-glucoside (**E**); and isorhamnetin-3-*O*-rutinoside (**F**)

Appendix k continued

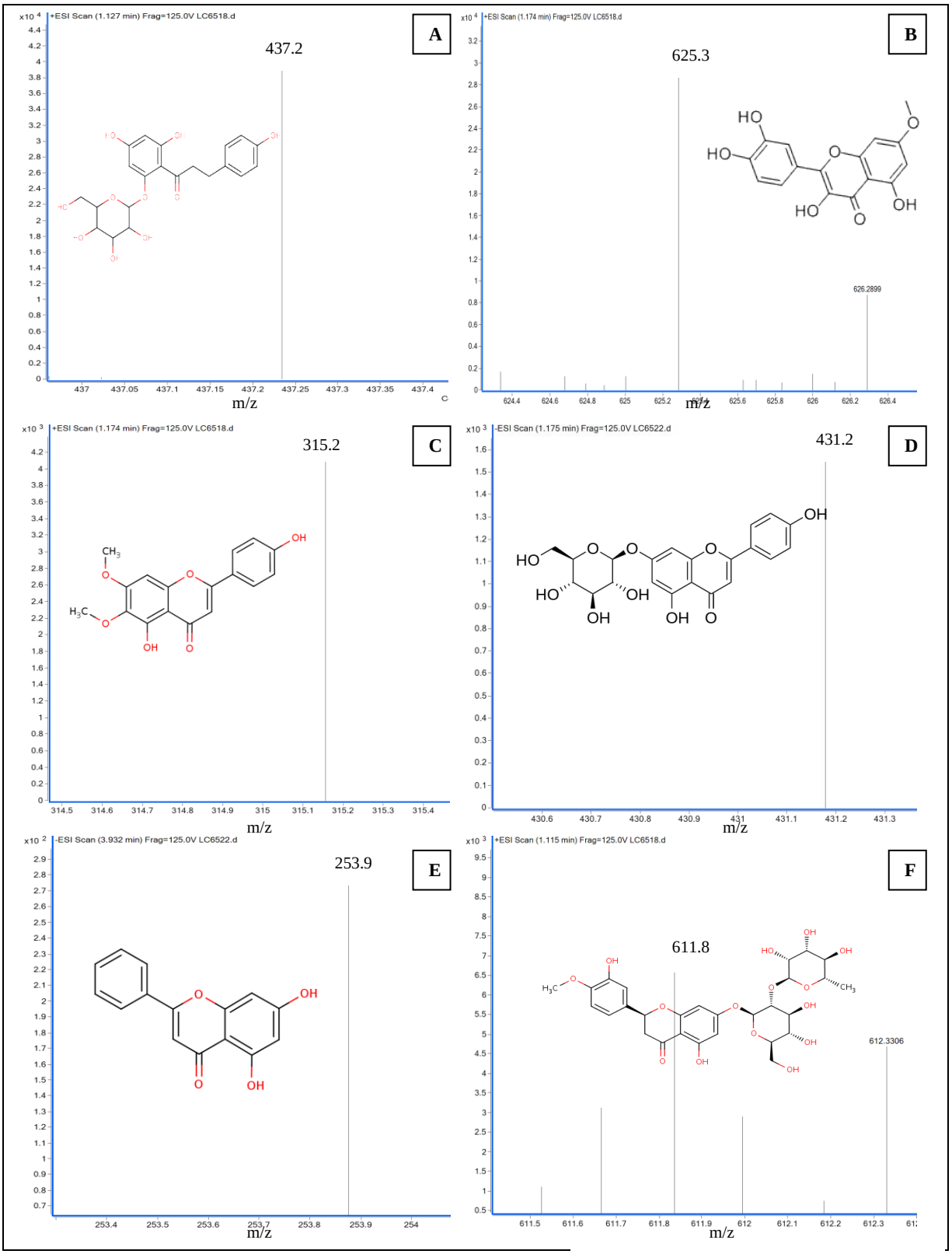

Figure 12e: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *V. amygdalina* methanolic extract.

