# 1. INTRODUCTION

Natural products from different sources such as plants and microorganism have played an important role in the prevention of infectious diseases and are of general use in health care. Bioactive compounds that are extracted from natural products are generally regarded as effective and safe compared to synthetic compounds. Synthetic substances such as antibiotics produced by the pharmaceutical industry have in many cases caused side effects such as nausea, vomiting, epigastric pain, abdominal discomfort, diarrhea and have caused the emergence of resistance by pathogenic bacteria, to such an extent that an increasing number of researchers are seeking to produce biologically active compounds from natural sources (De Gaudio *et al.*, 2012). One important source of natural products are medicinal plants which have been used as traditional medicine in different parts of the world for many applications such as the treatment of infectious diseases, diarrhea, hypertension, skin diseases and other conditions (Tripathi *et al.*, 2013).

In this study, *Morinda citrifolia*, *Annona squamosa*, *Alstonia angustiloba* and an Australian plant mixture were selected based on knowledge of their medicinal properties. *M. citrifolia* is one important plant used as a medicine in many countries of the world. The common names of this plant are *Noni*, Indian mulberry, *nuna*, and *mengkudu* (Nelson, 2006; Potterat and Hamburger, 2007). Noni is rich in phytochemical compounds which can be extracted from different parts of the plant (Singh, 2012). The compounds found in this plant have many bioactive properties such as antibacterial and antioxidant activities (Potterat and Hamburger, 2007). Past studies on the extracts from the fruit, leaves and roots of *M. citrifolia* have documented antibacterial activity against a wide spectrum of microorganisms (Natheer *et al.*, 2012; Selvam *et al.*, 2009) and antioxidant activity by using different assays such as the DPPH and SOD assays

(Pongnaravane *et al.*, 2006; Zin *et al.*, 2002). One of the major bioactive compounds of the plant is anthraquinones which are found in different parts of the plant (Chan-Blanco *et al.*, 2006; Singh, 2012). Until now, no studies have shown these compounds to possess antibacterial activity against *S. aureus* (RF 122), *E. coli* (UT181), *B. cereus* (ATCC 14579), *P. aeruginosa* (PA7), methicillin-resistant *S. aureus* (ATCC BA-43) and *H. pylori* ATCC 43504. Also the antioxidant activity and the mode of action of these bioactive extracts were not documented. This study isolated and tested anthraquinones extracted from this plant tissue for antibacterial and antioxidant activities along with LC-MS analysis and FTIR of the anthraquinones in this extract and postulated their mode of action. Another class of bioactive compound present in *M. citrifolia* are alkaloids and this is the first work documenting antibacterial and antioxidant activities with LC-MS analysis, FTIR and the mechanism of action of these bioactive extracts by SEM.

A second plant used in this study was *A. squamosa* which belongs to the Annonaceae family. Its common names are *Nona*, sugar apple, ata, gishta and sweet sop plant (Lim, 2012; Pareek *et al.*, 2011). It is used as a general tonic to enrich blood, relieve vomiting, cancer, as a vermicide, for skin complaints and also applied to wounds and ulcers (Pandey and Barve, 2011; Shah, 2011). Sugar apple contains many phytochemical compounds which may be extracted from different parts of the plant (Pandey and Barve, 2011). These phytochemical compounds have many bioactive properties such as antibacterial and antioxidant activities (Tomar, 2013). Previous studies have reported that the different extracts from the fruit, leaves and roots of *A. squamosa* have antibacterial activity against a wide spectrum of microorganisms (Padhi *et al.*, 2011) and antioxidant activity (Jagtap and Bapat, 2012; Mariod *et al.*, 2012). One of the important compounds in this plant found in the fruit are diterpenoid as 16 α hydroxy-(-)-kauran-19-oic acid, kauran-16-en-18-oic acid, annonosin A, annosquamosin C,

annosquamosin D, annosquamosin E, annosquamosin F, annosquamosin G, annosquamosins B (Andrade *et al.*, 2001; Hassan *et al.*, 2003; Yang *et al.*, 2002). Past studies reported that 16 α hydroxy-(-)-kauran-19-oic acid extracted from the fruit of this plant showed antibacterial activity against ATCC strains of *S. aureus* and *Streptococcus pneumoniae* (Wiart *et al.*, 2005). Other bioactive compounds found in different parts of the plant are alkaloids such as liriodenine**,** oxoanalobine**,** anonaine, isocorydine, norisocorydine, abenzooxyquinazoline, samoquasine A, anolobine and reticuline (Bhakuni *et al.*, 1972; Lebrini *et al.*, 2010; Morita *et al.*, 2000). In this study, these bioactive extracts, diterpenoid and alkaloids from this plant were isolated and tested for antibacterial and antioxidant activities with LC-MS analysis, FTIR while the mechanism action of these extracts by SEM.

The third plant used in this study is *A. angustiloba* which belongs to the Apocynaceae family. The local name of this plant is *pulai* or *pulai bukit* (Al-Adhroey *et al.*, 2010; Neo *et al.*, 2013; Valkenburg and Bunyapraphatsara, 2001). This plant is used as a medicine for skin sores and gynaecological diseases in Indonesia (Mulyoutami *et al.*, 2009). It has rich indole alkaloids in the different parts of this plant and these compounds showed cytotoxicity against KB cells, a cancer cell line (Ku *et al.*, 2011). Until now, there are no reports that demonstrate alkaloids extracted from the roots of this plant show antibacterial activity against pathogenic bacteria including MRSA and *H. pylori* and also show antioxidant activity. Alkaloids in this extract were identified by LC-MS and FTIR analysis.

The fourth sample was an Australian plant mixture that contained four indigenous Australian plants (*Backhousia citriodora*, *Terminalia ferdinandiana*, *Citrus australasica* and Australian wheatgrass sprouts). The plants in the mixture have phenolic compounds with significant amounts vitamin C (Konczak *et al.*, 2010; Lim,

2012b). These compounds are responsible for antibacterial and antioxidant activities (Dupont *et al.*, 2006; Konczak *et al.*, 2010). This study isolated and tested this mixture for antibacterial and antioxidant activities with LC-MS analysis of the components of this plant mixture.

Lactic acid bacteria are established as source of antibacterial peptides. An important genus of lactic acid bacteria is *Lactobacillus* which belongs to the Lactobacteriaceae family. *Lactobacillus* are Gram-positive, catalase negative, oxidase negative, non-sporing and rod shaped bacteria (Patil *et al.*, 2010; Salminen and Von Wright, 2011). *L. casei*, a bacteria is commonly used in probiotic and industrial applications (Kandler and Weiss, 1986). It is isolated from cheese, fermented products, the intestinal tract, the oral cavity and soil (Datta *et al.*, 2013; Herrero-Fresno *et al.*, 2012; Martinovic *et al.*, 2013; Randazzo *et al.*, 2006; Sumeri *et al.*, 2012). *L. casei* produces a type of bacteriocin known as lactacin. This substance has antibacterial activity against Gram-positive bacteria (Avonts *et al.*, 2004). In addition, this extract from bacteria shows antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid, Butylated Hydroxyl Toluene (BHT) and NADPH assays (Suman and Vibha, 2006).

Another member of this family, *L. paracasei* subsp. *paracasei* may be isolated from dairy products such as raw cow's milk, cheddar cheese, feta cheese and feta brine (Bergamini *et al.*, 2009; Floros *et al.*, 2012; Maragkoudakis *et al.*, 2006; Oliszewski *et al.*, 2013). Bacteriocin extracted from *L. paracasei* subsp. *paracasei* strain M3 has antibacterial activity against more than 60 strains of bacterial and yeasts strains (Atanassova *et al.*, 2003). Previous studies reported that the supernatant of *L. paracasei* subsp. *paracasei* TKU010 isolated from squid pen had antioxidant activity by using the DPPH assay (Liang *et al.*, 2010). In the current study, *L. paracasei* subsp. *paracasei* 8700:2 was isolated from cow's milk and antibacterial peptides were isolated by

different methods and tested for both antibacterial and antioxidant activity while the mode of action by was evaluated by SEM and TEM.

Peptides were also isolated from the plants used in this study. Plant peptides with antibacterial activity are known as defensins and are found in different plant families. They have antibacterial activity against bacteria at lower concentrations compared to other bioactive compounds from natural products (Barbosa Pelegrini *et al.,* 2011). These molecules have antioxidant activity because they have amino acid residues to reduce free radicals ions (Pownall *et al.*, 2010). Liu *et al.* (2010) reported that the peptide extracted from *Cornu bubali* seeds had antioxidant activity using DPPH assay. However, no study has isolated peptides from *M. citrifolia*, *A. squamosa*, *A. angustiloba* and Australian plant mixture tested for antibacterial and antioxidant activities or evaluated their mode of action by SEM or TEM.

The objectives of this research are to:

1- Study antibacterial and antioxidant activities of bioactive compounds from selected plants.

2- Identify bioactive compounds from selected plants and study their mechanism of action.

3- Isolate and identify lactic acid bacteria with antibacterial and antioxidant properties from dairy products.

4- Extract and determine antibacterial and antioxidant peptides from medicinal plants and study their mechanism of action.

# 2. LITERATURE REVIEW

## 2.1. *Morinda citrifolia*

### 2.1.1. Plant morphology

*M. citrifolia* belongs to the Rubiaceae family and comprises 80 species (Figure 2.1). *Noni*, Indian mulberry, *nuna*, and *mengkudu* are common names for this plant (Nelson, 2006; Potterat and Hamburger, 2007). A medium-sized tree, it is 3-10 metres tall with abundant wide elliptical leaves and small tubular white flowers, which are grouped together. The petioles leave ring-like marks on the stalks (Morton, 1992; Ross, 2001). The oval-shaped fruit of this plant has an embossed appearance. It is initially green to yellow in colour but the ripe fruit is white and covered with small reddish brown buds containing seeds (Chan-Blanco *et al.*, 2006). The seeds are medium sized, ovoid in shape, reddish brown and with a distinct air chamber at the end probably for widespread seed dispersal by water (Nelson, 2006; Potterat and Hamburger, 2007; Wang *et al.*, 2002). This plant is found in South East Asia, Caribbean countries, Australia and Central-South America (Morton, 1992; Nelson, 2006). *M. citrifolia* has been used as a medicine for many ailments such as dysentery, heartburn, liver diseases, diabetes, high blood pressure, muscle aches, headaches, heart diseases, cancer, gastric ulcers and arthritis. It has also been applied for the treatment of drug addiction. The ripe fruit of this plant is used to treat respiratory infections and tuberculosis (Singh, 2012). The roots and bark of *M. citrifolia* can be turned into dyes and medicine, while the leaves and fruit are sources of food and medicine (Nelson, 2006; Potterat and Hamburger, 2007).

Figure 2.1. *Morinda citrifolia*.

**Taxonomy of *Morinda citrifolia***

Kingdom: Planteae

Phylum: Magnoliophyta

Class: Magnoliopsida (dicot)

Order: Rubiales

Family: Rubiaceae (coffee family)

Genus: *Morinda*

Species: *Morinda citrifolia*

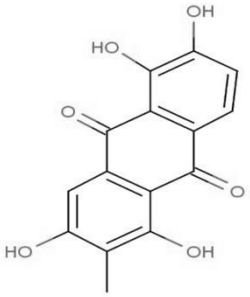
The juice from the fruit of this plant has a long medicinal history in places such as the Fiji Islands, the Pacific Islands, South-east Asia and India (Rethinam and Sivaraman, 2007). Through *in vivo* experiments, Glang *et al.* (2013) reported that noni juice was deemed to be effective in the treatment of gingivitis and periodontitis. It used twice daily as a mouthwash, this juice significantly reduced the gingival inflammation.

### 2.1.2. Chemical components and bioactive compounds of *M. citrifolia*

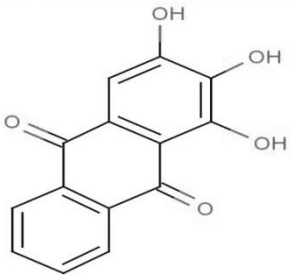
*M. citrifolia* has approximately 200 phytochemical compounds which are distributed throughout the plant (Singh, 2012). The chemical composition of these compounds is largely dependent on the part of the plant in which they are found. These compounds include scopoletins, coumarins, terpenoides, octanoic acid, carotene, linoleic acid, anthraquinones, capric acid, flavone glycosides, proxeronine, L-asperuloside, morindone, and rutin (Pawlus and Kinghorn, 2007; Ross, 2001). Anthraquinone, a major bioactive compound is present in different parts of the plant (Chan-Blanco *et al.*, 2006; Singh, 2012). Among the compounds found in the fruit of this plant are 2-methoxy-1,3,6-trihydroxyanthraquinone (1), anthragallol 1,3-di-*O*-methylether (2), 6-hydroxyanthragallol-1,3-di-*O*-methyl ether (3), austrocortinin (4), morindone-5-*O*-methylether (5), anthragallol-2-*O*-methylether (6), 5,15-dimethylmorindol (7), 1,6-dihydroxy-5-methoxy-2-methoxymethylanthraquinones (8), 1,5,7-trihydroxy-6-methoxy-2-methoxymethylanthraquinones (9), 1,3-dimethoxyanthraquinone (10), 1,2-dihydroxyanthraquinone (11), morindacin (12) and lucidin (13) Table 2.1 and Figure 2.2.

**Table 2.1.** Anthraquinones extracted from *M. citrifolia* fruit

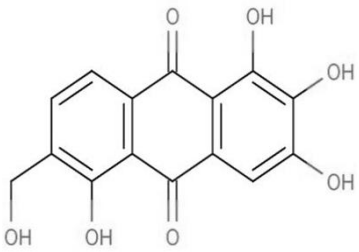
| **Name of the compound** | **Part of the plant** | **References** |
|---|---|---|
| 2-Methoxy-1,3,6-trihydroxyanthraquinone (1) | Fruit | Pawlus *et al.* (2005) |
| Anthragallol 1,3-di-*O*-methyl ether (2) | Fruit | Kamiya *et al.* (2005) |
| 6-Hydroxyanthragallol-1,3-di-*O*-methyl ether (3) | Fruit | Kamiya *et al.* (2005) |
| Austrocortinin (4) | Fruit | Kamiya *et al.* (2005) |
| Morindone-5-*O*-methyl ether (5) | Fruit | Kamiya *et al.* (2005) |
| Anthragallol 2-*O*-methyl ether (6) | Fruit | Pawlus *et al.* (2005) |
| 5,15-Dimethylmorindol (7) | Fruit | Deng *et al.* (2009) |
| 1,6-dihydroxy-5-methoxy-2-methoxymethyl anthraquinones (8) | Fruit | Lin *et al.* (2007) |
| 1,5,7-trihydroxy-6-methoxy-2-methoxymethyl anthraquinones (9) | Fruit | Lin *et al.* (2007) |
| 1,3-dimethoxyanthraquinone (10) | Fruit | Siddiqui *et al.* (2007) |
| 1, 2-dihydroxyanthraquinone (11) | Fruit | Siddiqui *et al.* (2007) |
| Morindacin (12) | Fruit | Kamiya *et al.* (2005) |
| Lucidin (13) | Fruit | Deng *et al.* (2009) |



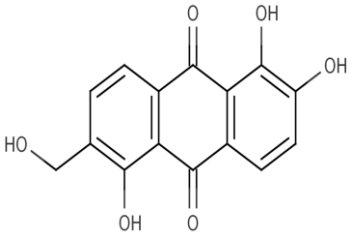

3-Hydroxymorindone.



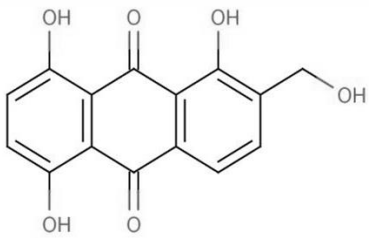

1,2,3-Trihydroxyanthraquinone



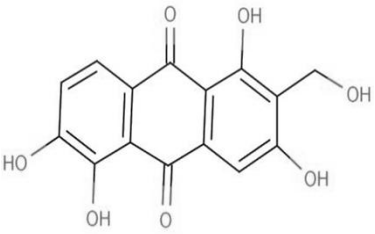

1,2,3,5-Tetrahydroxy-6-hydroxymethyl-9,10-anthracenedione.



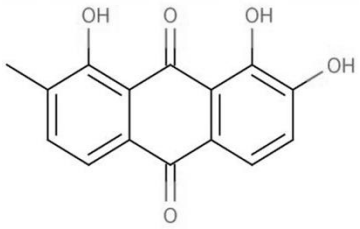

Morindol



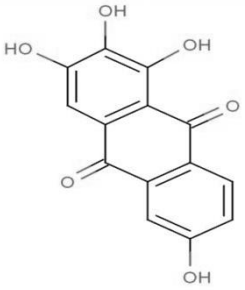

1,5,8-Trihydroxy-2-(hydroxymethyl)-9,10-anthracenedione



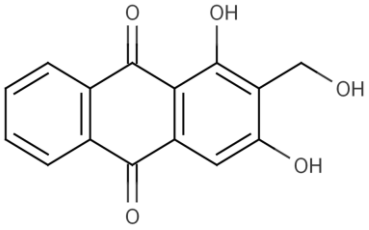

5,6-Dihydroxylucidin




Morindicinone




1,2,3,6-Tetrahydroxy-9.10-anthracenedione




1,3-Dihydroxy-2-hydroxymethylanthraquinone

**Figure 2.2.** Chemical structure of anthraquinones from *M. citrifolia* (Chan-Blanco *et al.*, 2006).

*M. citrifolia* leaves contain 2-methoxy-1,3,6-trihydroxyanthraquinone (14), 5,15-dimethylmorindol (15), 1,3-dihydroxy-2-methylol-9,10-anthraquinone (16), 1,2-dihydroxyanthraquinone (17) and 1,3-dihydroxy-2-methylanthraquinone (18). The many compounds found in the root of this plant include damnacanthal (19), 2-ethoxy-1-hydroxyanthraquinone (20), nordamnacanthal (21), tectoquinone (22), 2-formyl-1-hydroxyanthraquinone (23), 1-methoxy-3-hydroxyanthraquinone (24), 1,3-dimethoxy-2-methoxymethylanthraquinone (25), morindone-6-methylether (26), 1,7-dihydroxy-8-methoxy-2-methylanthraquinone (27), alizarin 1-*O*-methyl ether (28), morenone-1(29) and morenone-2 (30) Table 2.2.

In addition to the above, compounds of anthraquinones found in *M. citrifolia* root include morindone-5-methylether (31), rubiadin (32), morindone (33), anthragallol 1,2-di-*O*-methylether (34), ibericin (35), damnacanthal (36) and soranjidiol (37) Table 2.2.

**Table 2.2.** Anthraquinones extracted from *M. citrifolia* leaves and roots

| **Name of the compound** | **Part of the plant** | **References** |
|---|---|---|
| 1,5,15-Trimethylmorindol (14) | Leaves | Takashima *et al.* (2007) |
| 5,15-Dimethylmorindol (15) | Leaves | Takashima *et al.* (2007) |
| 1,3-dihydroxy-2-methylol-9,10-anthraquinone (16) | Leaves | Deng *et al.* (2009) |
| 1,2-Dihydroxyanthraquinone (17) | Leaves | Deng *et al.* (2009) |
| 1,3-dihydroxy-2-methylanthraquinone (18) | Leaves | Deng *et al.* (2009) |
| Damnacanthal (19) | Root | Lv *et al.* (2011); Thomson (1996) |

| | | |
|---|---|---|
| 2-ethoxy-1-hydroxyanthraquinone (20) | Root | Ee *et al.* (2009) |
| Nordamnacanthal (21) | Root | Ee *et al.* (2009) |
| Tectoquinone (22) | Root | Lv *et al.* (2011)<br>Sang and Ho (2006) |
| 2-formyl-1-hydroxyanthraquinone (23) | Root | Lv *et al.* (2011) |
| 1-methoxy-3-hydroxyanthraquinone (24) | Root | Lv *et al.* (2011) |
| 1,3-dimethoxy-2-methoxymethylanthraquinone (25) | Root | Lv *et al.* (2011) |
| Morindone-6-methylether (26) | Root | Ee *et al.* (2009) |
| 1,7-dihydroxy-8-methoxy-2-<br>methylanthraquinone (27) | Root | Lv *et al.* (2011) |
| Alizarin 1-*O*-methyl ether (28) | Root | Pawlus *et al.* (2005) |
| Morenone-1(29) | Root | Jain and Srivastava<br>(1992) |
| Morenone-2 (30) | Root | Jain and Srivastava<br>(1992) |
| Monndone-5-methylether (31) | Root | Lv *et al.* (2011) |
| Rubiadin (32) | Root | Lv *et al.* (2011) |
| Morindone (33) | Root | Ee *et al.* (2009) |
| Anthragallol 1,2-di-*O*-methyl ether (34) | Root | Singh (2012) |
| Ibericin (35) | Root | Ee *et al.* (2009) |
| Damnacanthol (36) | Root | Sang and Ho (2006) |
| Soranjidiol (37) | Root | Rethinam and Sivaraman (2007) |

Other bioactive compounds present in this plant are alkaloids (chemical compounds containing nitrogen atoms). Ralph Heinicke documented that the *M. citrifolia* fruit contains a natural precursor called proxeronine (Figure 2.3). This compound merges with an enzyme in the human intestine called proxeroninase to result in xeronine (Heinicke, 1985; Lim, 2013). Xeronine plays the part of directing the protein in the human body to fold into an appropriate conformation to perform properly. In this way, xeronine ensures the performance of protein in overcoming a variety of health problems (Wang *et al.*, 2002).