Mass spectra from full scan analysis by LC-MS and their molecular structure of phloridzin (**A**); rhamnetin-*O*-rutinoside (**B**); cirsimaritin (**C**); apigenin-7-*O*-glucoside (**D**); chrysin (**E**); and neohesperidin (**F**)

Appendix k continued

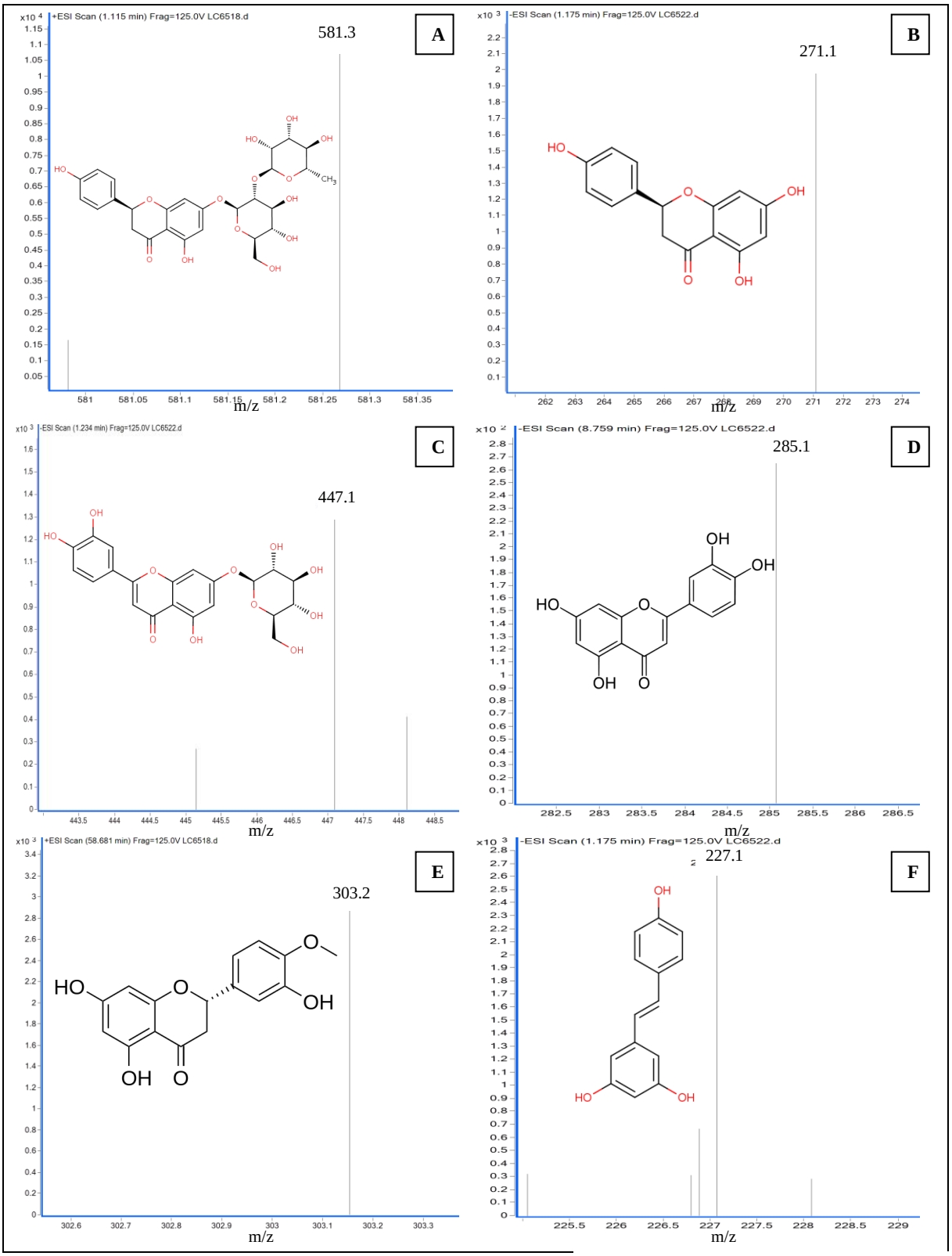

Figure 12f: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *V. amygdalina* methanolic extract.