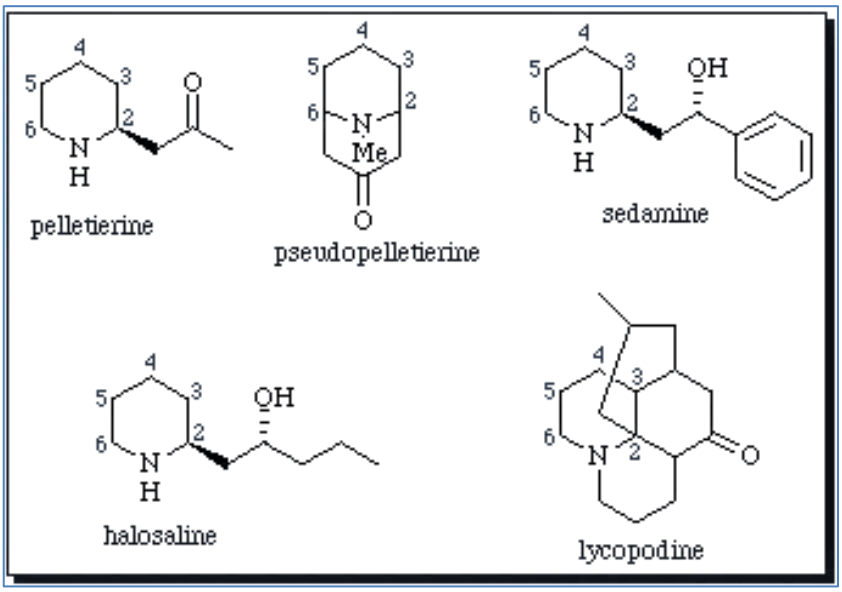



**Figure 2.3.** Chemical structure of xeronine from *M. citrifolia* fruit (Heincke, 1985).

### 2.1.3. Antibacterial activity of *M. citrifolia*

*M. citrifolia* contains many bioactive compounds including anthraquinones, alkaloids, and phenolic compounds. These compounds display antibacterial activity towards several pathogens. Atkinson (1956) documented that this plant has an antibacterial activity on microorganisms such as *S. aureus, P. aeruginosa, Proteus morgaii, B. subtilis, E. coli, H. pylori, Salmonella* and *Shigella*. Previous studies reported that acetonitrile extract from the dried fruit of the noni plant inhibits the growth of *P. aeruginosa, B. subtilis, E. coli* and *Streptococcus pyrogene* (Locher *et al.*, 1995; Singh, 2012). The ripe fruit of *M. citrifolia* inhibits the growth of *P. aeruginosa, M. pyrogenes, E. coli, Salmonella typhosa, S. montevideo, S. schottmuelleri* and *Shigella paradys* (Dittmar, 1993; Singh, 2012).

Ethanolic and hexane extracts from the fruit of the noni plant inhibits the growth of *Mycobacterium tuberculosis* because the hexane extracts contain E-phytol, cycloartenol and stigmasterol (Saludes *et al.*, 2002). Methanolic extract from the fruit and leaf of this plant exhibit antibacterial activity against *E. coli, S. aureus, Proteus vulgaris, P. aeruginosa, Klebsiella pneumoniae, Streptococcus sp., Shigella flexneri, Proteus mirabilis, P. diminuta, P. fluorescens, S. aureus* ATCC 6538, *E. coli* ATCC 25922 and *Enterobacter cloacae* (Natheer *et al.*, 2012; Selvam *et al.*, 2009). However, the ethyl acetate extract from *M. citrifolia* (fruit and leaves) showed inhibition zones against all test strains except *P. aeruginosa, P. fluorescens, Proteus mirabilis, P. diminuta* and *E. coli* ATCC 25922 which displayed resistant against these extracts (Natheer *et al.*, 2012). Rivera *et al.* (2011) reported that the juice extract from *M. citrifolia* inhibited the growth of *Mycoplasma pneumoniae, M. penetrans,* and *M. fermentas*. Sibi *et al.* (2012) reported that methanolic extracts of the whole leaves and roots inhibits the growth of *S. aureus, Streptococcus epidermidis, S. pyogens, E. coli, Serratia marsecens, P. aeruginosa,* and *K. pneumoniae.* The noni juice has antibacterial properties against

aerobic bacterial species isolated from subjects survived from gingival pouches such as *Klebsiella oxytoca*, *P. aeruginosa*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Glang *et al.*, 2013).

### 2.1.4. Antioxidant activity of *M. citrifolia*

Fruits and vegetables are the major sources of antioxidants which reduce the free radicals that cause oxidative damage in cancer cells (Kalt *et al.*, 1999; Prior and Wu, 2013). In view of this, *M. citrifolia* is a medical plant with the relevant properties to help the human body overcome a host of health problems. The juice from the fruit of this plant exhibited three times the antioxidant activities of vitamin C, making it more capable of reducing scavenge reactive oxygen free radicals (Wang and Su, 2001). Zin *et al.* (2002) reported that the ethyl acetate extract from the fruit, root and leaves of *M. citrifolia* showed significant antioxidant activity compared to α-tocopherol and butylated hydroxyltoluene (BHT). This was arrived at through ferric thiocyanate (FTC) and thiobarbituric acid (TBA) assays (Mkrtchyan *et al.*, 2010). It has been noted that the methanolic extract from noni roots showed higher antioxidant activity compared to the fruit and leaves.

Pongnaravane *et al.* (2006) documented that the pressurized hot water extraction from *M. citrifolia* roots exhibited higher antioxidant activity compared to other methods of extraction measured by DPPH assay. On the other hand, through the ferric thiocyanate and thiobarbituric methods, it was found that fractions of phenolic compounds from the fruit of this plant exhibited higher antioxidant activity than BHT or α-tocopherol. These phenolic fractions are related to antioxidant activity because they have hydroxyl groups in aromatic rings of the structure that are able to reduce agents such as hydrogen donating antioxidants and singlet oxygen quenchers (Zin *et al.*, 2002). Microparticle powder of the extract from the fruit produced by the spray drying process

exhibited high antioxidant activity when evaluated by the DPPH method (Krishnaiah *et al.*, 2012).

## 2.2. *Annona squamosa*

### 2.2.1. Plant morphology

The genus *Annona* comprises 120 species. An economically significant species is *A. squamosa* which belongs to the Annonaceae family (Figure 2.4). Its specific native range is indefinite because of widespread commercial cultivation but is generally deemed to originate from the Caribbean region (Egydio *et al.*, 2013). Common names for this plant are *Nona*, sugar apple, ata, gishta and sweet sop (Lim, 2012; Pareek *et al.*, 2011). It is a small semi-evergreen tree/shrub, 3-7 m tall, with irregular or crown branches. The leaves are oblong-lanceolate and pale green on both surfaces. The flowers are greenish-yellow and produced in single or short lateral clusters (Shah, 2011). The petioles are green and 0.6-1.3 cm in length. The fruit of this plant is round, heart shaped, ovate or conical. It is green-yellow in colour initially, but the ripe fruit is white with the sweetly aromatic pulp also white (Lim, 2012). The seeds are shiny, numerous, and blackish or dark brown in colour (Pino, 2010).

This plant is found in different parts of the world but native cultivation is extensive in the tropics and the West Indies. It is also a common sight in India and Thailand (Pandey and Barve, 2011). Other than a food source (fresh fruit), *A. squamosa* has been used by various cultures in a variety of ways. The pulp of this plant is used as flavouring in ice cream. A good tonic can be derived from the fruit which enriches blood and relieves vomiting (Ong and Norzalina, 1999). *A. squamosa* leaves are used for the treatment of cancer, vermicide and skin problems and also applied to wounds and ulcers (Pandey and Barve, 2011; Shah, 2011). The roots are used as a drastic purgative and the root bark is used for the relief of toothache (Pandey and Barve, 2011;

Raj *et al.*, 2009). Also, the seeds of this plant are used for the treatment of enema in children and for getting rid of head lice (Yang *et al.*, 2007).

**Figure 2.4.** *Annona squamosa*.

**Taxonomy of *Annona squamosa***

Kingdom: Planteae

Phylum: Spermatophyta

Class: Magnoliopsida (dicot)

Order: Magnoliales

Family: Annonaceae – Custard-apple family

Genus: *Annona*

Species: *Annona squamosa*

### 2.2.2. Chemical constitutes of *A. squamosa*

*A. squamosa* contains glycoside alkaloids, saponins, flavonoids, tannins, carbohydrates, proteins, phenolic compounds, amino acids and diterpenes (Pandey and Barve, 2011). The main bioactive compounds found in various parts of the plant are alkaloids which include liriodenine (1), oxoanalobine (2), anonaine (3), isocorydine (4), norisocorydine (5), benzo-oxyquinazoline (6), samoquasine (7), analobine (8) and reticuline (9) Table 2.3 and Figure 2.5.

**Table 2.3.** Chemical constitutes of alkaloids of *A. squamosa*

| **Name of the compound** | **Part of the plant** | **References** |
|---|---|---|
| Liriodenine (1) | Leaves and seeds | Lebrini *et al.* (2010) |
| Oxoanalobine (2) | Leaves and seeds | Lebrini *et al.* (2010) |
| Anonaine (3) | Leaves, tender stem, bark, roots, and seeds | Bhakuni *et al.* (1972) |
| Isocorydine (4) | Leaves, tender stem, bark and roots. | Bhakuni *et al.* (1972) |
| Norisocorydine (5) | Leaves, tender and stem | Bhakuni *et al.* (1972) |
| Liriodenine (6) | Roots | Bhakuni *et al.* (1972) |
| Abenzooxyquinazoline (7) | Seeds | Morita *et al.* (2000) |
| Samoquasine A (8) | Seeds | Morita *et al.* (2000) |
| Anolobine (9) | Roots | Bhakuni *et al.* (1972) |
| Reticuline (10) | Roots | Bhakuni *et al.* (1972) |

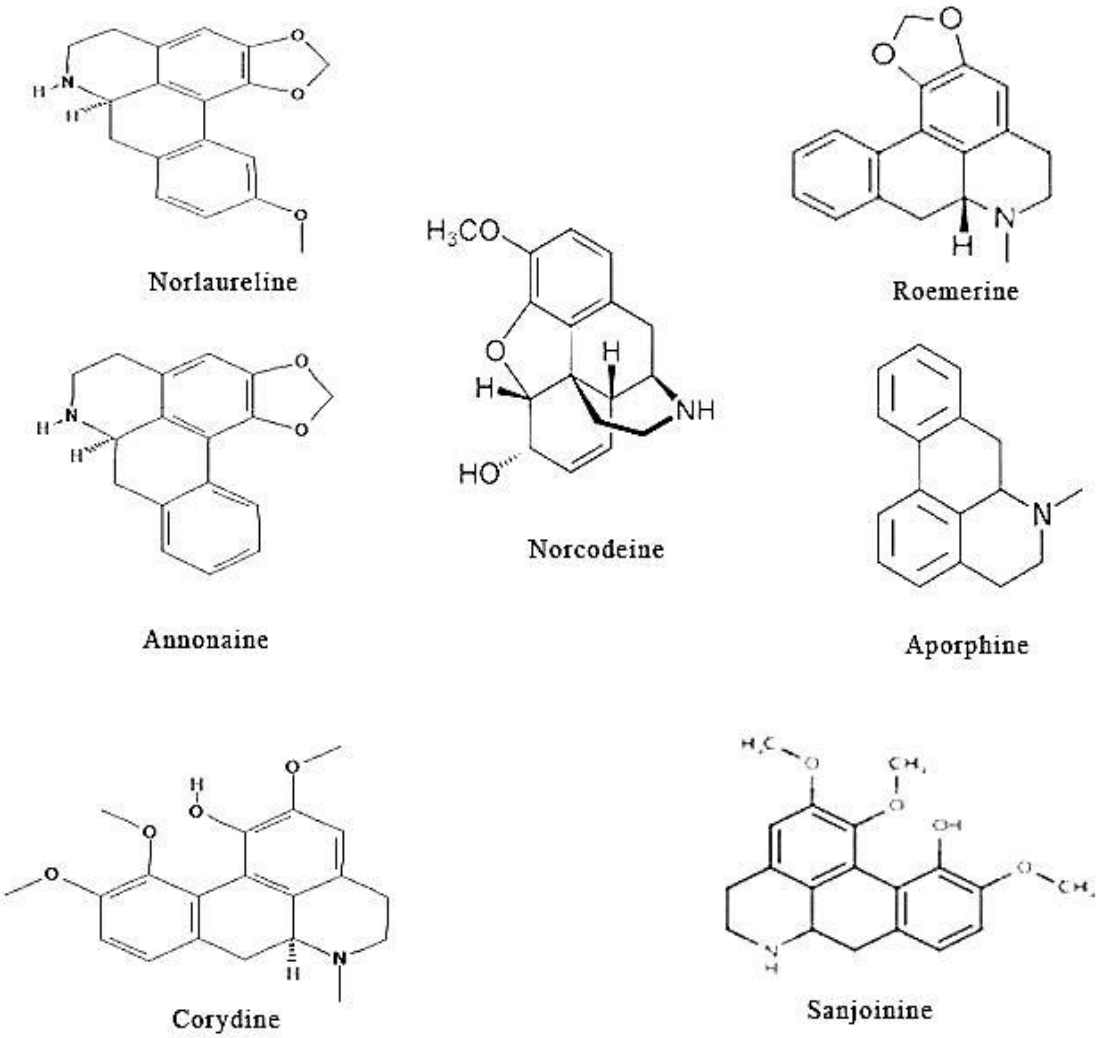



**Figure 2.5.** Chemical structure of the alkaloids from *A. squamosa* (Bhakuni *et al.*, 1972).

Terpenoids are very important compounds found in the fruit and stem of this plant. These terpenoids include 16 α hydroxy-(-)-kauran-19-oic acid (21), kauran-16-en-18-oic acid (22), annomosin A (23), annosquamosin C (24), annosquamosin D (25), annosquamosin E (26), annosquamosin F (27), annosquamosin G (28) and annosquamosins B (29) Table 2.4 and Figure 2.6.

**Table 2.4.** Chemical constitutes of diterpenes of *A. squamosa*

| **Name of the compound** | **Part of the plant** | **References** |
|---|---|---|
| 16 α Hydroxy-(-)-kauran-19-oic acid (22) | Fruit | Wiart *et al.* (2005) Lim (2012a) |
| kauran-16-en-18-oic acid (23) | Fruit | Andrade *et al.* (2001) |
| Annomosin A (24) | Stem | Yang *et al.* (2002) |
| Annosquamosin C (25) | Stem | Yang *et al.* (2002) |
| Annosquamosin D (26) | Stem | Yang *et al.* (2002) |
| Annosquamosin E (27) | Stem | Yang *et al.* (2002) |
| Annosquamosin F (28) | Stem | Yang *et al.* (2002) |
| Annosquamosin G (29) | Stem | Yang *et al.* (2002) |
| Annosquamosins B (30) | Stem | Mukhlesur Rahman *et al.* (2005) |

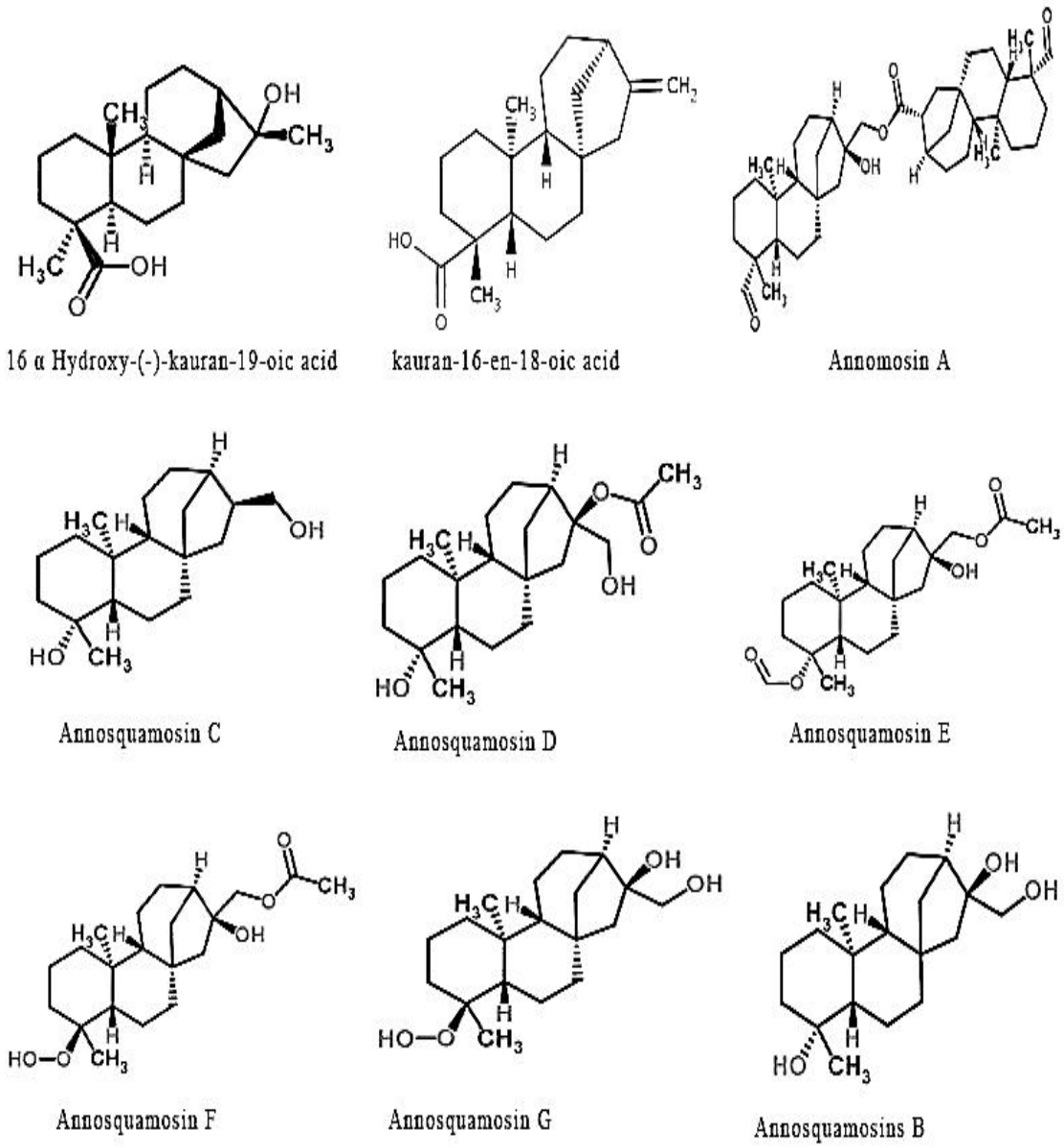



**Figure 2.6.** Chemical structure of the diterpenes from *A. squamosa* (Yang *et al*,. 2002).

### 2.2.3. Antibacterial activity of *A. squamosa*

*A. squamosa* extracts from several solvents have antibacterial activity against many strains of bacteria. Padhi *et al.* (2011) used different solvents for leaf extracts of *A. squamosa* to inhibit Gram-positive bacteria such as *S. aureus, B. subtilis, S. epidermidis* and Gram-negative bacteria including *E. coli, P. aeruginosa, S. typhi, Vibrio alginolyticus* and *V. cholera*. The silver nanoparticles of aqueous extract from the leaf of sweet apple exhibited antibacterial activity against *B. cereus* (NCIM 2703), *B. subtilis* (NCIM 2635), *S. typhimurium* (NCIM 2501), *S. aureus* (NCIM 2654), *P. aeruginosa* (NCIM 5032) and *Proteus vulgaris* (NCIM 2813) (Jagtap and Bapat, 2012). Leaf extracts from different solvents exhibited antibacterial activity against all test strains of *Neisseria gonorrhoeae* in patients infected with acute gonococcal urethritis (Shokeen *et al.*, 2005). Previous studies on ethanol and aqueous extracts from the same part of this plant have revealed significant antibacterial activity against Gram positive bacteria (*S. epidermidis, Streptococcus agalactiae*) and Gram negative bacteria (*E. coli, P. aeruginosa*) (Sailaja *et al.*, 2009).

Aher *et al.* (2012) reported that methanol, petroleum ether and chloroform extracts from the fruit of the nona plant have antibacterial properties against *S. aureus, E. coli, P. aeruginosa, B. subtilis* and *Klebsiella pneumonia*. The methanolic extract exhibited higher antibacterial activity against *S. aureus* compared to other bacterial strains whereas the methanolic extract from the same morphological part of the plant showed high antimicrobial activity against *K. pneumonia* and the lowest antibacterial activity against *B. subtilis.* The author documented that the chloroform extract from the fruit of this plant had significant antibacterial activity against *E. coli,* but the lowest antibacterial activity against *B. subtilis.* A previous study reported that ethanolic extract from the dried fruit of *A. squamosa* inhibited the growth of Gram-positive bacteria, Gram-negative bacteria, and yeast (Hassan *et al.*, 2003). 16 α hydroxy-(-)-kauran-19-oic

acid extracted from the fruit of this plant showed antibacterial activity against ATCC strains of *S. aureus* and *Streptococcus pneumonia* (Wiart *et al.*, 2005).

### 2.2.4. Antioxidant activity of *A. squamosa*

*A. squamosa* is medically significant as an antioxidant source to protect the body from cell damaging free radicals and many diseases including diabetes and cancer. Utilising the N.N. dimethyl-p-phenylendiamine (DMPD) radical scavenging assay, Jagtap and Bapat (2012) reported high antioxidant activity from the fruit pulp of the nona plant and the acetone extract revealed a high antioxidant level through the TPC and DPPH methods. Nandhakumar and Indumathi (2013) documented the high antioxidant activity level of the methanolic extract from the fruit pulp compared to the aqueous extract through the utilisation of DPPH, lipid peroxidation, nitric oxide, superoxide anion ($O_2^-$), hydroxyl radical (OH), reducing power assay and total antioxidant.

Oxygen radical absorbance capacity and MTT assays revealed the good antioxidant activity of the methanolic extract from the leaves, roots, bark and cake of *A. squamosa* (Mariod *et al.*, 2012). The ferric reducing power assay showed that the antioxidant activity of the hydroalcoholic extract from the leaf of this plant is depended on the concentration of the extract. This is due to the presence of phenolic compounds which are responsible for antioxidant activity (Tomar, 2013).

### 2.3. *Alstonia angustiloba* (plant morphology and chemical components)

*A. angustiloba* belongs to Apocynaceae family (Figure 2.7). It is a tropical tree that can grow up to 45 metres in height. The leaves are on stout petioles in whorls of 5-8, 8-16 cm. They are elliptical, subacuminate or obtuse with numerous secondary veins. The flower of the plant is bisexual, calyx histellous and corolla glabrous outside. The local name of this plant is *pulai* or *pulai bukit* (Al-Adhroey *et al.*, 2010; Neo *et al.*, 2013; Valkenburg and Bunyapraphatsara, 2001).