Mass spectra from full scan analysis by LC-MS and their molecular structure of naringin (**A**); (±)-naringenin (**B**); luteolin-7-*O*-glucoside (**C**); luteolin (**D**);hesperitin (**E**); and *trans*-resveratrol/*cis*-resveratrol (**F**)

Appendix k continued

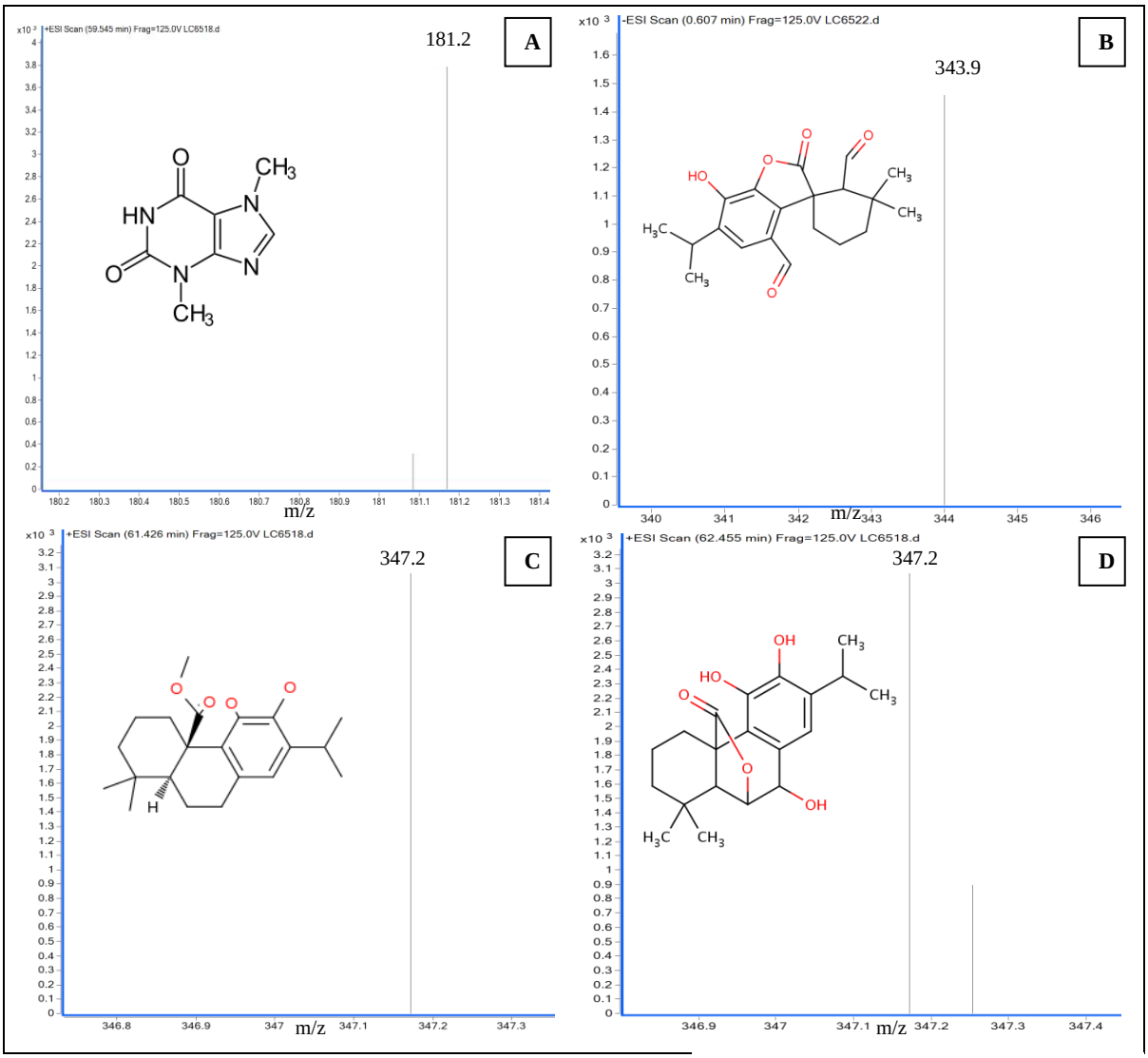

Figure 12g: Liquid Chromatography Mass Spectroscopy (LC-MS) mass spectrum of *V. amygdalina* methanolic extract.

Mass spectra from full scan analysis by LC-MS and their molecular structure of theobromine (**A**); rosmadial (**B**); methyl carnosate (**C**); and epirosmanol (**D**)

Appendix L. Protocol and Data Sheet in the Study

1. Protocol used for the plant DNA extraction kit

Quick-Start Protocol

DNeasy® Plant Mini Kit

The DNeasy Plant Mini Kit (cat. nos. 69104 and 69106) can be stored at room temperature (15–25°C) for up to 1 year.