**Figure 2.7.** *Alstonia angustiloba*

**Taxonomy of *Alstonia angustiloba***

Kingdom: Planteae

Phylum: Magnoliophyta

Class: Magnoliopsida (dicot)

Order: Gentiananae

Family: Apocyanaceae

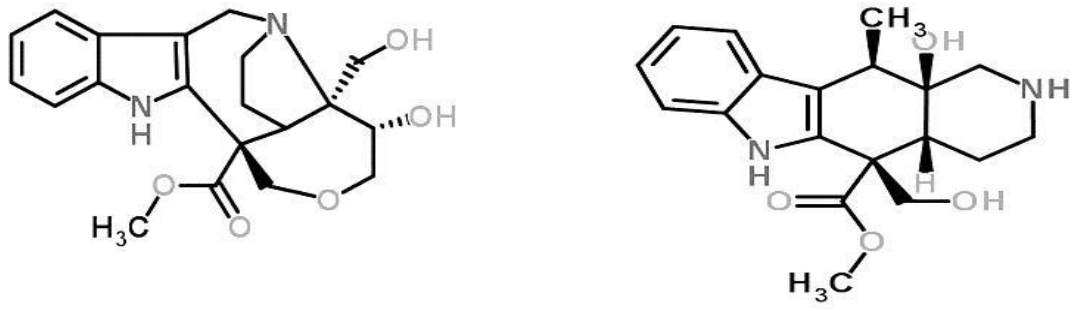
Genus: *Alstonia*

Species: *Alstonia angustiloba*

It is found in the tropical continents of Africa and Asia (Koyama *et al.*, 2008). In Indonesia, this plant is used as medicine for skin sores and gynaecological diseases (Mulyoutami *et al.*, 2009). While in Malaysia, the leaves of the pulai plant are used for the treatment of headache (Lin, 2005).

*A. angustiloba* is rich in indole alkaloids which are present in various parts of the plant. Goh *et al.* (1997) reported that this plant found in the lowland forests of Sabah, have bioactive alkaloids which can be extracted from its leaves and bark. Bark from the stem of *A. angustiloba* contains 20 alkaloids including angustilobine and andranginine (Figure 2.8). These compounds display cytotoxicity against KB cells (Ku *et al.*, 2011).

Koyama *et al.* (2008) reported that five indole alkaloids (alstilobanine A-E) were isolated from the leaves of this plant. These alkaloids display vasorelaxant activity. The methanolic extract from the leaves of this plant exhibit higher antitrypanosomal and cytotoxicity activities because it is richer in alkaloids compared to other plants (Norhayati *et al.*, 2013).




Angustilobine

Andranginine

**Figure 2.8.** Chemical structure of some alkaloids of *A. angustiloba* (Norhayati *et al.*, 2013).

## 2.4. Australian plant mixture

Australian native herbs have been used in the food industry (Ahmed and Johnson, 2000). These herbs have bioactive compounds such as aldehyde and phenolic that show evidence of antimicrobial activity against microorganisms (Duraipandiyan *et al.*, 2006; Gram *et al.*, 2002; Rauha *et al.*, 2000). An Australian Plant Mixture (APM) that contains four indigenous Australian plants (*B. citriodora*, *T. ferdinandiana*, *C. australasica* and Australian wheatgrass sprouts) was examined for synergistic antimicrobial activity using selective test microorganisms.

The lemon myrtle or lemon-scented iron wood (*B. citriodora*) plant of the Myrtaceae family is found in the subtropical rain forests of central and eastern Australia (Wilson *et al.*, 2000). While some trees may grow up to 20 metres in height, others are smaller. The leaves are opposite lanceolate, evergreen, with an entire margin. The creamy-white flowers are produced in clusters at the end of the branches between summer and autumn (Armesto *et al.*, 2001; Howard, 1994). *B. citriodora* has been used as a food ingredient, food flavouring agent, and consumed as herbal tea (Taylor, 1996).

It contains essential oils and two main isomeric aldehydes, *cis* and *trans*-citral (Buchaillot *et al.*, 2009; Burke *et al.*, 2004). Hayes and Markovic (2002) reported the essential oils of *B. citriodora* have the antibacterial activity against Gram-positive test bacterial strains and Gram-negative test bacterial strains in disc diffusion and MIC assays. The aqueous, ethanol and hexane extracts of *B. citriodora* leaves inhibited the growth of *Enterococcus faecalis*, *P. aeruginosa, E. coli, S. aureus, S. typhi, S. typhimurium* and *Listeria monocytogenes* (Dupont *et al.*, 2006).

The billy goat plum (*T. ferdinandiana*) of the Combretaceae family also goes by the names kakadu plum or murung. It is a tropical tree found in northern and north western Australia which can grow up to 32 metres (Bell, 1999). It has large, broadly oval, pale

green leaves and a flaky bark. The flowers are small and creamy-white and the fruit is yellowish green with a large seed. The plant is used as a natural source of vitamin C in dietary health supplements (Mohanty and Cock, 2012). A high level of total phenolic content is found in the kakadu plum fruit reaching 160 µmol gallic acid equivalence GAE/g. This enhances its antioxidant capacity along with a high level of vitamin C (Konczak *et al.*, 2010).

The third plant in the mixture is the finger lime, *C. australasica,* of the Rutaceae family. It is a small tree, 2-7 metres in height, with small leaves and white flowers. The shape of the fruit is cylindrical and comes in different colours such as pink and green. It is found in the southern and northern regions of New South Wales, Australia. It is used in cooking and the preparation of jams and drinks (Lim, 2012b). The fruit of finger lime contains a low level of total phenolic content of 9.12 µmol gallic acid equivalence (GAE/g) and a small amount of vitamin C resulting in a low antioxidant capacity (Konczak *et al.*, 2010). The essential oils of finger lime inhibited the growth of *S. epidermidis* in an electromagnetic field (Matewele, 2010). The Australian plant mixture also contains organic Australian wheatgrass sprouts which are rich in a wide range of vitamins, minerals, amino acid and carotenoids.

### 2.5. Mechanism action of bioactive compounds

The many bioactive compounds in plants include anthraquinones, alkaloids, diterpenes, and phenolic compounds. These compounds are found in plants as secondary metabolites to overcome a variety of pathogens. Researchers discovered that unlike antibiotics, the application of these compounds do not result in side effects. These compounds use different antibacterial activities such as membrane disruption, inhibition of protein synthesis, inhibition of DNA or RNA synthesis, inactive enzymes or block cell wall synthesis to destroy microorganisms (Cowan, 1999; Negi, 2012).

A significant bioactive compound is anthraquinones. Cationic anthraquinones can destroy *E. coli* and MRSA by membrane disruption. However this compound has a modest effect on Gram negative bacteria (*E. coli*) because Gram negative bacteria have negatively charged that greatly lower the inhibition rate. The mode of actions of these compounds inhibit the rodex processes inside cells and cause cell death (Chan *et al.* 2011). Lu *et al.* (2011) reported that with the utilisation of a transmission electron microscope, it was revealed that anthraquinones extracted from rhubarb cause breakage in the cell wall and membrane of *Aeromonas hydrophila* leading to leakage of the cellular cytoplasmic content. A previous study on anthraquinones extracted from *Polygonum cuspidatum* roots showed morphological changes in bacteria exposed to the binding of active compounds to the cell surface and the penetration of bacterial cells to affect the phospholipid of the cell membrane and block membrane-bonding enzymes (Shan *et al.*, 2008).

Alkaloids (berberine, piperine) are other significant compounds that cause intercalate of the cell wall and/or DNA (Atta-ur-Rahman and Choudhary, 1995). Through the utilisation of a TEM, Obiang-Obounou *et al.* (2011) found that sanguinarine extracted from the root of *Singuinaria canadensis* causes distorted septa with rare lysis of treated cells of MRSA.

The mode of action of terpenes is by membrane disruption of target cells (Cowan, 1999; Radulovic *et al.*, 2013). These compounds can be alternated to affect membrane permeability leading to the draining of the inner contents of treated bacterial cells (*S. enteritidis*). These distorted treated bacterial cells can be differentiated from bacterial cells without compounds which have regular cell walls and cell membranes (Tyagi *et al.*, 2013).

The most important bioactive compounds are phenolic compounds which are found in most plants. These compounds have an effect on membranes and there is a correlation between the toxicity and hydrophobicity of phenolic compounds (Negi, 2012). Borges *et al.* (2013) reported that ferulic and gallic acids treated with different bacterial strains of Gram positive and Gram negative bacteria can lead to changes in the charge, the intra and extracellular permeability, and the physicochemical properties of membranes. These then leads to rupture or pore-formation in the cell membranes which consequently leak essential intracellular constituents. The mode of action of phenolic compound is related to the inactivation of cellular enzymes. This mechanism depends on the rate of penetration of the compound into the cell or membrane permeability changes. These alterations in permeability result in the death of the cell (Moreno *et al.*, 2006).

## 2.6. Lactic acid bacteria

There are 20 genera of lactic acid bacteria found in different environmental conditions such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Lactococcus* (Florou-Paneri *et al.*, 2013; Ghanbari and Jami, 2013). *Lactobacillus* has 125 species compare to other genus of lactic acid bacteria (Kandler and Weiss, 1986). It belongs to Lactobacteriaceae family, which is Gram-positive, catalase negative, oxidase negative, non-sporing and rod shape bacteria (Patil *et al.*, 2010; Salminen and Von Wright, 2011). These bacteria are found in several sources such as plants, animals and fermented dairy products (Giraffa *et al.*, 2010; Stiles, 1996).

*Lactobacillus casei*, a species of this genus, is Gram positive, long or short rod-shaped with square ends, catalase negative, and oxidase negative. It thrives in a wide temperature (15-45°C) and pH range. The colonies appear white to light yellow with a smooth lens or diamond shape. It ferments glucose, fructose, galactose, mannose, ribose, sorbitol, sorbose, terholose and, at a slower rate, maltose and sucrose (Almståhl *et al.*, 2012; Banwo *et al.*, 2013; Kandler and Weiss, 1986; Wang *et al.*, 2012). This bacteria is utilised as a starter culture in milk fermentation and cheese production (Koutinas *et al.*, 2009; Martinovic *et al.*, 2013; Pogačić *et al.*, 2013; Settanni and Moschetti, 2010). Specific strains of these bacteria have been identified as probiotic bacteria (Douillard *et al.*, 2013; Settanni and Moschetti, 2010).

*L .casei* is isolated from different sources such as raw and fermented dairy products, plant materials and the gastrointestinal tracts of animals and humans (Chang *et al.*, 2010; Datta *et al.*, 2013; Herrero-Fresno *et al.*, 2012). Among the methods used for the identification and characterization of *L. casei* are biochemical tests, antimicrobial susceptibility, bacteriophage types, 16S rRNA and DNA sequences (Korhonen, 2010; Muñoz-Atienza *et al.*, 2013).

Another group of lactic acid bacteria from the Lactobacteriaceae family is *L. paracasei.* This bacteria, which is Gram- positive and rod shaped, is isolated from dairy products such as raw cow milk, cheddar cheese, feta cheese and feta brine (Floros *et al.*, 2012; Maragkoudakis *et al.*, 2006; Oliszewski *et al.*, 2013; Pavlidou *et al.*, 2011; Settanni and Moschetti, 2010). It is at home in a temperature range between 30 and 37°C and a pH range of 5.5-6.5. The colonies are cream-white in colour and this bacterium is catalase negative, non-spore forming, oxidase negative and non $H_2S$ forming. It ferments D-glucose, fructose, galactose, mannose, arabinose, sorbitol, cellobiose, terholose, rhamnose, salicin, esculin and maltose (Forouhandeh *et al.*, 2010; Mercanti *et al.*, 2012; Pérez-Sánchez *et al.*, 2011; Terzic-Vidojevic *et al.*, 2009).

### 2.7. Antibacterial peptide from lactic acid (bacteriocins)

*Lactobacillus spp.* produces a natural antimicrobial peptide or protein called "Bacteriocins". The term refers to antibiotic-like compounds which inhibit bacterial strains (Deshmukh and Thorat, 2013; Siamansouri *et al.*, 2013). In 1925, A. Gratia identified bacteriocin as a substance that killed bacteria (Nishant, 2012). These compounds produced by lactic acid bacteria, are small peptides (> 10-KDa) which are cationic charged and heat stable (Zacharof and Lovitt, 2012). Bacteriocins are applied in a variety of ways including for food preservation and as a safe antibacterial compound to replace antibiotics in the spheres of medical veterinary applications and crop management (Figure 2.9) (Dicks *et al.*, 2011; Montalban-Lopez *et al.*, 2011; Yusuf and Hamid, 2013).

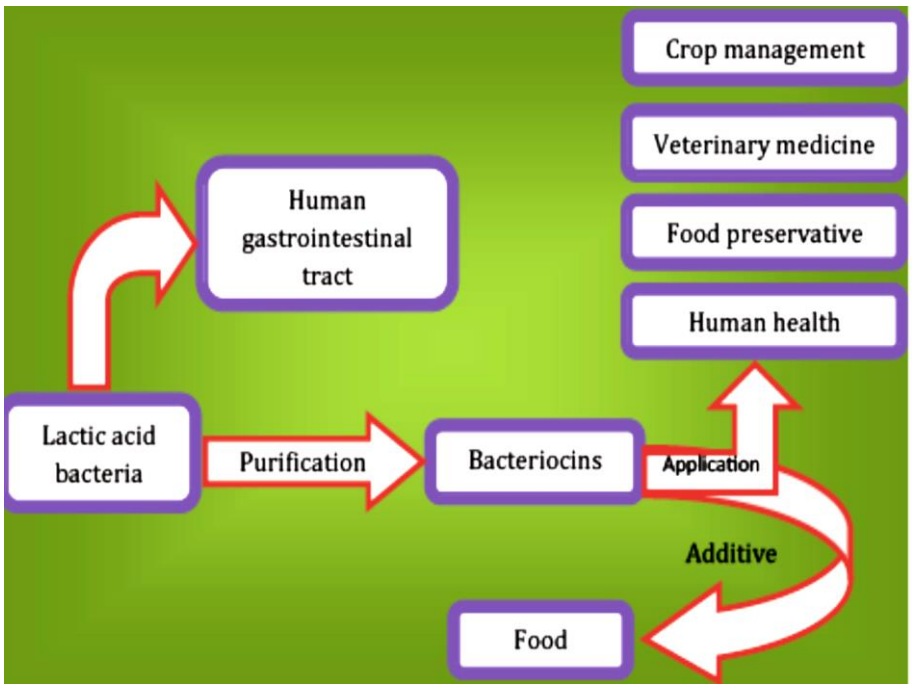



**Figure 2.9.** The application of bacteriocin produced by lactic acid bacteria (Nishant, 2012).

Bacteriocin produced from lactic acid bacteria are separated into four classes (Table 2.5). Each class defined by characterizations such as structure, size, primary sequence, biosynthesis and mode of action (Cotter *et al.*, 2012; Kawai and Saito, 2011; Papagianni, 2012).

**Table 2.5.** Classes of bacteriocins of main producers of lactic acid bacteria

| **Class** | **Bacteriocin** | **Lactic acid bacteria producers** |
|---|---|---|
| I | Nisin | *Lactococcus lactis* subsp. *lactis* |
| | Lactococcin | *Lactococcus lactis* ADr 185 L030 |
| | Lacticin 3147A1 | *Lactococcus lactis* subsp. *lactis* |
| | Lactocin S | *Lactobacillus sake* |
| | Mersacidin | *Bacillus* sp. strain HIL Y-85,54728 |
| II | Lactocin 705 | *Lactobacillus casei* |
| | Paracin C | *Lactobacillus paracasei* CICC 20241 |
| | Lactobin A | *Lactobacillus amylovorus* |
| | Mesentericin Y105 | *Leuconostoc mesenteroides* |
| | Pediocin A | *Pediococus pentosaceous* |
| | Pediocin F | *Pediococcus acidilactici* |
| | Enterocin A | *Enterococcus faecium* |
| | Curvacin A | *Lactobacillus curvatus* |
| | Acidolin | *Lactobacillus acidophilus* |
| | Plantaricin A, B, | *Lactobacillus plantarum* |
| | Lactolin | *Lactobacillus plantarum* |
| III | Helveticin J | *Lactobacillus helveticus* |
| | Enterolysin | *Enterococcus fecalis* |
| IV | Acidocin B | *Lactobacillus acidophilus* |
| | Gassericin A | *Lactobacillus gasseri* LA39 |

1- Class I bacteriocin : Lantibiotics

These compounds, which have a low molecular weight of less than 5-kDa, are heat stable and enzymatic sensitive. They are characterized by a thioester bond between side chains of amino acids in their structure (Bierbaum and Sahl, 2009; Deshmukh and Thorat, 2013; Islam *et al.*, 2012). Based on the structure, the two types of these bacteriocins are A and B lantibiotic. Lantibiotic A which weighs between 2 to 4 kDa is positively charged, elongated, screw-shaped and amphipatiac. The major groups of these bacteriocins are nisin and laticin (Piper *et al.*, 2009; Wilson-Stanford and Smith, 2011; Zacharof and Lovitt, 2012). Lantibiotic B weighs between 2 to 3 kDa, is globular in structure with rigid and negative or neutral peptides. The major groups of these bacteriocins are lacticin 3147 A1 and mersacidin (Lee, 2011; Rattanachaikunsopon and Phumkhachorn, 2010; Siamansouri *et al.*, 2013). These bacteriocins exhibit antibacterial activity towards Gram positive bacteria such as lactic acid bacteria, *Clostridium sp.*, *L. monocytogenes*, *Bacillus sp.* and *Micrococcus sp.* (Asaduzzaman and Sonomoto, 2011; Campion *et al.*, 2013; Field *et al.*, 2012).

2- Class II:Non-Lantibiotics

This type of bacteriocin, with a molecular weight of <10-kDa, is heat stable with a possible disulfide bond in its structure (Dicks and Botes, 2010; Siamansouri *et al.*, 2013). These bacteriocins have three subgroups (a, b and C). Class IIa bacteriocins are pediocin-like bacteriocins with 37 to 48 amino acid residues. They exhibit cationic and antibacterial activity towards *L. monocytogenes*. Class IIb Non-Lantibiotics comprise two peptides weighing between 2 and 4-kDa with each peptide containing 33 amino acids (Kjos *et al.*, 2009; Šuškovi *et al.*, 2010; Zacharof and Lovitt, 2012). Lactocin 705, which belongs in Class IIb, is a product of *Lactobacillus casei* CRL705. This bacteriocin, with a small molecular weight of 3357.80 Da, has rich glycine residues and an isoelectric point of 10.03. It exhibits antibacterial activity towards *L. monocytogenes*

(Castellano *et al.*, 2003; Nissen-Meyer *et al.*, 2010). Another Class IIb bacteriocin named paracin C is produced from *Lactobacillus paracasei* CICC 20241. This peptide, weighing 5000 Da, is heat-stable and displays antibacterial activity towards 17 strains of *Alicyclobacillus acidoterrestris* (Pei *et al.*, 2013). Type IIc non-lantibiotics is small, heat-stable and thiol active peptides. They are found in subgroups such as divergicin A and acidocin B (Siamansouri *et al.*, 2013).

3- Class III Bacteriocins

Bacteriocins belonging to this group are a large molecular mass weighing > 30-kDa and heat-stable. This group is isolated from different strains of *Lactobacillus spp.* (Deshmukh and Thorat, 2013; Nishant, 2012; Siamansouri *et al.*, 2013) and is found in *Lactobacillus helveticus* and *Enterococcus faecium* (Zacharof and Lovitt, 2012).

4- Class IV Bacteriocins

This class of bacteriocins has a larger peptide than the others. Produced from lactic acid bacteria, the circular peptide of this group contains 58 to 70 amino acid residues in its structure (Maqueda *et al.*, 2008; Masuda *et al.*, 2012; Rattanachaikunsopon and Phumkhachorn, 2010). This type of bacteriocin is complex and requires carbohydrate or lipid moieties to be active against microorganisms. This class comprises gassericin A, isolated from *Lactobacillus gasseri* LA39, and acidocin B, from *Lactobacillus acidophilus* (Balciunas *et al.*, 2012; Nishant, 2012).

## 2.8. Mechanism of action of antibacterial peptides from lactic acid bacteria

Bacteriocins produced from most lactic acid bacteria are cationic peptides as a result of the presence of amino acids (lysine, arginine and histidine) and their hydrophobic and amphiphilic nature as they contain 20 to 60 amino acids. These properties make possible the binding of these bacteriocins with the negatively charged phospholipid in the cytoplasmic membrane of cells. These compounds then destroy the target cells after

a series of reactions (Carmona-Ribeiro and de Melo Carrasco, 2013; Yeamn and Yount, 2003). Lactic acid bacteria produce Class I, II, III and IV bacteriocins. All classes of bacteriocins inhibit bacteria by pore formation through different mechanisms (Balciunas *et al.*, 2012; Deshmukh and Thorat, 2013).

Class I bacteriocins have a dual model to destroy bacterial cells. These compounds bind with lipid II of the cytoplasmic membrane in bacterial cells to block synthesis of the cell wall resulting in cell death or utilize lipid II of the membrane of bacterial cells to form cell-destroying pores (Figure 2.10) (Bierbaum and Sahl, 2009; Rea *et al.*, 2011; Yusuf and Hamid, 2013).

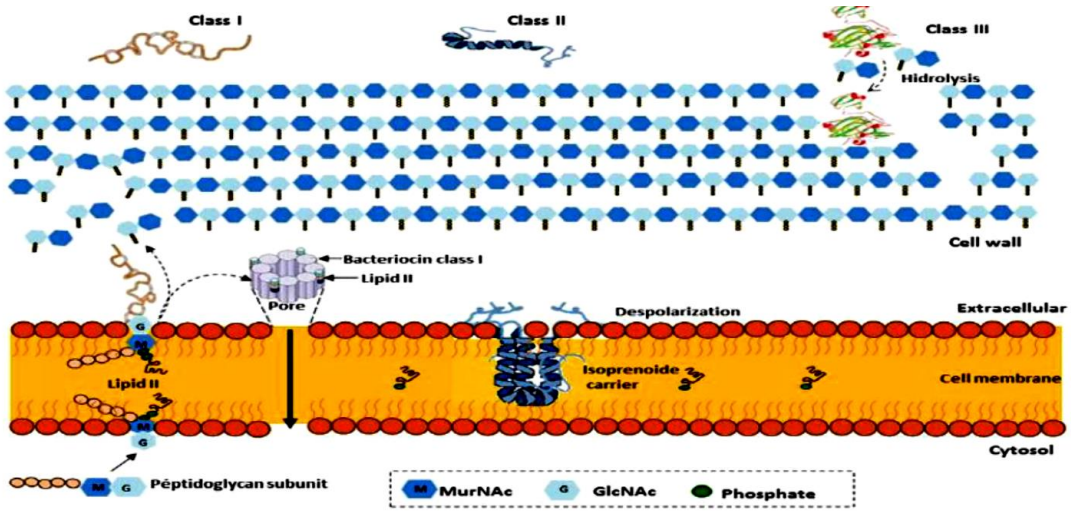



**Figure 2.10.** Mode of action of bacteriocins produced from lactic acid bactetria (Yusuf and Hamid, 2013).

The mechanism of class II bacteriocins are amphiphilic in nature affecting the anionic surface of the cytoplasmic membrane of bacterial cells by electrostatic interactions (Drider *et al.,* 2006). The hydrophilic N-terminal end of this peptide is connected to the hydrophobic C-terminal end as a hinge region. The hydrophobic C-terminal ends of these bacteriocins then penetrate the cell membrane and the resulting pores lead to the destruction of the bacterial cells (Cotter *et al.,* 2012; Nissen *et al.*, 2009).

Pangsomboon *et al.* (2006) reported that bacteriocin purified from *L. paracasei* HL32 and treated with *Prophyromonas gingivalis* causes distortion in the morphological shape of bacteria. Furthermore, this peptide can be the source of pore formation in the rod-shaped bacterial cells. The mechanism of action of class III bacteriocins such as enterolysin A is by hydrolysis of the peptide bonds in the peptidoglycan of bacterial cells because these peptides have two catalytic domains in N terminal and C terminal ends. The catalytic activity of this peptide hydrolyses the peptidoglycan of sensitive bacterial cells and kills the bacteria (Khan *et al.*, 2013; Nilsen *et al.*, 2003; Šušković *et al.*, 2010).

## 2.9. Antibacterial peptides from plants

Antibacterial peptides and protein are important compounds for plants as they are a part of the defence against of infections by a range of harmful pathogens (Kovalskaya *et al.*, 2011; Wong *et al.*, 2013). These compounds, known as defensins, are found in all plant species. They display antibacterial activity towards bacteria at low concentrations compared to other bioactive compounds from natural sources (Barbosa Pelegrini *et al.*, 2011). The first plant source from where antibacterial peptides were isolated is wheat flour (*Triticum aestivum*) which has the ability to inhibit the growth of *Pseudomonas solanacearum, Xanthomonas campestris* and *Corynebacterium michiganese* (Caleya *et al*., 1972). Mandal *et al.* (2009) reported that three purified peptides (Cn-AMP1, Cn-AMP2, Cn-AMP3) extracted from green coconut water demonstrated antibacterial activity towards *S. aureus*, *E. coli*, *B. subtilis* and *P. aeruginosa*. The peptides extracted from the seeds of *Pisum sativum* using through different methods such as ammonium sulphate precipitates and purified gel filtration, showed antibacterial activity towards *Micrococcus luteus, S. aureus, S. epidermidis, E.coli, K. pneumoniae, S. typhi, Proteus vulgaris, Pasterurella multocida* and *P. aeruginosa.* Two purified peptide fractions of this plant exhibited antibacterial activity (Rehman and Khanum, 2011).

Astafieva *et al.* (2012) identified three purified peptides from the flowers of *Taraxacum officinale* which had antibacterial activity towards *Pseudomonas syringae* VKM B-1546, *B. subtilis*VKM B-1053, *Clavibacter michiganense* subsp. *Michiganense* VKMAc-1144, and *Xanthomonas campestris*VKM B-608.

Previous studies reported that the primary and tertiary structures of these peptides from plants show that 33% of them possess antibacterial activity. They have 30 to 50 amino acid residues in 59% of their structure (Barbosa Pelegrini *et al.*, 2011; Hammami and Hancock, 2009).

The plant defensins utilised a structural fold known as a cysteine-stabilized αβ motif (CS αβ) or a disulphide bridge to attain structural stability (Carvalho and Gomes, 2009; Franco, 2011). The structure of antibacterial peptides from plants has multifunctional activities (Figure 2.11). The activities of these compounds are related to lethal pathogens such as bacteria and fungi due to their effects on digestive enzymes such as proteinases and α-amylases (de Oliveira and Moreira, 2011).

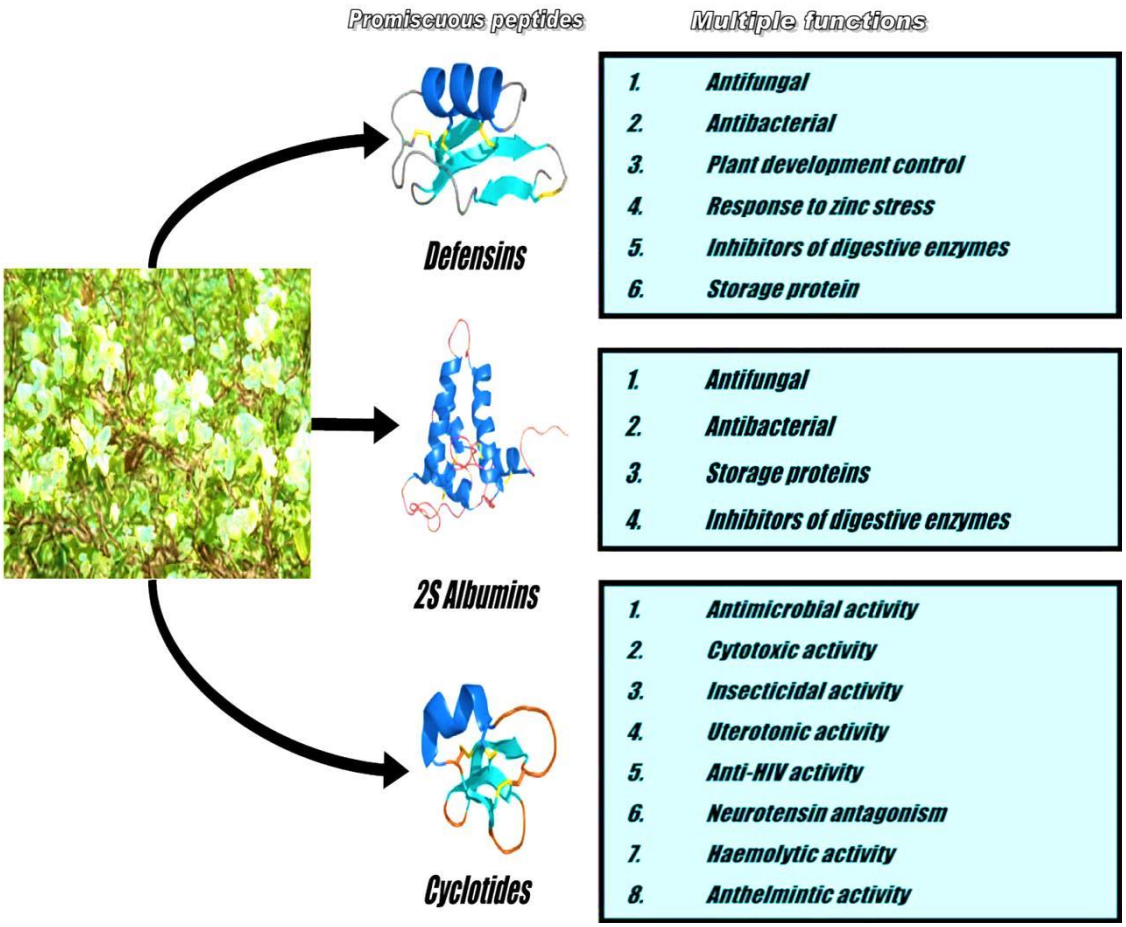



**Figure 2.11**. The multiple functions of plant defensins (Franco, 2011).

Other groups of peptides have rich glycine isolated from *Psidium guajava* seeds, and recombinant *E. coli* displays antibacterial activity towards Gram-positive such as *S. aureus* and *S. epidermides*, and Gram-negative such as *E. coli*, *K. pneumonia* and *P. aeruginosa* (Tavares *et al.*, 2012). These molecules have antioxidant activity because they contain amino acid residues to reduce free radical ions (Pownall *et al.*, 2010).

## 2.10. Mechanism of action of antibacterial peptides from plants

Studies conducted on the mechanism action of antibacterial peptides isolated from plants revealed that these compounds have an amphipathic structure and a cationic charge at physiological pH which are responsible for their antibacterial properties (Yeamn and Yount, 2003). These molecules have the ability to destroy the membranes of bacterial cells by interacting with their lipid layer surfaces (Joshi *et al.*, 2010; Park *et al.*, 2011). Based on the hypothesis, the cationic peptides were attracted to the negative charge of teichoic acid from Gram positive bacterial cells and lipopolysaccharides, or phospholipids from Gram negative bacterial cells. The peptide then binds with the membrane of bacterial cells (Haney *et al.*, 2010; Morris *et al.*, 2012).

There are two models to explain the mechanism action of these molecules. One of these models is the barrel-stave mechanism (Figure 2.12). According to this model, the peptides interact with the hydrophobic portion of the membrane to promote the formation of pores by binding with phospholipid acyl chains. These pores result in the membrane being permeable (Dong *et al.*, 2012; Rahnamaeian, 2011). The peptides then invade the bacterial cells through these pores to cause cell death. The concentration of pores formed is dependent on the concentration of peptides (Okorochenkov *et al.*, 2011).

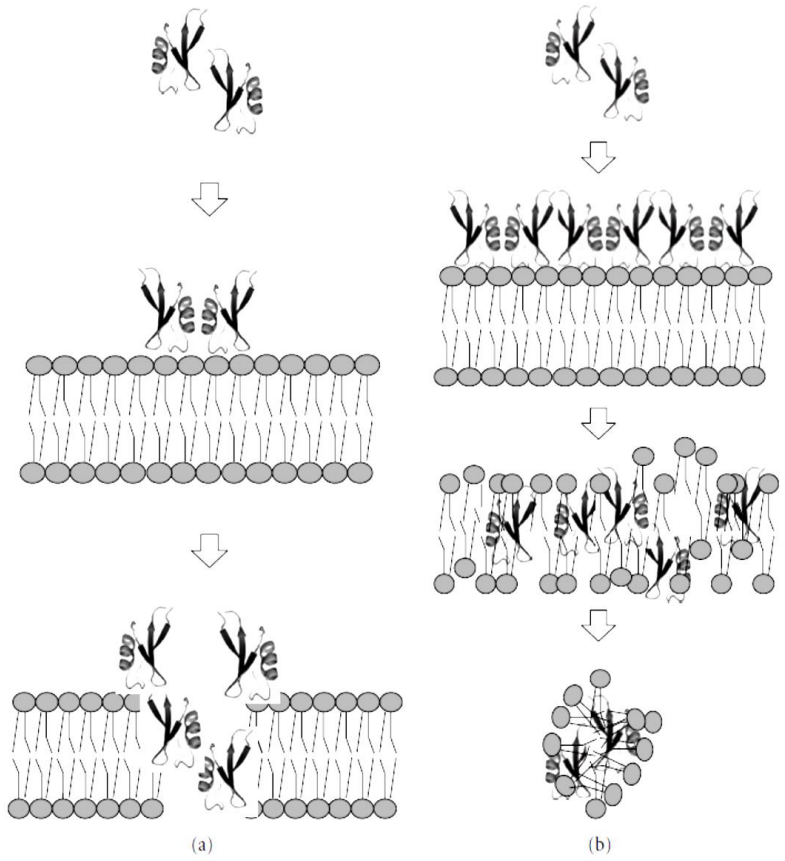



**Figure 2.12.** Mechanism of action of antibacterial peptides (a) barrel-stave model (b) carpet model (Barbosa Pelegrini *et al.*, 2011).

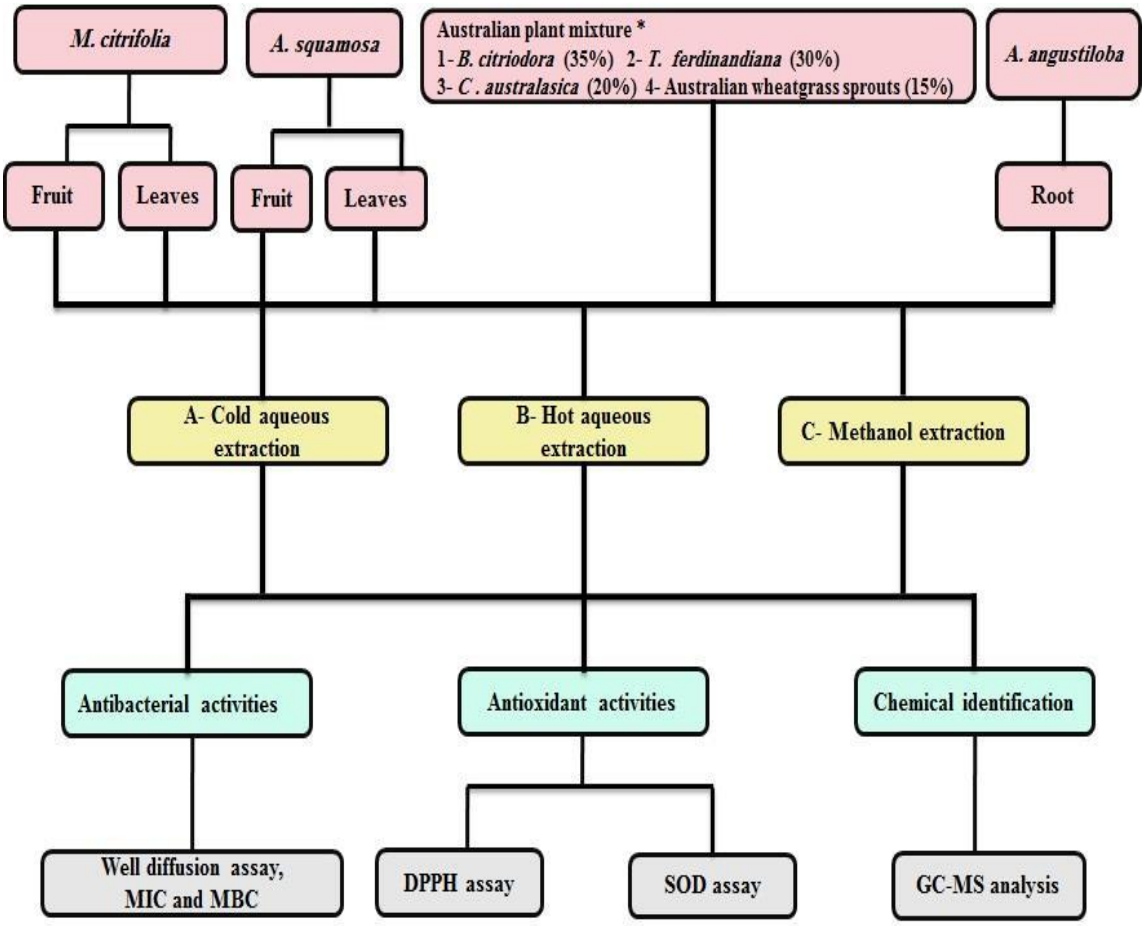
Another model to describe the mechanism of antibacterial peptides is the carpet mechanism. According to this mechanism, the peptides bind to the cell surface and cover the membrane completely. This changes the fluidity and reduces the properties of the bacterial cell membrane leading to membrane disintegration and cell death (Lee *et al.*, 2011; Park *et al.*, 2011).

Kyle *et al.* (2012) reported that peptide recombinant from some plant species (soybean, barley, wheat and *Solanum nigrum*) showed changes in morphology of some bacteria cells (*E. coli*) with cell division caused by the inhibition of cell proportion. Unlike untreated bacterial cells with these peptides, treated cells tend to be elongated and non-septated.

# 3. METHODOLOGY

## 3.1. Part 1: Crude extracts

**Flow chart of experiment done on crude extract**




*** Refer to Table 3.1**

### 3.1.1. Plant collection

The fresh ripe fruit and leaves of *M. citrifolia* were collected from Sendayan Valley, Seremban, *A. squamosa* was collected in November, 2010, from Juasseh, Kuala Pilah, and *A. angustiloba* roots were collected from the herbarium of the University of Malaya. These plants were identified at the herbarium under the registration numbers KLU 22480, KLU 047368 and KLU 33380 respectively. The mixture (APM) used in the study contains four plants namely *B. citriodora*, *T. ferdinandiana*, *C. australasica* and *L. ponticum* (Table 1). The plants were identified by the Australian Tropical Herbarium where voucher specimens were deposited with reference numbers QRS 34595 for *B. citriodora*, CANB 812161.1 for *T. ferdinandiana*, CANB 673743.1 for *C. australasica* and HO 410713 for *L. ponticum.* The leaves of these plants and sprouts of *L. ponticum* were harvested in October 2010. The leaves and sprouts of the constituent plants in the APM originally sourced from Australia.

All samples were washed under tap water and dried in an oven at 40ºC for 3 days. The plant materials were then put through a grinder with a mesh size of 2 mm.

**Table 3.1.** Profile contents of Australian plant mixture

| **S.N** | **Botanical name** | **Common name** | **Family** | **Plants' part** | **% composition** |
|---|---|---|---|---|---|
| 1- | *Backhousia citriodora* | Lemon myrtle | Myrtaceae | Dried leaves | 35% (52.5 g/150 g) |
| 2- | *Terminalia ferdinandiana* | Kakadu plum | Combretaceae | Dried leaves | 30% (45 g/150 g) |
| 3- | *Citrus australasica* | Finger lime | Rutaceae | Dried leaves | 20% (30 g/150g) |
| 4- | *Lophopyrum ponticum* | Australian wheatgrass | Poaceae (syn. Gramineae) | Dried sprouts | 15% (22.5 g/150 g) |

### 3.1.2. Cold aqueous extraction

Five grams of dried powder dried from each plant material was thoroughly mixed with 50 ml of distilled water in a blender and then filtered with Whatman No. 1 filter paper to obtain a concentration of 100 mg/ml. The positive control was 10 mg/ml of tetracycline while distilled water was used as the negative control.

### 3.1.3. Hot aqueous extraction

Five grams of dried powder dried from each plant material was deposited into 50 ml of boiling distilled water for five minutes. Upon cooling, the mixture was filtered with Whatman No.1 filter paper and a concentration of 100 mg/ml was obtained. The positive control used was 10 mg/ml of tetracycline while distilled water was used for the negative control.

### 3.1.4. Methanol extraction

The extraction was based on the method of Hassan et al. (2003). Five grams of dried powder dried from each plant material was mixed with 50 ml of 99% methanol with the aid of a magnetic stirrer at room temperature for three days. After filtration with a Whatman No. 1 filter paper, the solvent was removed with the utilisation of a rotary evaporator at 40ºC (Heidolph WB2000, Germany). The extract was weighed to 0.1g and dissolved in 1 ml of 5% dimethyl sulfoxide (DMSO). The final concentration of 100 mg/ml was diluted to 50 and 25 mg/ml.

### 3.1.5. Determination of antimicrobial activities of plants

#### 3.1.5.1. Well diffusion assay

For this study, four species of bacteria were used. *S. aureus* (RF 122), *E. coli* (UT181), *B. cereus* (ATCC 14579) and *P. aeruginosa* (PA7) were procured from cultures maintained at the Fermentation Technology Laboratory in the Microbiology Division, Institute of Biological Sciences, University of Malaya, Malaysia. Other strains

used in this study included methicillin-resistant *S. aureus* (MRSA) (ATCC BA-43) and *H. pylori* ATCC 43504. Well diffusion assay was performed based on the method of Selvam *et al* (2009). The strains of bacteria, except for *H. pylori*, were inoculated into Mueller-Hinton agar (Difco, Detroit, MI, USA) using cotton swabs. *H. pylori* was inoculated into tryptic soy agar (Difco, Detroit, MI, USA) with 5% defibrinated sheep blood for 3 days at 37°C under microaerophilic conditions. Wells of 6 mm in diameter were made on the media surface in petri plates. All extracts were dispensed into the wells (50 µl) and incubated overnight at 37ºC. Inhibition zones were observed in triplicate plates. The positive control used was 10 mg/ml of tetracycline and the negative control 5% DMSO.

### 3.1.5.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

All extracts were determined for their MIC values using a standard protocol (Andrews, 2001). Nutrient broth (Difco, Detroit, MI, USA) was used as the medium to culture bacteria. One ml of this broth was added to the numbered tubes 1-9. One ml of the stock culture was added to tube 1 and successively diluted until tube number 7. The last 1 ml of tube 7 was discarded. Tube number 8 was used as a negative control and the tube 9 as a positive control. The bacterial inoculum was cultured in nutrient broth and incubated overnight, but *H. pylori* was inoculated into tryptic soy broth with 5% fetal calf serum (Sigma Aldrich GmbdH, Germany) and incubated at 37°C under microaerophilic conditions. All the tubes were inoculated with 1 ml of the test bacteria media, except tube number 8, and incubated for 24 hr. at 37ºC. MIC values were determined based on the tube which showed no growth. MBC values were determined by sub-culturing from the MIC assay tubes onto Muller- Hinton agar (Difco, Detroit, MI, USA) and then determining the dilution at which growth was detected. McFarland 0.5 turbidity standards was used to determine the amount of colony forming units

(CFU) of the bacteria in nutrient broth (1 x $10^8$ CFU/ml) based on optical density measurement at 620 nm.

### 3.1.6. Determination of antioxidant activities of plants

#### 3.1.6.1. DPPH radical scavenging assay

Free radical scavenging activities were determined by using the method of Bozin *et al.* (2008) with some modifications which included the number of samples and conditions of incubation (dark, 25°C for 2 hr.). The reagent of the assay is 2,2-diphenyl-1- picrylhydrazyl solution (Sigma Aldrich GmbdH, Germany) (950 µl) that was added to 50 µl of the extract (10 mg/ml) and the volumes of the solutions made up to 4 ml by adding 95% ethanol. This mixture was shaken vigorously and incubated at room temperature for two hours in the dark. All samples were measured at 515 nm using a Genesys 20 Thermo Scientific (USA) spectrophotometer. The percentage of DPPH radical scavenging activity of the resulting solutions was calculated using the following equation:

DPPH radical scavenging activity (%) = $[(A_{control} - A_{sample)} / A_{control}] \times 100$

Ascorbic acid (10 mg/ml) was used as a positive control of the assay.

$IC_{50}$ was calculated using linear regression plots. The $IC_{50}$ values represent the concentrations of the sample that is required to scavenge 50% of DPPH free radicals.

#### 3.1.6.2. Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was determined using a SOD Assay Kit-WST (Dojindo Molecular Technologies, Gaithersburg). The protocol used in this study was modified from Sakudo *et al.* (2005). The modifications included varying the amount of samples used and the incubation period. The samples (20 µl) were mixed with the reaction mixture in the kit. Then, the mixtures were gently shaken and incubated at 37ºC for 20 min. Antioxidant activity was measured at 450 nm using a Genesys 20 Thermo Scientific (USA) spectrophotometer. The positive control was ascorbic acid (10

mg/ml). The negative control to measure inhibition rates of SOD-like activity used all treatments without sample.