For more information, please refer to the *DNeasy Plant Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If necessary, redissolve any precipitates in Buffer AP1 and Buffer AW1 concentrates.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Preheat a water bath or heating block to 65°C.

1. Disrupt samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the TissueRuptor®, the TissueLyser II, or a mortar and pestle.
2. Add 400 µl Buffer AP1 and 4 µl RNase A. Vortex and incubate for 10 min at 65°C. Invert the tube 2–3 times during incubation.

   Note: Do not mix Buffer AP1 and RNase A before use.
3. Add 130 µl Buffer P3. Mix and incubate for 5 min on ice.
4. **Recommended**: Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm).
5. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x g.
6. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.

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7. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x *g* (≥8000 rpm). Discard the flow-through. Repeat this step with the remaining sample.
8. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 1 min at ≥6000 x g. Discard the flow-through.
9. Add another 500 µl Buffer AW2. Centrifuge for 2 min at 20,000 x g.

   Note: Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.
10. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
11. Add 100 µl Buffer AE for elution. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g.
12. Repeat step 11.

2. Manual for DNA Purification Kit.

2

Kit Information

DESCRIPTION

- The MEGAquick-spin™ Total Fragment DNA Purification Kit is designed to extract and purify DNA fragments of 60 bp ~ 20 kb from normal or low-melt agarose gels in either Tris acetate (TAE) or Tris borate (TBE), or to purify PCR products directly from a PCR amplification and DNA cleanup from other enzymatic reactions. Recovery is achieved up to 95%. PCR products are commonly purified to remove excess nucleotides and primers. This membrane-based system, which can bind up to 45 µg DNA, allows recovery of isolated DNA fragments or PCR products in as little as 20 minutes, depending on the number of samples processed and the protocol used. The purified DNA can be used for automated fluorescent DNA sequencing, cloning, labeling, restriction enzyme digestion or in vitro transcription/translation without further manipulation.
- The BNL Buffer are optimized for efficient recovery of DNA and removal of contaminants. As an added convenience from gel extraction procedures, the BNL Buffer contains a color indicator that allows easy monitoring of the solution pH for optimal DNA binding.
- The MEGAquick-spin™ Total Fragment DNA Purification Kit is ideal multi-functional (Gel extraction, PCR purification and DNA clean-up) product for isolation of fragment DNA.

CHARACTERISTICS

- **Multifunction**
  Gel extraction, PCR purification and DNA clean-up
- **Improved recovery**
  Up to 95% recovery of ready-to-use DNA
- **Simple and easy process**
  Fast procedure, Cleanup of DNA up 60 bp ~ 20 Kb in three easy steps
- **Prevention of error**
  Easy determination of the optimal pH for DNA binding

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Kit Information

KIT CONTENTS

Label	Description	Contents 50 Columns	Contents 200 Columns
BNL Buffer[1]	Agarose gel lysis buffer	40 ml	140 ml
Washing Buffer (concentrate) [2]	Washing buffer	10 ml	40 ml
Elution Buffer	Elution buffer	20 ml	20 ml
MEGAquick- spin™ column (Blue column w/o Cap)	Nucleic acid binding column	50 Col. (17286)	200 Col. (17287)
MEGAquick- spin™ column (Clear Column with cap & dark blue O-ring)	Nucleic acid binding column		200 Col. (17288)
Collection tube	2 ml polypropylene tube	50 tubes	200 tubes

1 BNL Buffer contains chaotropic salts which are irritants. Take appropriate laboratory safety measures and wear gloves when handling.

2 Washing Buffer is supplied as concentrate. Add 40 ml (50 columns) or 160 ml (200 columns) per each bottles of ethanol (96~100%) according to the bottle label before use.

STORAGE

MEGAquick-spin™ Total Fragment DNA Purification Kit should be stored dry at room temperature (15–25°C). Under these conditions, MEGAquick-spin™ Total Fragment DNA Purification Kit can be stored for up to 24 months without showing any reduction in performance and quality. Check buffers for precipitate before use and redissolve at 37°C if necessary. The entire kit can be stored at 2–8°C, but in this case the buffers should be redissolved before use. Make sure that all buffers and spin columns are at room temperature when used. The term of validity is marked on the box.