### 3.1.7. GC-MS analysis

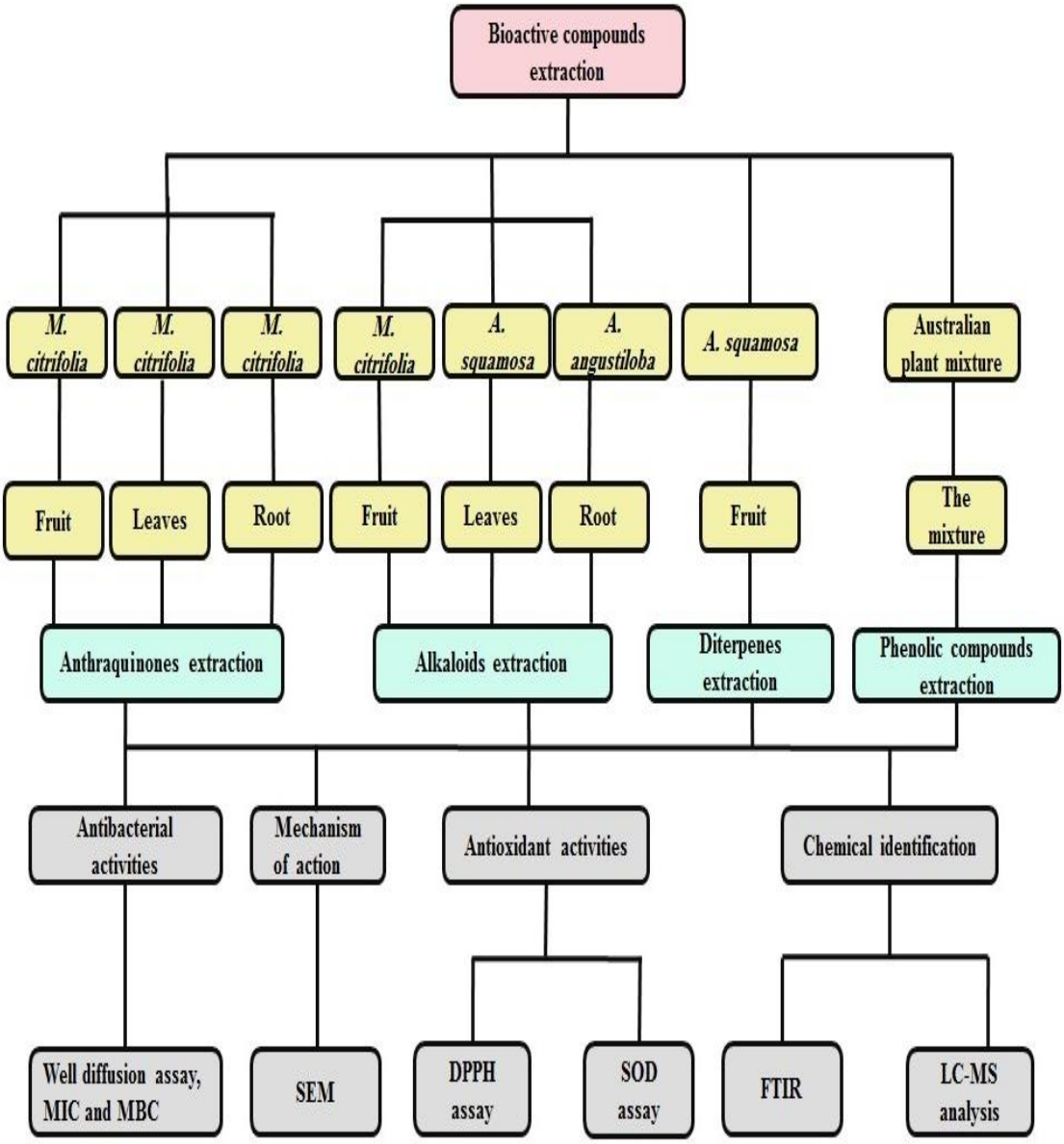
Hot and cold aqueous extracts from the fruit of *A. squamosa*, methanolic extracts from the fruit of *M. citrifolia,* and methanolic extracts from the fruit and leaves of *A. squamosa* were analysed by the system of GC MS-QP2010 PLUS (SCHIMADZU-JAPAN). The mass spectrometer for detection of the electron ionization mode used 400 V and the mass range between *m/z* 50-600 in this assay. The gas chromatography column (DB ms 30 m x 0.25 mm, 025 µm) and the temperature of this column were started at 50°C to 280°C with the rate 2°C/ min. For this experiment, the flow rate of the gas was set at 1.2 ml/min and the volume of sample injection was of 1 µl. The compounds of these extracts were analysed by retention times and mass fragmentation patterns with the standard data in the NIST library provided with these facilities.

### 3.1.8. Statistical analysis

Data is expressed as mean ± SD. Statistical analyses were carried out using SPSS version 17. One-way ANOVA followed by Duncan's multiple comparison were used to compare the values of samples with control. A $P$ value < 0.05 was deemed as indicating significant differences. Each treatment was three replicates and each experiment was repeated at least twice.

### 3.2. Part 2: Bioactive compounds extraction

**Flow chart of experiment done on bioactive compounds extracts**



### 3.2.1. Anthraquinones extracts from *M. citrifolia* fruit and leaves

This method is based on Smita and Sushma (2010). The dried powder of the fruit and leaves of this plant (50 g) were added to 100 ml of methanol and 150 ml of distilled water and refluxed for 3 hours. Then, the extract was added to 4 ml of concentrated HCl with 5% of methanolic solution and refluxed for 6 hr. Extraction was conducted with chloroform and filtered. Chloroform was then evaporated at 40ºC using a rotary evaporator until the solvent was removed (Heidolph WB2000, Germany). The product yield was 0.47% of the original material. These extracts were weighed to 0.1g and dissolved in 1 ml of dimethyl sulfoxide 5% (DMSO). They were diluted to 100, 50 and 25 mg/ml and tested for antibacterial activity using well diffusion, MIC and MBC assays. Antioxidant activities were assessed using DPPH and SOD assays.

### 3.2.2. Anthraquinones extracts from *M. citrifolia* root

The dried powder of the root of this plant was extracted with 200 ml of ethanol in the Soxhlet apparatus for 4 hours. The extract was filtered and ethanol removed at 40ºC using a rotary evaporator (Heidolph WB2000, Germany). The SEP-PAK C18 column was used to purify the product. The sample was eluted from the column using 100% ethanol as the mobile phase. The ethanol in the elute was evaporated to dryness under reduced vacuum at 40ºC, weighed (0.1) and dissolved in 1 ml of 5% Tween 80 and diluted to 50, 25 mg/ml to be tested for antibacterial and antioxidant activities.

### 3.2.3. Alkaloid extracts from *M. citrifolia* fruit

This method is based on Smita and Sushma (2010). One hundred grams of the dried fruit powder was added to the mixture of ethanol-chloroform 1:3 with 2% of strong ammonia solution and refluxed for 6 hr. Extraction was conducted with 2N HCl and the extract was made alkaline with strong ammonia. The solution was extracted with chloroform and washed with distilled water. Chloroform was then evaporated until the

solvent was removed at 40ºC using a rotary evaporator (Heidolph WB2000, Germany). The product yield was 0.1% of original material. These extracts were weighed to 0.1g and dissolved in 1 ml of 5% dimethyl sulfoxide (DMSO). They were diluted to 100, 50 and 25 mg/ml and tested for antibacterial activity using well diffusion, MIC and MBC assays. Antioxidant activities were assessed using DPPH and SOD assays.

### 3.2.4. Alkaloid extracts from *A. squamosa* leaves and *A. angustiloba* roots

The method is based on Hadi and Bremner (2001) with some modifications The modifications included varying the quantity of samples used and the incubation period of extraction. Two hundred and fifty grams of the dried powder of the parts of the plants was immersed in 100% of cold distilled methanol. The extracts were filtered with a Whatman No.1 filter paper and methanol was removed at 40ºC using a rotary evaporator (Heidolph WB2000, Germany). They were then added to 5% acetic acid. The liquids were extracted with dichloromethane and the aqueous layer was basified with 10% sodium carbonate to regulate the pH to 10. Further extractions of their compounds were conducted with dichloromethane. The extracts were concentrated under reduced vacuum at 40ºC to 0.04 g of alkaloids, weighed to 0.1g, and dissolved in 1 ml of dimethyl sulfoxide 5% (DMSO). They were then diluted to 100, 50 and 25 mg/ml and analysed for antibacterial activity using well diffusion, MIC and MBC assays. DPPH and SOD assays were used for antioxidant activities.

### 3.2.5. Diterpens extracts of *A. squamosa* fruit

This method is based on Wu *et al.* (1996). One kg of the dried fruit was extracted five times with methanol. The combined methanolic extracts were evaporated under reduced vacuum at 40ºC. Chloroform solution was added to 3% HCl and the extracts were dried by evaporation under reduced vacuum at 40ºC. The product yield was 0.01% of the original sample. These extracts were weighed to 0.1g and dissolved in 1 ml of

dimethyl sulfoxide 5% (DMSO). They were diluted to 100, 50 and 25 mg/ml and analysed for antibacterial activity using well diffusion, MIC and MBC assays. Antioxidant activities were assessed using DPPH and SOD assays.

### 3.2.6. Phenolic compounds of Australian plant mixture

Five grams of dried powder of this mixture was extracted with 50 ml of 100% ethanol at room temperature for 3 days with the aid of a magnetic stirrer. The mixture was then filtered with a Whatman No.1 filter paper. The liquid was left to evaporate at 40ºC with the aid of a rotary evaporator until the solvent was removed (Heidolph WB2000, Germany). The product was purified with the utilisation of a SEP-PAK C18 column. The sample was eluted from the column using 100% ethanol as the mobile phase. The ethanol in the elute was evaporated to dryness under reduced vacuum at 40ºC, weighed and dissolved in 1 ml of 5% Tween 80 to be analysed for antibacterial and antioxidant activities.

### 3.2.7. Total phenolic contents

Total phenolic contents of the hot aqueous and ethanol extracts of the APM were determined by the Folin-Ciocalteau method based on Slinkard and Singleton (1977). In brief, 20 µl of the samples were added to 100 µl of 2 M of Folin- Ciocalteau reagent. The final volume of the mixture was adjusted to 1600 µl using distilled water. Then, 300 µl of 10% sodium carbonate was added and incubated at 37ºC for 45 minutes. These solutions were measured using a Genesys 20 Thermo Scientific (USA) spectrophotometer at 760 nm wavelength. Gallic acid at different concentrations of 50 to 1000 mg/ml was used to plot a standard curve to calibrate total phenolic contents in the extracts.

### 3.2.8. Thin layer chromatography (TLC) and IR spectrometry

TLC chromatography based on the method (Smita and Sushma, 2010). Anthraquinones fractions of all parts of the bioactive compounds were loaded on TLC plates 60 F254 (Merck, Germany). The mobile phase dichloromethane: methanol (9:1) and spray by using KOH reagent to get the red colour of the bands of anthraquinones fractions. Dragendroff's reagent gets the orange bands for alkaloids, and the reagent anisaldehyde $H_2SO_4$ to get the blue or violet spot for diterpenes and phenolic compounds. All TLC plates were visualized under UV light at wavelength 245 nm and 356 nm. Then, the IR spectrum of these compounds was recorded by FTIR (Perkin Elmer spectrum 400 FT-IR, UK) at room temperature from 400 to 4000 $cm^{-1}$ for scanning directly.

### 3.2.9. LC-MS analysis of bioactive compounds

All bioactive compounds extracted from *M. citrifolia, A. squamosa, A. angustiloba* and APM were identified through the Agilent 6530 quadrupole time-of-flight liquid chromatography mass spectrometer (Agilent Technologies, USA) with binary pump and automatic sampler. All extracts were filtered by 0.22 µM filters before injection. The solvents used A were 2% acetonitrile in water with 0.1% formic acid and B: 2% water in acetonitrile with 0.1% formic acid. A step gradient of solvent B was used to run the column-SL as follows: 2-30% for 0-30 min, 30-98% for 30-40 min and 98-8% for 55-60 min and the volume of injection 5µl. The flow rate was 100 µl/ min. The mass ranged between 50 to 3100 *m/z* and electrospray ionisation with positive ion polarity, the capillary voltage 3.5 KV, gas temperature 300°C, nebulizer pressure to 40 psi, sheathgas temperature 350°C and gas flow 8 L/min. The data was analysed by Agilent MassHunter Workstation Software B.01.03.

### 3.2.10. Effect of bioactive extracts from selected plants by SEM

Bacterial culture (*B. cereus*) was inoculated into nutrient broth and then incubated overnight at 37°C. This culture (1 ml) was added to one milliliter of bioactive extracts of anthraquinones from *M. citrifolia* fruit, alkaloids from *A. squamosa* leaves, and diterpens from *A. squamosa* fruit. All treated and untreated samples were kept for 4 hr at 37°C. This mixture was then centrifuged at 6500 g at 4°C for 10 min. The pellet was washed twice with 50 mM sodium phosphate buffer (pH 7). The bacterial cells were re-suspended with buffer and 1 µl of suspension deposited on a membrane filter. Bacterial cells were fixed with 8% glutaraldehyde for 1 hr. The fixed cells were washed with buffer in distilled water in a ratio of 1:3 for 15 min. The bacterial cells were dehydrated in ascending concentrations of ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 100 and 100%) with a 15 min exposure period for each concentration. The bacterial cells were further dehydrated in different ratios of ethanol: acetone (3:1, 1:1 and 1:3) for 20 min for each mixture and then washed with pure acetone four times each for 20 min. These bacterial cells were subjected to critical point drying using liquid $CO_2$ and the cells mounted on a stub. The cells were then coated with gold and examined through a scanning electron microscope (Model: JEOL JBM 7001F, UK). The control used in this experiment is normal, untreated bacterial cells which were compared with *B. cereus* cells treated with bioactive extracts from medicinal plants.

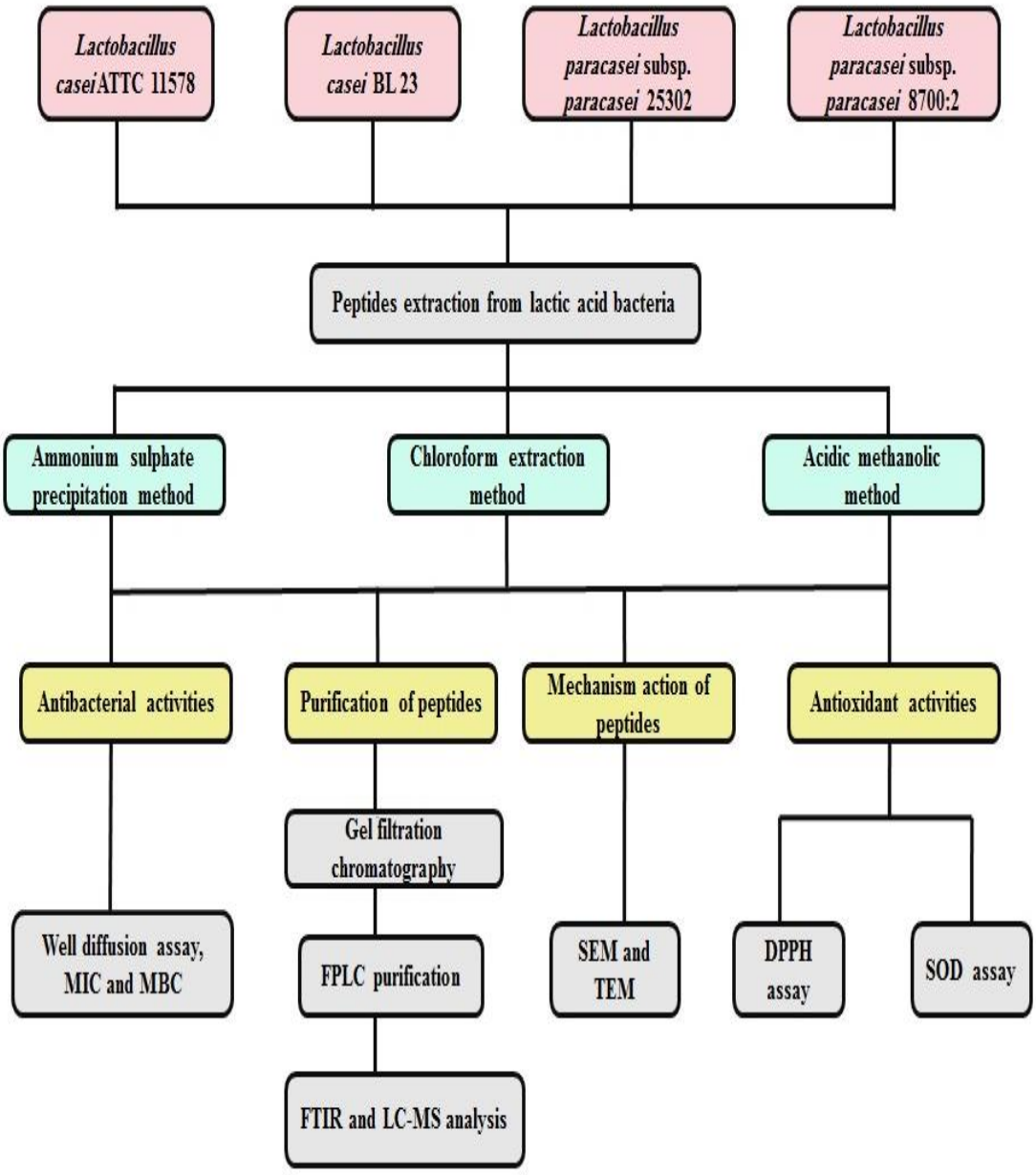
### 3.2.11. Statistical analysis

Data is expressed as mean ± SD. Statistical analyses were carried out using SPSS version 17. One-way ANOVA followed by Duncan's multiple comparison were used to compare the values of samples with the control. A $P$ value < 0.05 was deemed as indicating significant differences. Each treatment was three replicates and each experiment was repeated at least twice.

### 3.3. Part 3: Peptide extracts

#### 3.3.1. Peptide extracts of lactic acid bacteria

**Flow chart of experiment done on peptide extracts of lactic acid bacteria**



#### 3.3.1.1. Isolation of lactic acid bacteria

These bacteria were isolated from fermented soy milk samples purchased from the local market in Kuala Lumpur. Cow milk was randomly collected on November 2010 from farms in Juasseh, Kuala Pilah, Malaysia. These samples were left to ferment for 24 hours. One milliliter of the sample was added to 10 ml of MRS broth (Difco, Detroit, MI, USA) at 30°C for 24 hrs aerobically. Another sample isolated from cow milk was incubated at 37°C for 24 hours anaerobically in the same medium. After incubation, the bacteria were inoculated into MRS agar (Difco, Detroit, MI, USA) under the conditions cited above.

#### 3.3.1.2. Identification of lactic acid bacteria

In this study, identification of bacteria was carried out by Gram stain, catalase and oxidase tests, and 16S rRNA technique. In the 16S rRNA technique, the culture of bacteria was inoculated into MRS broth overnight. Bacterial DNA was extracted by (i-genomic kit BYF, Belgium). PCR mixtures were exposed to a temperature of 94°C for 5 minutes, 35 cycles of denaturation, annealing at 52°C for 1 minute and extension at 72°C for 10 minutes. These products were analysed by 1% agarose gel electrophoresis in 0.05% TBE buffer, following which the gel was stained with ethidium bromide. The bands were visualized under UV illumination. DNA sequences of the PCR product was carried out by BIO NEER Company, Malaysia. The 16S rRNA sequence was blasted with NCBI database and the bacterial species identified on the basis of similarity to the database of 16S rRNA sequences.

#### 3.3.1.3. Peptide extraction by ammonium sulphate precipitation

Peptide extract from *Lactobacillus spp*. was based on the method by Callewaert *et al.* (1999) with some modifications. The cultures of aerobic lactic acid bacteria were inoculated into 1 L of MRS broth at 30°C for 24 hours and anaerobic bacteria were

inoculated into the same broth at 37°C. These cultures were centrifuged at 5500 g for 30 minutes at 4°C. The supernatant was filtered through a 0.22 µm pore-size filter. The peptides were precipitated with 40% saturated ammonium sulphate at 4°C overnight. The supernatant was centrifuged and the pellet was collected and analysed against selected test bacterial strains and then examined for antibacterial and antioxidant activities.

### 3.3.1.4. Chloroform extraction method

The cultures of aerobic lactic acid bacteria were inoculated into 1 L of MRS broth at 30°C for 24 hours and anaerobic bacteria were inoculated at 37°C. This method was modified for optimal results by Burianek and Yousef (2001). These cultures were centrifuged (17000 g for 10 min at 4°C). The supernatant was filtered through a 0.22 µm pore-size filter. These supernatants were extracted with one volume of chloroform for 1 hour with continuous stirring at 4°C. The interphase layer was collected and centrifuged (17000 g for 10 min at 4°C). The chloroform residue was removed by rotary evaporator under vacuum at 40°C. The pellet was collected and analysed for antibacterial and antioxidant activities.

### 3.3.1.5. Acidic methanolic method

This method Wescombe *et al.* (2006) for peptide extraction was used with some modifications. All bacteria were inoculated into MRS broth under the conditions mentioned above. These cultures were then centrifuged (17000 g for 10 min at 4°C). The cells of the bacteria were washed with 0.1 N of NaCl and then centrifuged. The washed cells were extracted with 100 ml of 95% methanol and pH adjusted to 2 by concentrated HCl. This mixture was kept overnight at 4°C, stirred and centrifuged at 9500 g at 4°C for 30 minutes. The pellet was removed and the supernatant evaporated at

40°C until the solvent was reduced to 4 ml and then freeze-dried and kept at -20°C for later use. All peptides were tested for antibacterial and antioxidant activities.

### 3.3.1.6. Purification of peptides from lactic acid bacteria by gel filtration chromatography

The crude peptides extracted from *L. paracasei* subsp. *paracasei* strain 8700:2 cells were purified by Sephadex G-25 (Sigma Aldrich GmbdH, Germany). The freeze-dried sample was loaded on to the column (1.5 x 73 cm). It was eluted with 20 mM Tris-buffer, pH 7.6. The flow rate of gel filtration chromatography was adjusted to 1.5 ml/5 min. The volume of fractions 1.5 ml were collected and measured at 280 nm with a spectrophotometer (Thermo Scientific 2000, USA). Three fractions were tested for antibacterial activity.

### 3.3.1.7. Anion-exchange chromatography of peptides from lactic acid bacteria by fast protein liquid chromatography FPLC

The partially purified sample by gel filtration chromatography was applied to an anion-exchange column (2x 5 cm) (HiTrap Q HP) and the sample eluted to 20 mM Tris-HCl buffer (pH 7.6) as a flow rate of 1.5 ml/min. Elution was carried out using a linear gradient of NaCl up to 0.5 M (from 100% 20 mM Tris-HCl pH 8 at 0 min to 100% 0.5 M NaCl at 180 min). The fraction were collected and tested for antibacterial activity against *B. cereus*. LC-MS analysis of this fraction were identified by Agilent 6530 quadrupole time-of-flight liquid chromatography mass spectrometer (Agilent Technologies, USA) with binary pump and automatic sampler. The active fraction 2 was analysed by FTIR (Perkin Elmer spectrum 400 FT-IR, UK) at room temperature from 400 to 4000 $cm^{-1}$.

### 3.3.1.8. Effect of peptide extracts from *L. paracasei* subsp. *paracasei* strain 8700:2 examined by SEM

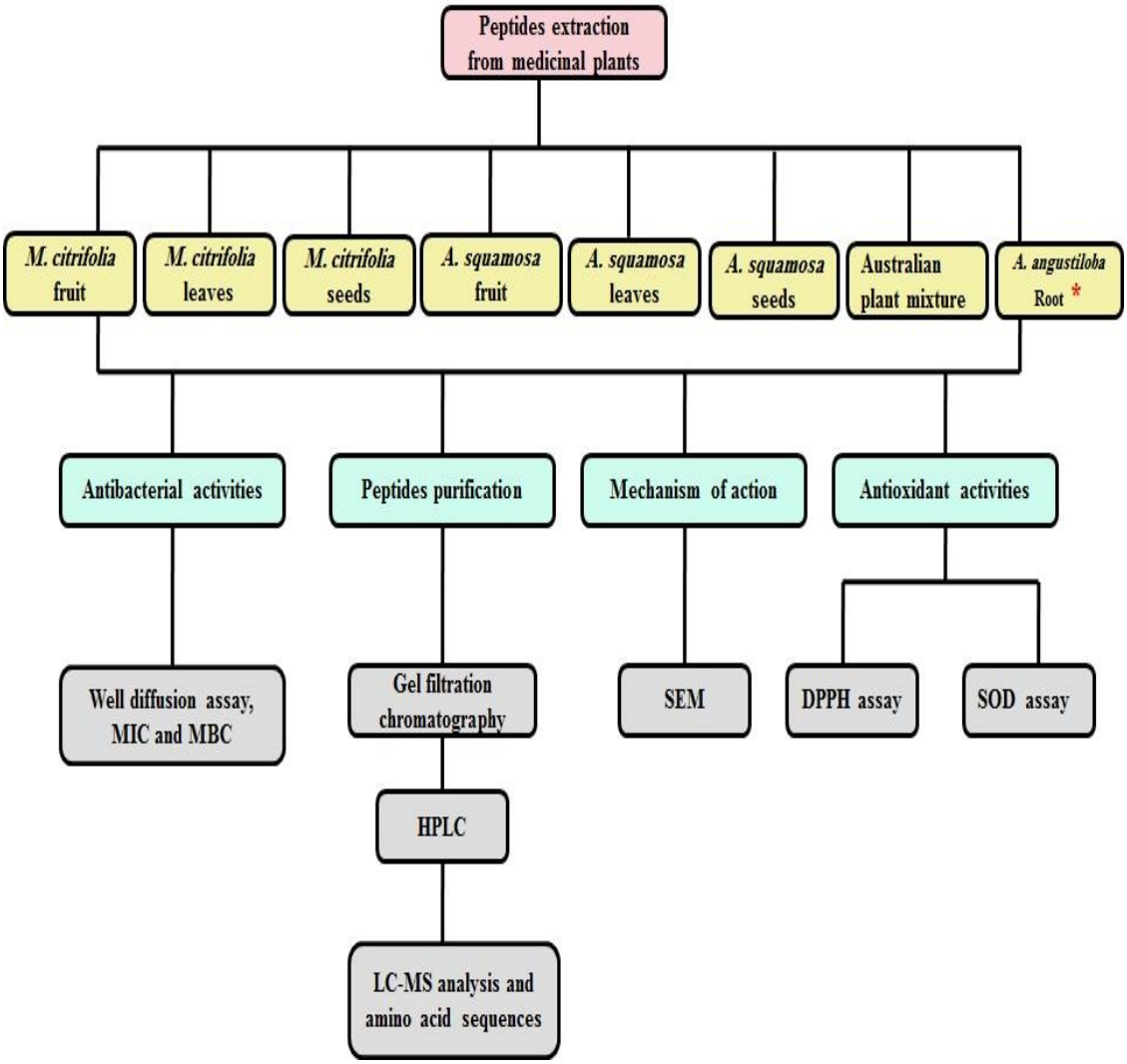
Bacterial cultures (*B. cereus*, MRSA and *H. pylori*) were incubated overnight at 37°C. This culture (1 ml) was added to one milliliter of a bioactive fraction of peptide extracts of *L. paracasei* subsp. *paracasei* strain 8700:2 and kept for 4 hours at 37°C. Then, this mixture was centrifuged at 6 500 g at 4°C for 10 minutes. The pellet was washed twice with sodium phosphate buffer (pH 7). The bacterial cells were suspended with buffer and 1 µl of suspension were deposited on a membrane filter. Bacterial cells were fixed with 8% glutaraldehyde for 1 hr. The bacterial cells were dehydrated in ascending concentrations of ethanol and ethanol: acetone (3:1, 1:1 and 1:3). They were then washed with pure acetone. The bacterial cells were subjected to critical point drying using liquid $CO_2$ and the cells mounted on a stub. These cells were coated with gold and examined through a scanning electron microscope (Model: JEOL JBM 7001F, UK). The control in this experiment is normal bacterial cells that were compared to *B. cereus*, MRSA and *H. pylori* treated with the active peptide. The active fraction was treated with *H. pylori* and tested by TEM.

### 3.3.1.9. Statistical analysis

Data is expressed as mean ± SD. Statistical analyses were carried out using SPSS version 17. One-way ANOVA followed by Duncan's multiple comparison were used to compare the values of samples with the control. A $P$ value < 0.05 was deemed as indicating significant differences. Each treatment was three replicates and each experiment was repeated at least twice.

### 3.3.2. Peptide extraction from medicinal plants

**Flow chart of experiment done on peptide extracts from medicinal plants**




***** Peptide extracts of *A. angustiloba* root did not show antibacterial and antioxidant properties.

#### 3.3.2.1. Peptide extraction from *A. squamosa* and *M. citrifolia* fruit

This method is based on Mandal *et al.* (2011) with some modifications. In brief, the dried powders of the fruit of this plant were added to 100 ml of 5% acetic acid and kept overnight at 4°C. The mixture was filtered and centrifuged at 5000 g for 30 minutes at 4°C. The supernatant was then precipitated with an equal volume of acetone for 24 hours with continuous stirring at 4ºC. The acetone was removed using a rotary evaporator at 40°C. The extract was finally freeze dried. The pellet was collected and analysed for antibacterial and antioxidant activities.

#### 3.3.2.2. Peptide extraction from the leaves of *M. citrifolia* and *A. squamosa*

This method is based on Ningappa *et al.* (2010) with some modifications. In brief, 10 g of dried powders of the fruit were added to 100 ml of 20 mM of Tris-HCl buffer (pH 8) with polyvinylpyrrolidone 20 mg/ml. This mixture was filtered and centrifuged at 4500 g for 30 min at 4ºC. The supernatant was precipitated with a gradual addition of ammonium sulphate until saturated and then left overnight at 4ºC. The supernatant is centrifuged under the same conditions. The pellet was collected and analysed against selected pathogenic bacteria and antioxidant assays.

#### 3.3.2.3. Peptide extraction from the seeds of *M. citrifolia* and *A. squamosa*

This method was based on Chan *et al.* (2009) with some modification. The dried powders of the seeds were added to 100 ml of 20 mM sodium acetate (pH 5). This mixture was filtered and centrifuged at 5343 g for 30 min at 4ºC. The supernatant was precipitated overnight using 65% ammonium sulphate at 4°C. The supernatant was centrifuged under the same conditions. The pellet was collected and analysed against pathogenic bacteria and antioxidant assays.

### 3.3.2.4. Peptide extraction from the selected plant mixture

This method is based on Ningappa and Srinivas (2008) with some modification. Ten grams of dried powder of APM was added to a solution of 20 mM of Tris-HCl (pH 7.6) buffer and polyvinylpyrrolidone (PVP) at a concentration of 20 mg/ml. This mixture was filtered and centrifuged at 4500 g for 30 minutes at 4ºC. The supernatant was removed and precipitated with gradually saturated ammonium sulphate at 4ºC overnight. The supernatant was separated by centrifuging at 4500 g for 30 minutes at 4ºC, and the resulting pellet was collected and analysed for antibacterial and antioxidant activities.

### 3.3.2.5. Total Protein estimation

The samples were measured for total protein concentration by the Bradford method (Schleicher and Wieland, 1978). In brief, 100 µl of the samples were added to 3 ml of Bradford reagent (containing Coomassei Brilliant Blue G-250) and then incubated at room temperature for 15 minutes. Absorbance of the samples was measured at 595 nm. Bovine serum albumin at different concentrations was used to plot a standard curve to calibrate protein concentration of the extracts as a measure of the peptide amount.

### 3.3.2.6. Purification of peptides of selected plant mixtures by gel filtration and HPLC

The crude peptide extracts from APM were purified by Sephadex G-75 (Sigma Aldrich GmbdH, Germany). The freeze-dried sample was loaded onto a column of dimension 1.5 x 73 cm. It was eluted with 20 mM Tris-buffer, pH 7.6. The flow rate was maintained at 1.5 ml/5 min. The fractions were collected and measured at 280 nm. All fractions were tested for antibacterial activity. The active fraction (F1) was dissolved in 0.1% aqueous trifluoroacetic acid (TFA) solution (3 ml) and fractionated by reverse phase-HPLC (Agilent 1100 series) with Zorbax 300SB-C18 column (4.5 x

150 µm, 50 µm, 300A°) at a flow rate of 1.0 ml/ min. The solvents used were: (A) 0.1% TFA in water, and (B) 0.1% TFA in acetonitrile. A step gradient of solvent A was used to run the column as follows: 0-60% for 0-45 min, 60-80% for 45-55 min and 80-100% for 55-60 min. The fractions of the mixture were detected at 245, 215 and 280 nm. Active fraction of APM was collected at 4.181 min and then lyophilized and stored at -20°C.

**3.3.2.7. LC-MS/MS analysis**

LC-MS analysis was carried out using an active fraction of APM that was rehydrated to 500 µL of 2% acetonitrile with 0.1% TFA centrifuged for 3 minutes at 13,000 rpm. The chromatography system was coupled to an LTQ Orbitrap Velos mass spectrometer equipped with a Nanospray II source (Thermo Fisher Scientific). Solvents were A: 2% Acetonitrile, 0.1% formic acid, and B: 90% Acetonitrile, 0.1% Formic acid. After a 249 bar (~ 5µL) pre-column equilibration and a 249 bar ( ~ 8µL) nanocolumn equilibration, samples were separated by a 55 minute gradient for IT-CID method (0 min: 5% B; 45 min: 45% B; 2 min: 95% B; 8 min: 95% B) or 70 minute gradient for FT-CID method (0 min: 5% B; 60 min: 40% B; 2 min: 80% B; 8 min: 80% B). The LTQ Orbitrap Velos parameters were as follows: Nano-electrospray ion source with spray voltage 2.2 kV, capillary at temperature 225°C. Survey MS1 scan *m/z* range 400-2000 profile mode, resolution 60,000 or 30,000 *-400m/z* with AGC target 1E6, and one microscan with maximum inject time 200 ms.

Raw files were analysed with Proteome Discoverer 1.3.0.339 software suite (Thermo Scientific). The peak lists were submitted to an in-house Mascot 2.2 against the UniProt-Swissprot 20110104 (523,151 sequences; 184,678,199 residues) database as follows: precursor tolerance 8 ppm; MS/MS tolerance 0.6 Da (Iontrap), 0.020 Da (FT) ; No enzyme; FT-ICR ESI instrument type; variable modifications: deamidation (N,Q);

oxidation (M). Percolator settings: Max delta Cn 0.05; Target FDR strict 0.01, Target FDR relaxed 0.05 with validation based on q-Value.

### 3.3.2.8. Effect of peptide extracts from APM by SEM

Bacterial cultures (*B. cereus*, MRSA) were incubated into nutrient broth and then incubated overnight at 37°C. This culture (1 ml) was added to one milliliter of a bioactive fraction of peptide extracts of APM and kept for 4 hours at 37°C. This mixture was then centrifuged at 6500 g at 4°C for 10 minutes. The pellet was washed twice with 50 mM sodium phosphate buffer (pH 7). The bacterial cells were re-suspended with buffer and 1 µl of suspension deposited on a membrane filter. Bacterial cells were fixed with 8% glutaraldehyde for 1 hr. The fixed cells were washed with buffer in distilled water in a ratio of 1:3 for 15 minutes. The bacterial cells were dehydrated in ascending concentrations of ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 100 and 100%) with 15 minutes of exposure for each concentration. The bacterial cells were further dehydrated in different ratios of ethanol: acetone (3:1, 1:1 and 1:3) for 20 minutes for each mixture and then washed with pure acetone four times each for 20 minutes. These bacterial cells were subjected to critical point drying using liquid $CO_2$ and the cells mounted on a stub. These cells were coated with gold and examined through a scanning electron microscope (Model: JEOL JBM 7001F, UK).

### 3.3.2.9. Statistical analysis

Data is expressed as mean ± SD. Statistical analyses were performed using SPSS version 17. One-way ANOVA followed by Duncan's multiple comparison were used to compare the values of samples with control. A $P$ value < 0.05 was deemed as indicating significant differences. Each treatment was three replicates and each experiment was repeated at least twice.

# 4. Results

## 4.1. Part 1: Crude extracts

### 4.1.1. Antibacterial activity of crude extracts

### A. Aqueous extracts

In this study, the plants *A. squamosa*, *M. citrifolia*, *A. angustiloba* and an Australian plant mixture were subjected to hot and cold aqueous extractions. In well diffusion assay, the cold and hot aqueous extracts of the *M. citrifolia*, *A. angustiloba* and an Australian plant mixture did not exhibit any inhibition zones against *S. aureus*, *E. coli*, *B. cereus*, *P. aeruginosa*, MRSA and *H. pylori*. On the other hand, except for *P. aeruginosa* and *H. pylori*, the cold and hot aqueous extracts of the *A. squamosa* fruit inhibited all the strains of bacteria tested (Figures 4.1, 4.2 and 4.3).

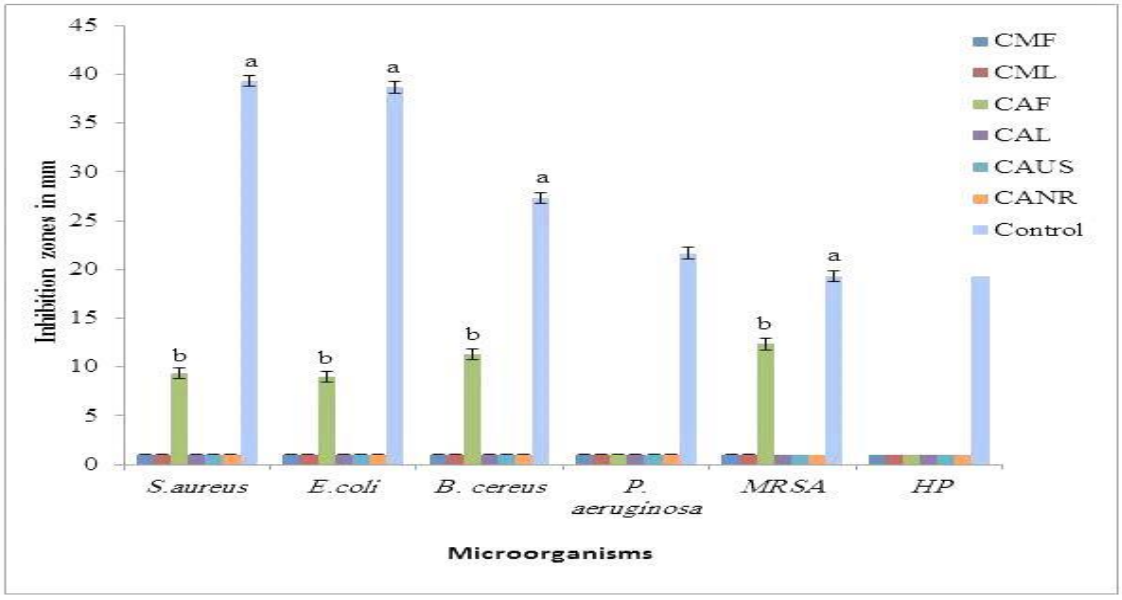



**Figure 4.1.** Inhibition zones of cold aqueous extracts (100 mg/ml) of selected plants on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). ■ **CMF**-Cold extract of *M. citrifolia* fruit, ■ **CML**-Cold extract of *M. citrifolia* leaves, ■ **CAF**-Cold extract of *A. squamosa* fruit, ■ **CAL**-Cold extract of *A. squamosa* leaves, ■ **CAUS**- Cold extract of Australian plant mixture, ■ **CANR**-Cold extract of *A. angustiloba* root and ■ Control. Samples represented with different letters (a, b) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All experiments were done in triplicates and values represent means ± SD.

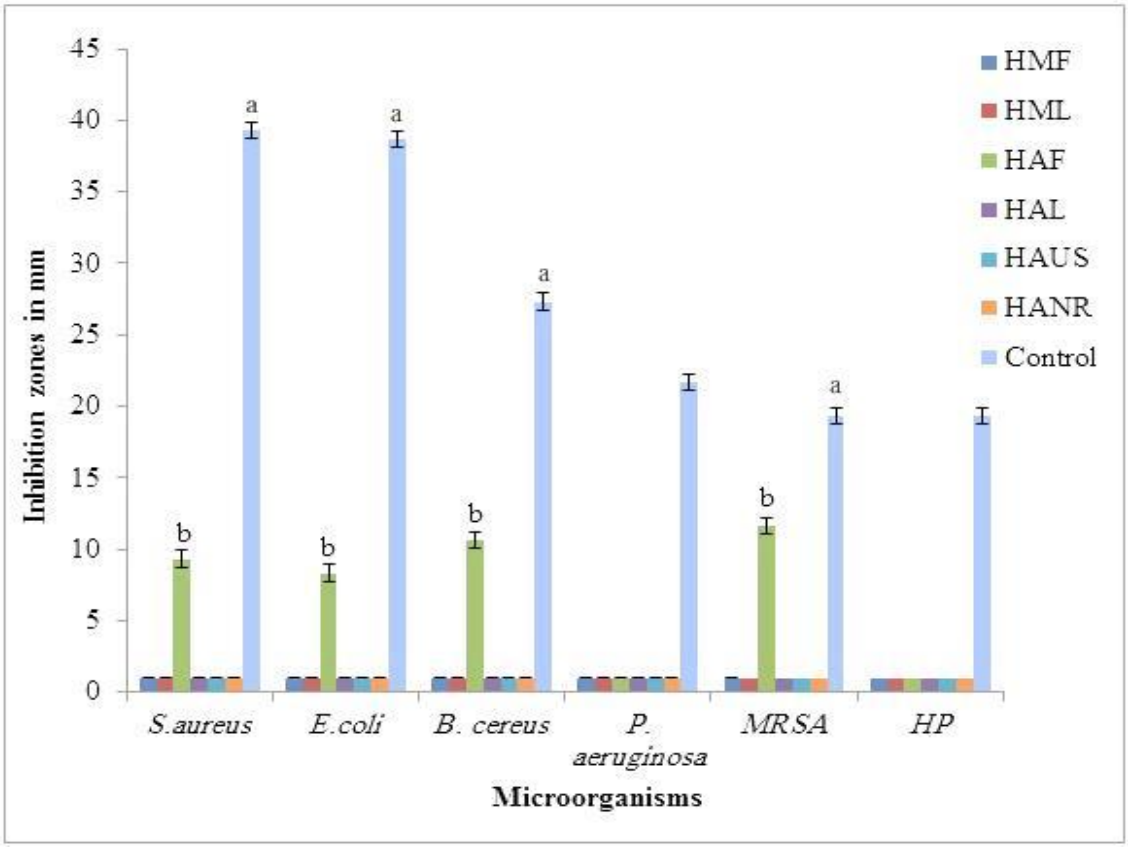



**Figure 4.2.** Inhibition zones of hot aqueous extracts (100 mg/ml) of selected plants on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). ■ **HMF**-Hot extract of *M. citrifolia* fruit, ■ **HML**-Hot extract of *M. citrifolia* leaves, ■ **HAF**-Hot extract of *A. squamosa* fruit, ■ **HAL**-Hot extract of *A. squamosa* leaves, ■ **HAUS**-Hot extract of Australian plant mixture, ■ **HANR**-Hot extract of *A. angustiloba* root and ■ Control. Samples represented with different letters (a, b) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All experiments were done in triplicates and values represent means ± SD.