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Kit Information

CONSIDERATION BEFORE USE

- A typical agarose gel slice is solubilized by adding 3 volumes of BNL Buffer to 1 volume of gel (e.g., 300 µl of BNL Buffer is added to 100 mg gel slice) and incubating at 55°C for 10 minutes. The high concentration of a chaotropic salt in BNL Buffer disrupts hydrogen bonding between sugars in the agarose polymer, allowing solubilization of the gel slice. In addition, the high salt concentration dissociates DNA binding proteins from the DNA fragments.
- If water is used for elution, make sure that its pH is between 7.0 and 8.5. Elution efficiency is dependent on pH and the maximum elution efficiency is achieved within this range. A pH <7.0 can decrease yield.
  Note : Store DNA at –20°C when eluted with water, as DNA may degrade in the absence of a buffering agent.

SAFETY INFORMATION

All chemicals should be considered as potentially hazardous. When working with chemicals, always wear a suitable lab coat and disposable glove. Some buffers contain the chaotropic salt which may be an irritant and carcinogen, so appropriate safety apparel such as gloves and eye protection should be worn. If a spill of the buffers occurs, clean with a suitable laboratory detergent and water.
If the liquid spill contains potentially infectious agents, clean the affected area first with laboratory detergent and water, then with a suitable laboratory disinfectant. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle these products.

DO NOT add bleach or acidic solutions directly to the sample preparation waste.

ADDITIONAL REQUIRED EQUIPMENT

- Agarose(iNtRON, Cta. No. 32032); scalpel
- Gel running buffer: TAE buffer or TBE buffer Electrophoresis Sterile
- Absolute ethanol
- Standard tabletop micro-centrifuge
- Micro-centrifuge tubes, sterile (1.5 ml)
- TE buffer (10 mM Tris-HCl, 0.1 mM EDTA; pH 8.0 - 8.5)

PROTOCOL B (PCR Purification, DNA Clean-up)

1. **Amplify target sample using standard amplification conditions. (or Prepare the DNA mixture enzymatically reacted for clean-up)**

2. **Add an 5 volume of BNL Buffer to the PCR reaction product, and mix well by vortexing. If the PCR product is 20 µl, add 100 µl of BNL buffer to the PCR tube directly.**
   Note : Centrifuge the tube briefly at room temperature to ensure that the contents are at the bottom of the tube.
   Note : If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to the MEGAquick-spin™ membrane is efficient only at pH ≤7.5. BNL Buffer contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

3. **(Optional) For < 200 bp, Add 1.5 volume of isopropanol to the sample and mix well by pipetting several times. Do not centrifuge after mixing well.**
   Note : For < 200 bp, Add 1.5 volume of isopropanol, and mix well. If the PCR product is 20 µl, add 100 µl of BNL Buffer and 150 µl of isopropanol. This step increases the yield of DNA fragment.

4. **Place one MEGAquick-spin™ column in a Collection Tube for each DNA gel mixture.**

5. **Transfer the DNA mixture to the MEGAquick-spin™ column assembly.**

6. **To bind DNA, apply the sample to the MEGAquick-spin™ column, and centrifuge for 1 min. Discard the flow-through after centrifuging and place the MEGAquick-spin™ column back in the same 2 ml collection tube.**
   Note : The maximum volume of the MEGAquick-spin™ column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

7. **Add 700 µl of Washing Buffer to column and centrifuge at 13,000 rpm for 1 min. Discard the flow-through after centrifuging and place the MEGAquick-spin™ column back in the same 2 ml collection tube.**
   Note : If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, repeat the step 4 using 500 µl of Washing buffer.

8. **Centrifuge for 1 min at 13,000 rpm to dry the spin membrane.**
   Note : It is important to dry the spin membrane since residual ethanol may interfere with other reactions.

10 Protocols

9. **Place the MEGAquick-spin™ column to a clean 1.5 ml microcentrifuge tube (not provided). Apply 30 ~ 100 µl of the Elution Buffer directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute. Centrifuge for 1 minute at 13,000 rpm.**

10. **Discard the MEGAquick-spin™ column and store the microcentrifuge tube containing the eluted DNA at -20℃.**

Note : It is suggested to use at least 30 µl of the Elution Buffer to obtain best result.
Note : Ensure that the elution buffer is dispensed directly onto the MEGAquick-spin™ membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.
Note : Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Quick Guide