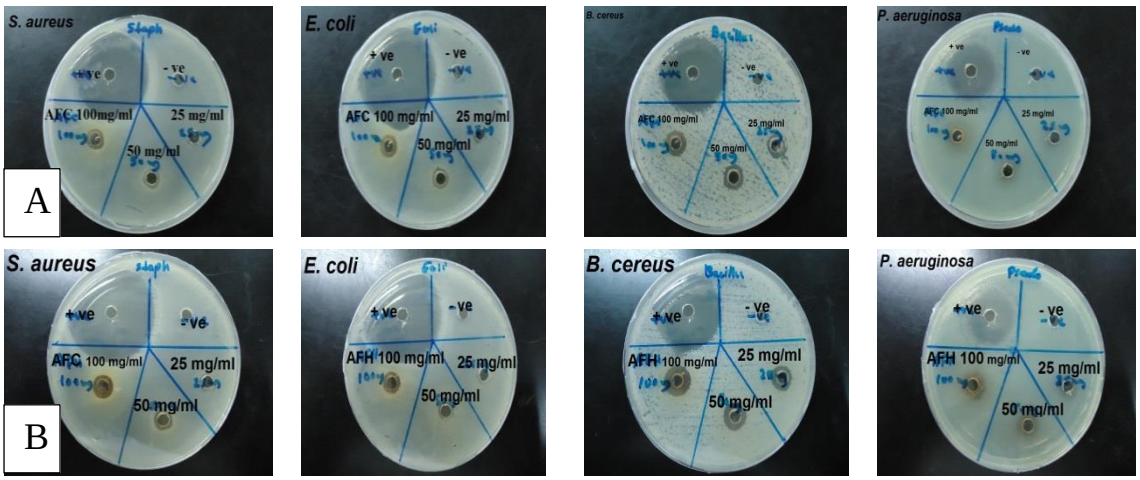



**Figure 4.3.** Effect of different concentration 100, 50 and 25 mg/ml of (A) cold aqueous extracts of *A. squamosa* fruit (AFC) and (B) hot aqueous extracts of *A. squamosa* fruit against the test microorganisms compared to positive control (10 mg/ml of tetracycline) and negative control (distilled water). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

**B. Methanolic extracts**

Figures 4.4, 4.5 and 4.6 show the zones of inhibition for the methanolic extract from the *A. squamosa* fruit for *S. aureus* (15.66 mm), *E. coli* (14.66 mm), *B. cereus* (15.66 mm), *P. aeruginosa* (10.00 mm) and MRSA (15.33 mm). The methanolic extract of the *M. citrifolia* fruit inhibited all strains of the test bacteria with *B. cereus* exhibiting the highest sensitivity towards extract. Methanolic extract from the leaves of *A. squamosa* showed mean inhibition zones against bacteria ranging from 18.33 mm to 9.00 mm while the tetracycline (10 mg/ml) as positive control had inhibition zones ranging from 19.33 mm to 39.66 mm.

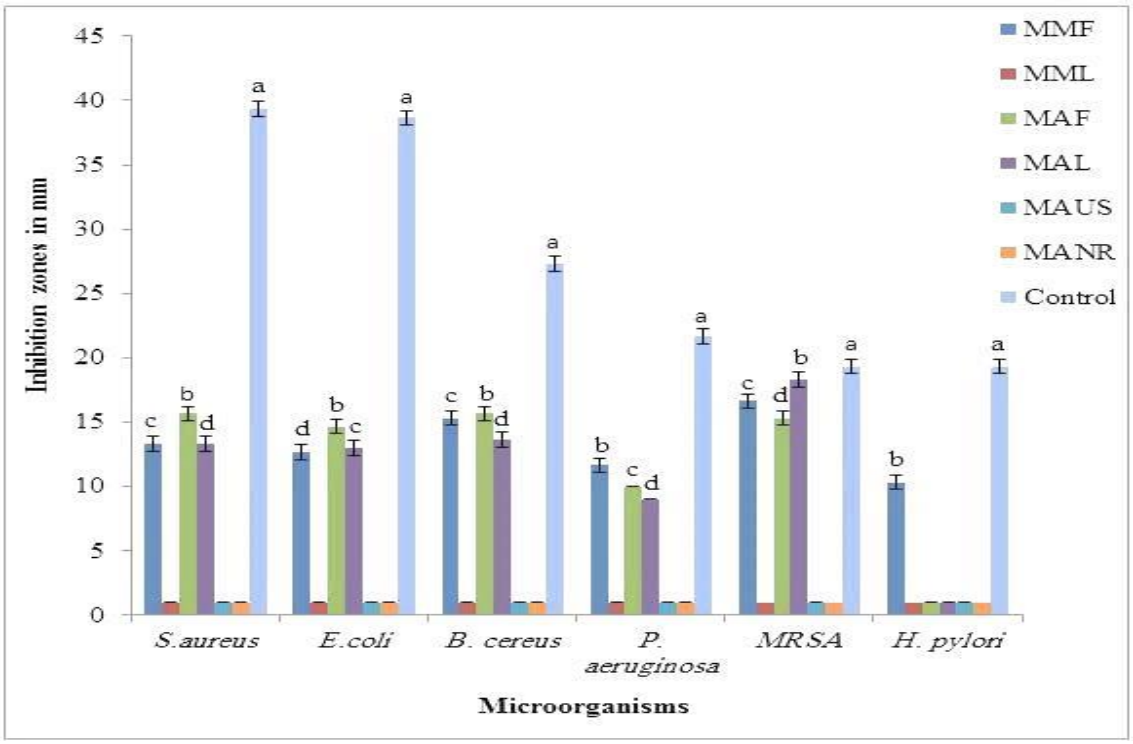



**Figure 4.4.** Inhibition zones of methanolic extracts (100 mg/ml) of selected plants on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). ■ **MMF**- Methanolic extract of *M. citrifolia* fruit, ■ **MML**- Methanolic extract of *M. citrifolia* leaves, ■ **MAF**- Methanolic extract of *A. squamosa* fruit, ■ **MAL**- Methanolic extract of *A. squamosa* leaves, ■ **MAUS**- Methanolic extract of Australian plant mixture ■ **MANR**- Methanolic extract of *A. angustiloba* roots and ■ Control. Samples represented with different letters (a, b, c and d) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All experiments were done in triplicates and values represent means ± SD.

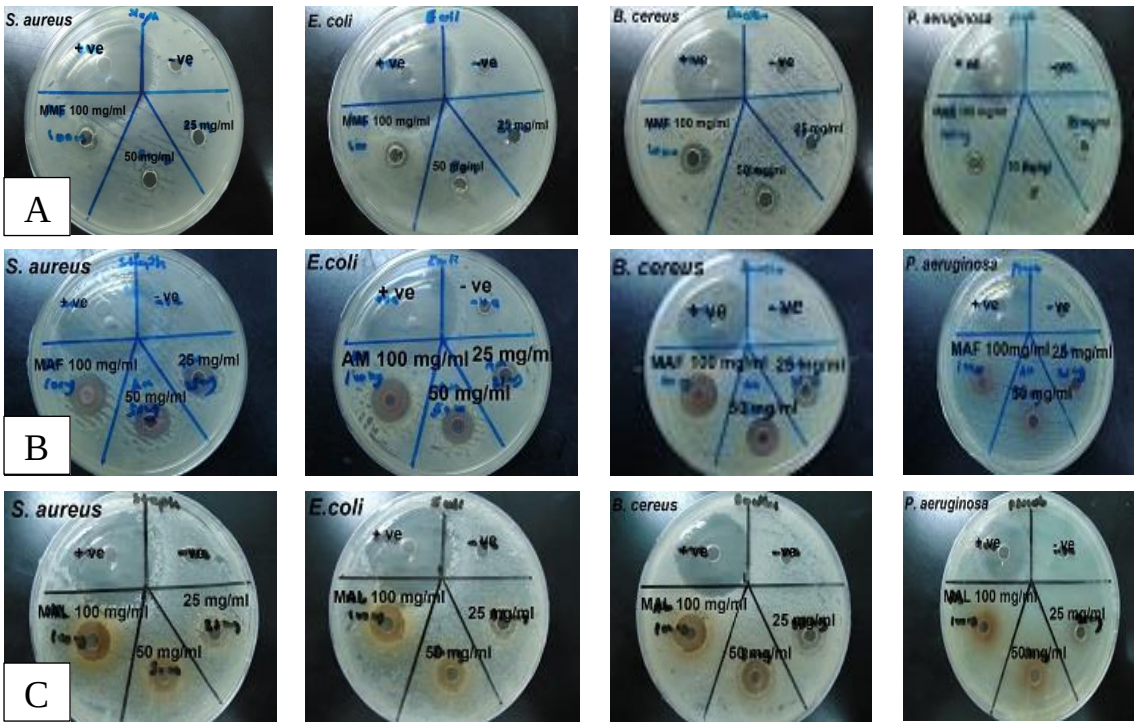



**Figure 4.5.** Effect of different concentration 100, 50 and 25 mg/ml of (A) methanolic (MMF) extracts from *M. citrifolia* fruit, (B) methanolic extracts of *A. squamosa* fruit (E) methanolic (MAL) extract from *A. squamosa* leaves against the test microorganisms compared to positive control (10 mg/ml of tetracycline) negative control (distilled water). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

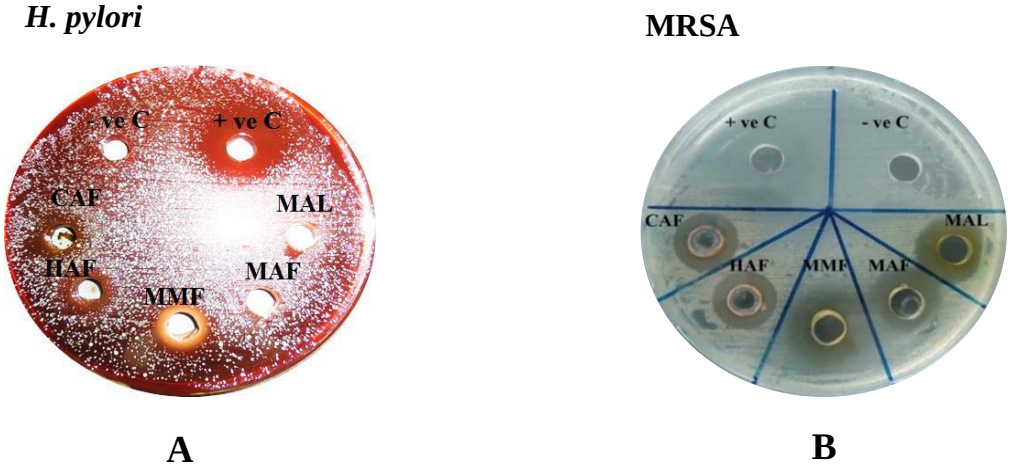



**Figure 4.6.** Effect of cold aqueous (AFC), hot aqueous (AFH), (MMF) methanolic extracts (100 mg/ml) from *M. citrifolia* fruit, methanolic extracts of *A. squamosa* fruit methanolic and (MAL) extract from *A. squamosa* leaves against (A) MRSA, (B) *H. pylori* compared to positive control (10 mg/ml of tetracycline) and negative control (distilled water). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

MIC and MBC values for the aqueous extracts of *M. citrifolia* fruit did not yield any result for all the test bacteria. However, the methanolic extract in the same study showed MIC and MBC values of 100 mg/ml for *S. aureus, E. coli*, *P. aeruginosa*, MRSA and *H. pylori* while *B. cereus* exhibited inhibition at 25 mg/ml. The MIC and MBC values for cold and hot aqueous extracts of *A. squamosa* fruit for *S. aureus*, MRSA and *E. coli* was at 50 mg/ml while for *B. cereus* was at 25 mg/ml. Methanolic extract of *A. squamosa* fruit and leaves showed MIC and MBC values at a concentration of 25 mg/ml for *S. aureus, E. coli* and *P. aeruginosa,* while for *B. cereus* inhibition was observed at a concentration of 12.5 mg/ml (Tables 4.1 and 4.2).

**Table 4.1.** MIC of cold, hot aqueous and methanolic extracts of *A. squamosa* and *M. citrifolia* on the test microorganisms

| | **Plant extracts (mg/ml)** | | | | |
|---|---|---|---|---|---|
| **Bacteria** | | **HAF** | **MMF** | **MAF** | **MAL** |
| ***S. aureus*** | 50.00 | 50.00 | 100.00 | >25.00 | 25.00 |
| ***E. coli*** | 50.00 | >50.00 | 100.00 | 25.00 | 25.00 |
| ***B. cereus*** | 25.00 | 25.00 | 25.00 | 12.50 | 12.50 |
| ***P. aeruginosa*** | Na | Na | 100.00 | 25.00 | >25.00 |
| **MRSA** | 50.00 | 50.00 | 100.00 | Na | Na |
| ***H. pylori*** | Na | Na | 100.00 | Na | Na |

**CAF**-Cold aqueous extracts of *A. squamosa* fruit, **HAF**-Hot aqueous extracts of *A. squamosa* fruit, **MMF**- Methanolic extract of *M. citrifolia* fruit, **MML**- Methanolic extract of *M. citrifolia* leaves, **MAF**-Methanolic extract of *A. squamosa* fruit, **MAL**- Methanolic extract of *A. squamosa* and **Na**- non active at high concentration (100 mg/ml).

**Table 4.2.** MBC of cold, hot aqueous and methanolic extracts of *A. squamosa* and *M. citrifolia* on the test microorganisms

| | Plant extracts ( mg/ml) | | | | |
|---|---|---|---|---|---|
| **Bacteria** | **CAF** | **HAF** | **MMF** | **MAF** | **MAL** |
| ***S. aureus*** | 50.00 | 50.00 | 100.00 | 25.00 | 25.00 |
| ***E. coli*** | 50.00 | 50.00 | 100.00 | 25.00 | 25.00 |
| ***B. cereus*** | 25.00 | 25.00 | >25.00 | 12.50 | <12.50 |
| ***P. aeruginosa*** | Na | Na | 100.00 | <25.00 | 25.00 |
| **MRSA** | 50.00 | >50.00 | 100.00 | Na | Na |
| ***H. pylori*** | Na | Na | 100.00 | Na | Na |

**CAF**-Cold aqueous extracts of *A. squamosa* fruit, **HAF**-Hot aqueous extracts of *A. squamosa* fruit, **MMF**- Methanolic extract of *M. citrifolia* fruit, **MML**- Methanolic extract of *M. citrifolia* leaves, **MAF**-Methanolic extract of *A. squamosa* fruit, **MAL**- Methanolic extract of *A. squamosa* and **Na-** non active at the highest concentration (100 mg/ml).

### 4.1.2. Antioxidant activity of crude extracts

Figure 4.7 shows the DPPH radical scavenging activity for the cold aqueous extract of *A. squamosa* fruit as 93.86% ($IC_{50}$ 5.33 mg/ml), the hot aqueous extract as 91.64% ($IC_{50}$ 5.46 mg/ml), the methanolic extract as 76.18% ($IC_{50}$ 6.57 mg/ml), the methanolic extract of *A. squamosa* leaves as 75.76% ($IC_{50}$ 6.60 mg/ml) and the methanolic extract of *M. citrifolia* fruit as 96.37% ($IC_{50}$ 5.19 mg/ml). These were compared to ascorbic acid as a positive control with radical scavenging activity of 96.59% ($IC_{50}$ 5.18 mg/ml). The SOD-like activity for the cold aqueous extract of *A. squamosa* fruit was 93.49%, but for the hot aqueous extract it was 91.23%. The methanolic extract of this fruit displayed antioxidant activity at 75.28% while for the leaves of *A. squamosa* it was at 76.25%t (Figure 4.8). The methanolic extract of the *M. citrifolia* fruit showed significant antioxidant activity at 96.36%.

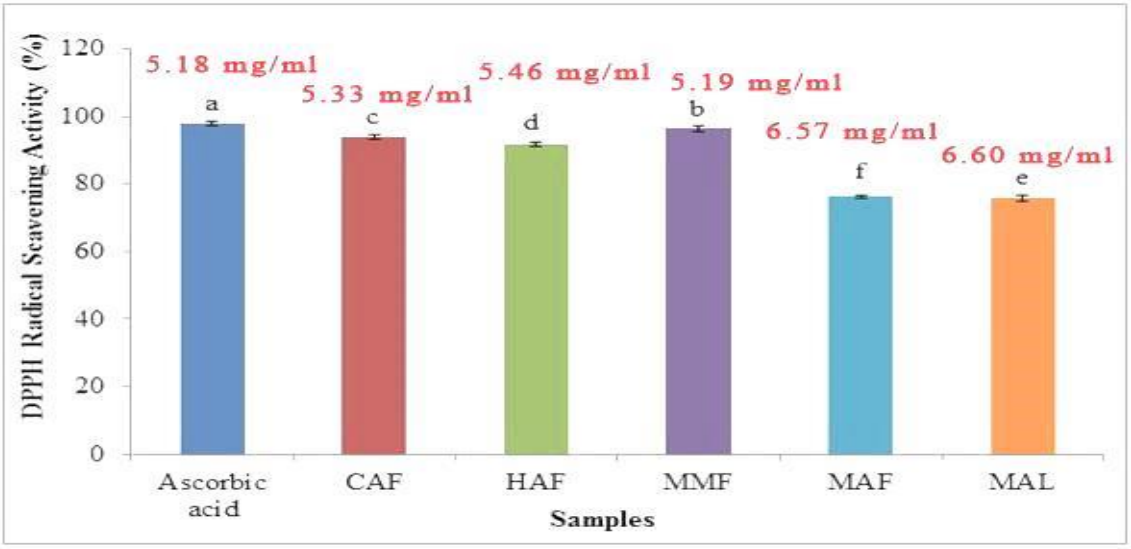



**Figure 4.7.** DPPH scavenging activity with $IC_{50}$ values (in red) of crude extracts of selected plants. **CAF**- Cold extract of *A. squamosa* fruit, **HAF**- Hot extract of *A. squamosa* fruit, **MMF**- Methanolic extract of *M. citrifolia* fruit, **MAF**-Methanolic extract of *A. squamosa* fruit and **MAL**- Methanolic extract of *A. squamosa* leaves. Samples represented with different letters (a, b, c, d, e and f) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of ascorbic acid).

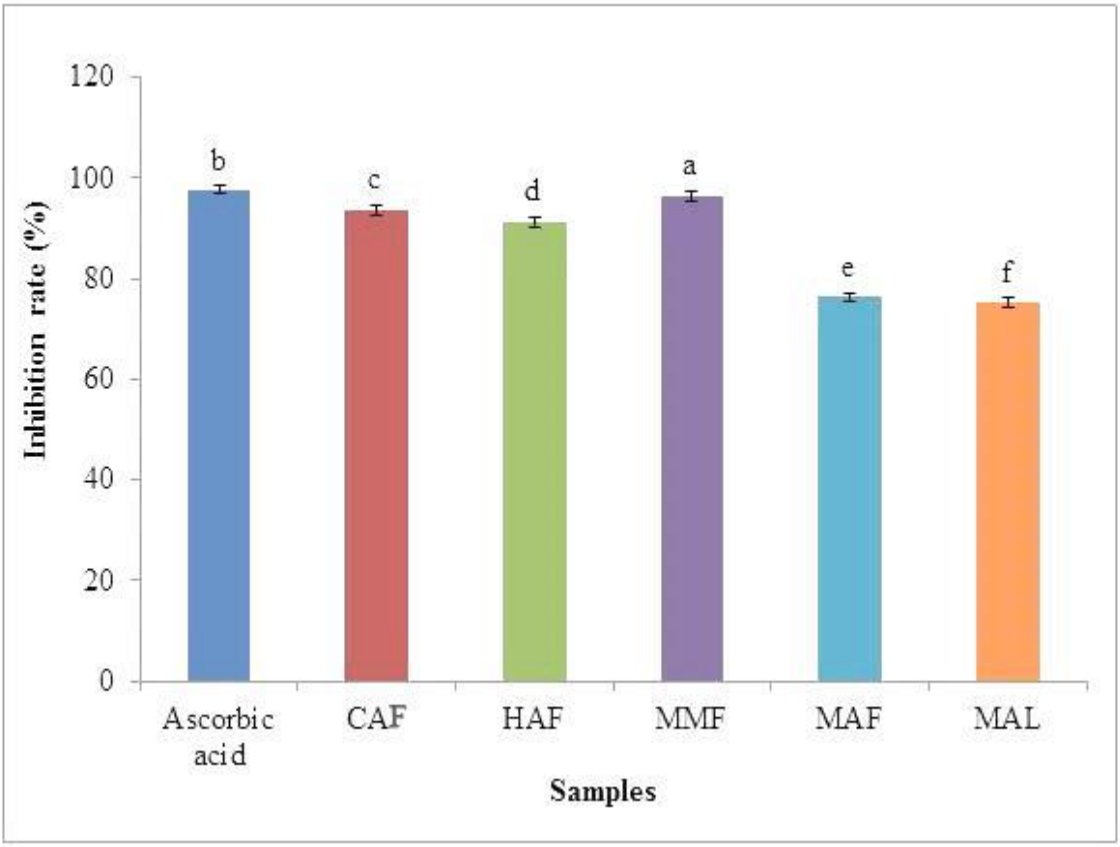



**Figure 4.8.** The rate of inhibition of SOD-like activities of crude extracts of selected plants. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b, c, d, e and f) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ml of ascorbic acid. **CAF**- Cold extract of *A. squamosa* fruit, **HAF**- Hot extract of *A. squamosa* fruit, **MMF**- Methanolic extract of *M. citrifolia* fruit, **MAF**- Methanolic extract of *A. squamosa* fruit and **MAL**- Methanolic extract of *A. squamosa* leaves.

### 4.1.3. GC-MS analysis

### A. Aqueous extracts

GC-MS results of the aqueous extracts of *A. squamosa* fruit showed the presence of 5 compounds that were identified and compared with a similar mass fragmentation in the NIST database library (Table 4.3, Appendix 1). These compounds are ent-spathulenol (2.96%), kauran-18-al (5.41%), kaur-16-ene (7.21%), kauren-18-ol (7.99%) and trihydroxycholan-24-oic acid (5.56%) with traces of unknown compounds (peaks 1,3,4,5,6,7,9,10,12,13,14,16,18,19,20).

**Table 4.3.** GC-MS analysis of the aqueous extract of the fruit of *A. squamosa*

| **S. N** [a] | **Peak RT** [b] **(min)** | **Peak area** | **Peak area %** | **Compound detected*** | **Hit** [c] | **SI**[d] | **CAS No** [e] | **Molecular Formula** | **Mol. Wt.** [f] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 19.53 | 161726 | 2.96 | Ent-spathulenol | 5 | 85 | 77171-55-2 | $C_{15}H_{24}O$ | 220 |
| 2 | 26.79 | 295814 | 5.41 | Kauran-18-al | 5 | 81 | 55902-84-6 | $C_{22}H_{34}O_3$ | 346 |
| 3 | 27.61 | 394308 | 7.21 | Kaur-16-ene | 5 | 81 | 20070-61-5 | $C_{20}H_{32}$ | 272 |
| 4 | 31.30 | 436897 | 7.99 | Kauren-18-ol | 5 | 80 | 72150-74-4 | $C_{22}H_{34}O_2$ | 330 |
| 5 | 32.87 | 303912 | 5.56 | Trihydroxychola-n-24-oic acid | 5 | 77 | 2955-27-3 | $C_{24}H_{40}O_5$ | 408 |

*The compounds that were identified and compared with a similar mass fragmentation in the NIST database library. [a] S.N: Serial number; [b] R.T: Retention time; [c] Hit: Numbers of expected compounds in the NIST database library; [d] SI: International system of units. [e] CAS No: Chemical abstracts service registry number; [f] Mol. Wt.: Molecular weight.

**B. Methanolic extracts**

The methanolic extract of the fruit of *A. squamosa* revealed the presence of three compounds which were identified as 2-isoxazolidine (14.06%), 4,5-octanediol (14.40%) and 2,3-hexanediol (9.69%) as shown in Table 4.4 and Appendix 2 with traces of unknown compounds (peaks 2,3,6,7,8,9,10,11,12).

**Table 4.4.** GC-MS analysis of the methanolic extract of the fruit of *A. squamosa*

| **S. N.** [a] | **Peak RT**[b] **(min)** | **Peak area** | **Peak area %** | **Compound detected *** | **Hit** [c] | **SI**[d] | **CAS No.** [e] | **Molecular Formula** | **Mol. Wt.** [f] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 15.56 | 10803 | 14.06 | 2-Isoxazolidi-ne | 5 | 80 | 504-72-3 | $C_3H_7NO$ | 73 |
| 2 | 21.54 | 11064 | 14.40 | 4,5-Octanediol | 5 | 89 | 22520-40-7 | $C_8H_{18}O_2$ | 146 |
| 3 | 23.07 | 7446 | 9.69 | 2,3-Hexanediol | 5 | 91 | 82360-67-6 | $C_6H_{14}O_2$ | 118 |

*The compounds that were identified and compared with a similar mass fragmentation in the NIST database library. [a] S.N: Serial number; [b] R.T: Retention time; [c] Hit: Numbers of expected compounds in the NIST database library; [d] SI: International system of units. [e] CAS No: Chemical abstracts service registry number; [f] Mol. Wt.: Molecular weight.

Table 4.5 and Appendix 3 show the major compounds in the methanolic extract of *A. squamosa* leaves which were identified by GC-MS as isoquinoline (21.62%), 1,6-methylphenazine (2.19%) and 1,2,3,4-tetrahydroisoquinoline (1.98%) with traces of unknown compounds (peaks 1,2,3,4,5,6,7,8, 10,12,14) .

**Table 4.5.** GC-MS analysis of the methanolic extract of the leaves of *A. squamosa*

| **S. N.**[a] | **Peak RT**[b] **(min)** | **Peak area** | **Peak area %** | **Compound detected *** | **Hit**[c] | **SI**[d] | **CAS No.** [e] | **Molecular Formula** | **Mol. Wt.**[f] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 29.13 | 833717 | 21.62 | Isoquinoline | 5 | 66 | 71783-56-7 | $C_{13}H1_9NO_2$ | 221 |
| 2 | 31.73 | 84566 | 2.19 | 1,6-Dimethylphe-nazine | 5 | 72 | 58718-43-7 | $C_{14}H_{12}N_2$ | 208 |
| 3 | 34.32 | 76356 | 1.98 | 1,2,3,4-Tetrahydro-isoquinoline | 5 | 66 | 0-00-0 | $C_{19}H_{23}NO_3$ | 313 |

*The compounds that were identified and compared with a similar mass fragmentation in the NIST database library. [a] S.N: Serial number; [b] R.T: Retention time; [c] Hit: Numbers of expected compounds in the NIST database library; [d] SI: International system of units. [e] CAS No: Chemical abstracts service registry number; [f] Mol. Wt.: Molecular weight.

Table 4.6 and Appendix 4 show the GC-MS results of the methanolic extract of *M. citrifolia* fruit. The major compounds detected were 1-butanecarboxylic acid (19.06 %), butyric acid (8.49%), N-acetylisoxazolidine (10.15%), propanoic acid (14.85%), carbamimidic acid (6.03%), 1,3-propanediol (6.61%), 4,5-octanediol (2.22%), 1,3-oxazine (0.64%) and semicarbazone (0.64%) with traces of unknown compounds (peaks 8, 9, 10, 11, 12, 14,15, 16, 17, 18, 19, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, 32).

**Table 4.6.** GC MS analysis of the methanolic extract from the fruit of *M. citrifolia*

| **S. N[a]** | **Peak RT[b] (min)** | **Peak area** | **Peak area %** | **Compound detected *** | **Hit[c]** | **SI[d]** | **CAS No [e]** | **Molecular Formula** | **Mol. Wt. [f]** |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 13.76 | 45859 | 19.06 | 1-Butanecarboxy-lic acid | 5 | 86 | 109-52-4 | $C_5H_{10}O_2$ | 102 |
| 2 | 13.81 | 20427 | 8.49 | Butyric acid | 5 | 91 | 107-92-6 | $C_4H_8O_2$ | 88 |
| 3 | 13.87 | 24416 | 10.15 | N-Acetylisoxazol-dine | 5 | 83 | 115615-36-6 | $C_5H_9NO_2$ | 115 |
| 4 | 13.95 | 35719 | 14.85 | Propanoic acid | 5 | 92 | 2786-22-3 | $C_3H_7NO_3$ | 105 |
| 5 | 14.10 | 14500 | 6.03 | Carbamimidic acid | 5 | 98 | 57-13-6 | $CH_4N_2O$ | 60 |
| 6 | 14.20 | 15897 | 6.61 | 1,3-Propanediol | 5 | 95 | 534-03-2 | $C_3H_9NO_2$ | 91 |
| 7 | 14.25 | 5333 | 2.22 | 4,5-Octanediol | 5 | 100 | 22520-40-7 | C8H18O2 | 146 |
| 8 | 30.40 | 1544 | 0.64 | 1,3-Oxazine | 5 | 100 | 109086-73-9 | $C_{10}H_{12}N_2O_3$ C | 208 |
| 9 | 40.02 | 1534 | 0.64 | Semicarbazone | 5 | 100 | 4581-65-1 | $C_{11}H1_7N_3O$ | 207 |

*The compounds that were identified and compared with a similar mass fragmentation in the NIST database library. [a] S.N: Serial number; [b] R.T: Retention time; [c] Hit: Numbers of expected compounds in the NIST database library; [d] SI: International system of units. [e] CAS No: Chemical abstracts service registry number; [f] Mol. Wt.: Molecular weight.

### 4.2. Part 2: Bioactive extracts

#### 4.2.1. Antibacterial activities of bioactive extracts

#### A. Anthraquinones

Figures 4.9, 4.10 and 4.11 identify the zones of inhibition for the anthraquinone extract from the fruit of *M. citrifolia* as 21.00 mm for *S. aureus*, 19.66 mm for *E. coli*, 21.33 mm for *B. cereus*, 21.66 mm for MRSA and 13.66 for *P. aeruginosa* while the inhibition zone for *H. pylori* was achieved at 26.83 mm.

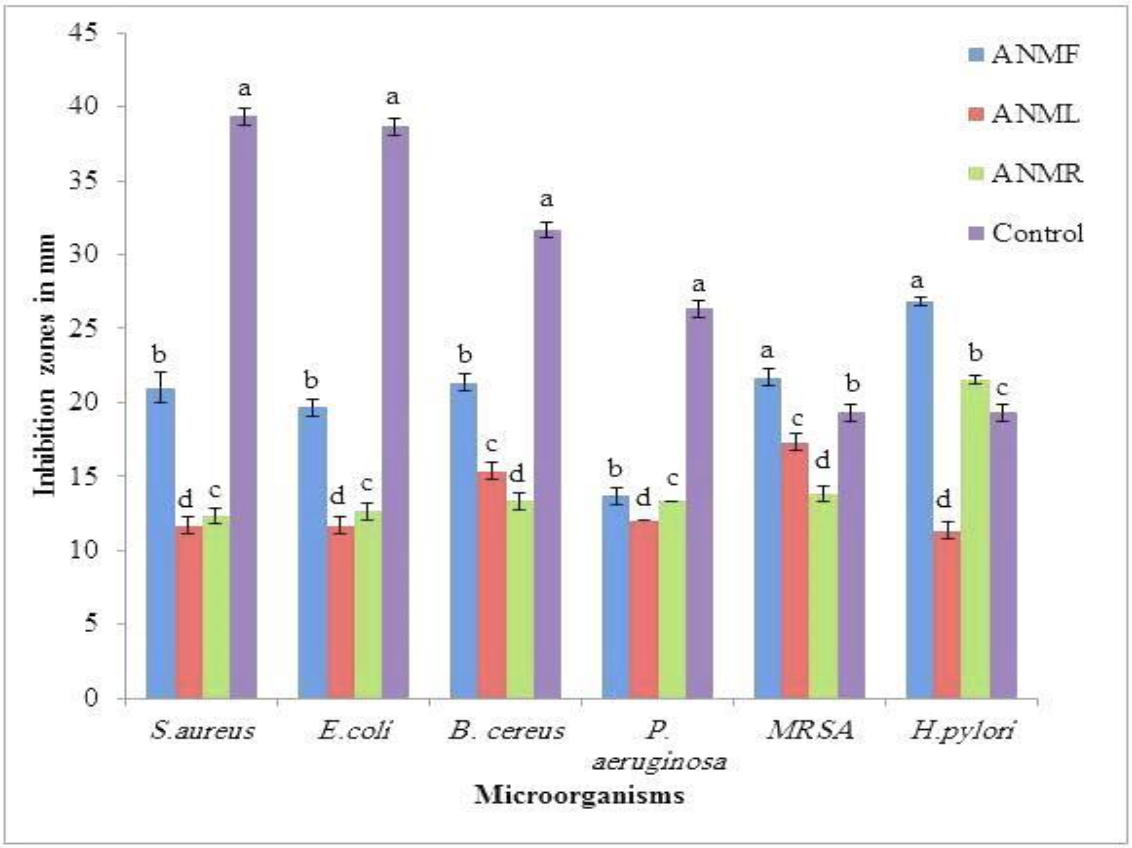
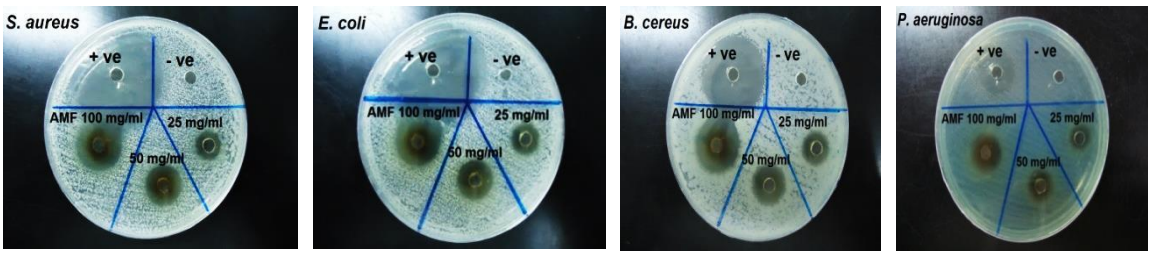



**Figure 4.9.** Inhibition zones of anthraquinones extracts (100 mg/ml) of different parts from *M. citrifolia* on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). Samples represented with different letters (a, b, c and d) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. ■ **ANMF**- Anthraquinones extracts of *M. citrifolia* fruit, ■ **ANML**- Anthraquinones extracts of *M. citrifolia* leaves, ■ **ANMR**- Anthraquinones extracts of *M. citrifolia* roots and ■ Control. All experiments were done in triplicates and values represent means ± SD.




A B C D

**Figure 4.10.** Inhibition zones of different concentrations 100, 50 and 25 mg/m of anthraquinones extracts of *M. citrifolia* fruit on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* compared to positive control (10 mg/ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

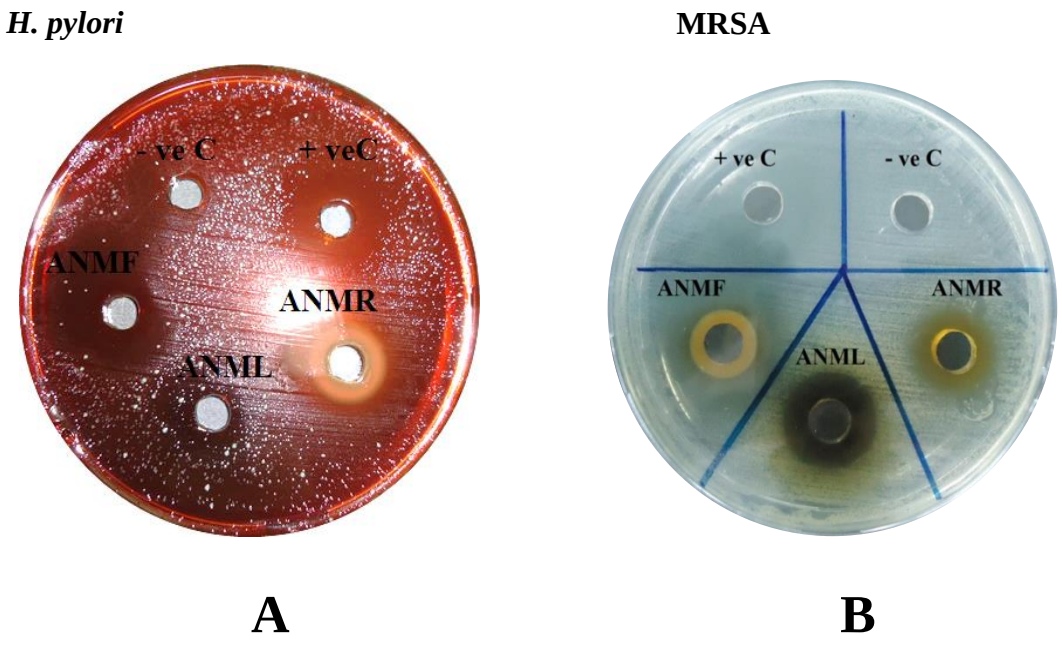
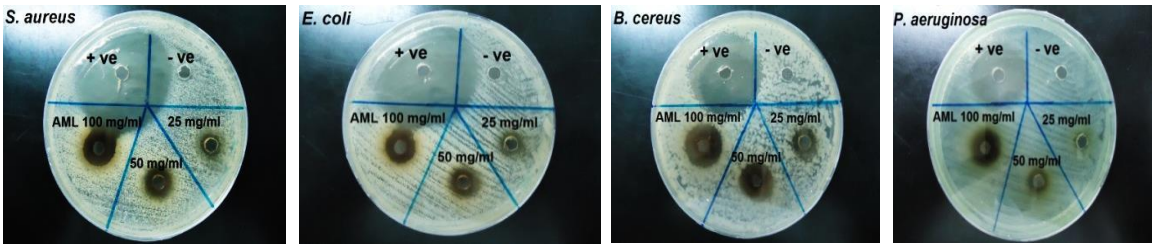


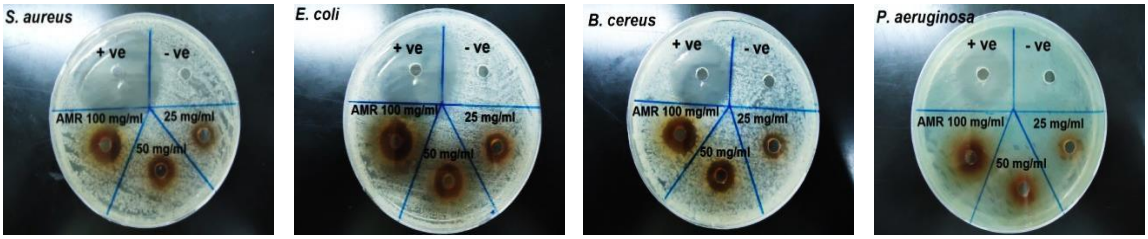

**Figure 4.11.** Inhibition zones of anthraquinones extracts (100 mg/ml) of different morphological parts of *M. citrifolia* against (A) *H. pylori* and (B) MRSA. **ANMF**- Anthraquinones extracts of *M. citrifolia* fruit, **ANML**- Anthraquinones extracts of *M. citrifolia* leaves and **ANMR**- Anthraquinones extracts of *M. citrifolia* root. Positive control (10 mg/ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

The anthraquinone extract from the leaves of the plant inhibited all test bacteria with the mean inhibition zones ranging from 17.33 mm to 11.66 mm compared to the positive control (tetracycline 10 mg/ml) with zones of inhibition ranging from 39.44 mm to 19.33 mm (Figures 4.9, 4.11 and 4.12). The anthraquinone extract from *M. citrifolia* roots displayed inhibition zones against S. *aureus, E. coli, B. cereus, P. aeruginosa* and *H. pylori* (Figures 4.9, 4.11 and 4.13).




A B C D

**Figure 4.12.** Inhibition zones of different concentration 100, 50 and 25 mg/ml of anthraquinones extracts of *M. citrifolia* leaves on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* compared to positive control (10 mg/ ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).




A B C D

**Figure 4.13.** Inhibition zones of different concentration 100, 50 and 25 mg/ml of anthraquinones extracts of *M. citrifolia* roots on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* compared to positive control (10 mg/ ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

MIC and MBC values for anthraquinone extracts of the fruit and leaves from this plant were 25 mg/ml for *S. aureus*, *E. coli* and *H. pylori* while *B. cereus* had MIC/MBC of 12.5 mg/ml. However, MIC/MBC for *P. aeruginosa* was at 50 mg/ml. MIC and MBC values for the roots of this plant were at 25 mg/ml for *S. aureus*, *E. coli* and *H. pylori*, and while for *B. cereus* it was at 12.5 mg /ml. However, for *P. aeruginosa* it was at 50 mg/ml (Table 4.7).

**Table 4.7.** MIC and MBC of anthraquinones extracts of the fruit of *M. citrifolia* on the microorganisms

| | **MIC and MBC of plant extracts (mg/ml)** | | |
|---|---|---|---|
| **Bacteria** | **ANMF** | **ANML** | **ANMR** |
| ***S. aureus*** | < 25 */ < 25 | 25/ 25 ** | 25/ 25 |
| ***E. coli*** | < 25/ > 25 | 25/ > 25 | 25/ 25 |
| ***B. cereus*** | 12.5/ ≤12.5 | 12.5/ >12.5 | 12.5/ 12.5 |
| ***P. aeruginosa*** | 50/50 | 50/50 | 50/ 50 |
| **MRSA** | 25/ 25 | 25/25 | 25/ >25 |
| ***H. pylori*** | 25/ 25 | 25/25 | 25/ 25 |

**ANMF**-Anthraquinones extracts of *M. citrifolia* fruit, **ANML**-Anthraquinones extracts of *M. citrifolia* leaves and **ANMR**-Anthraquinones extracts of *M. citrifolia* root. * refers to MIC, **refers to MBC.

**B. Alkaloids**

The results of well diffusion assay with alkaloid extract of *Morinda citrifolia* fruit showed defined inhibition zones for *S. aureus*, *E. coli*, *B. cereus*, and *H. pylori*. No result was obtained for *P. aeruginosa* (Figures 4.14, 4.15 and 4.16).

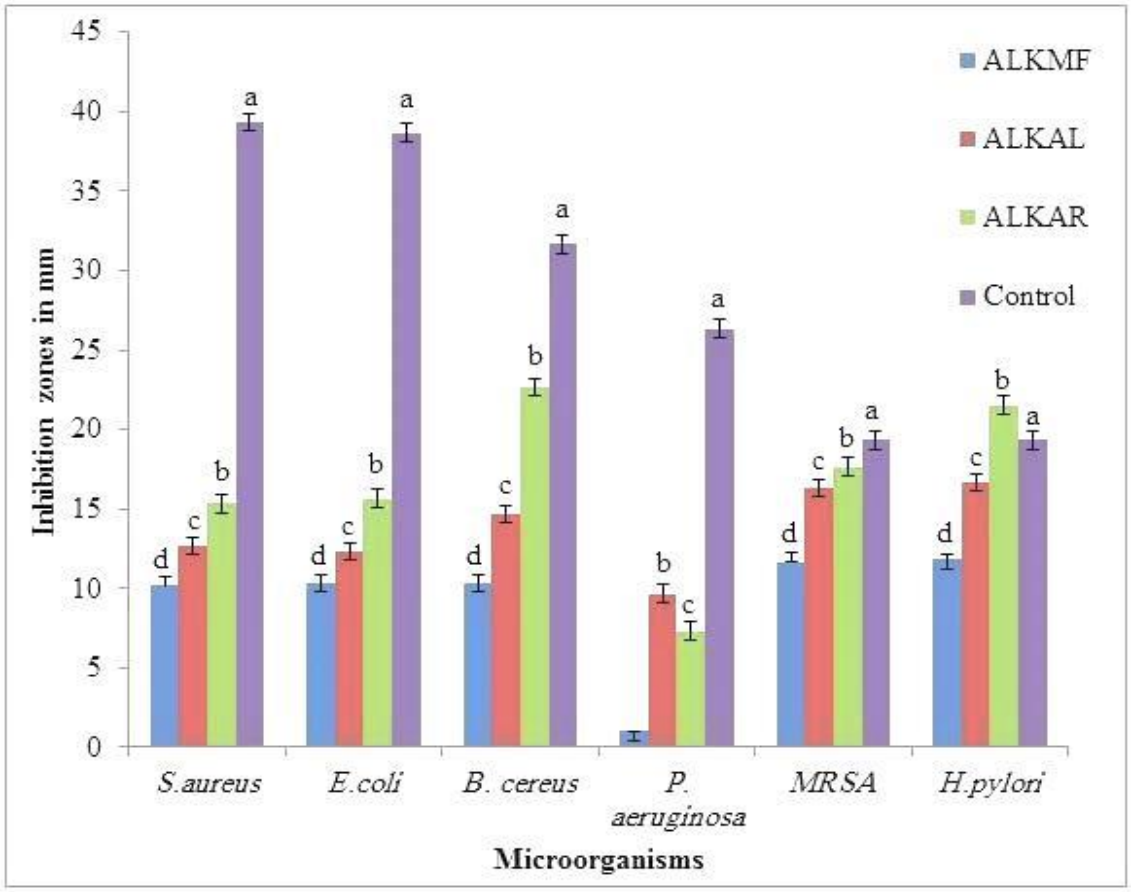
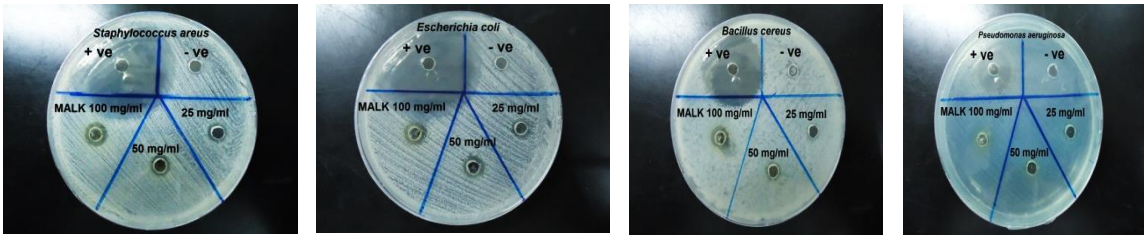



**Figure 4.14.** Inhibition zones of alkaloid extracts (100 mg/ml) of different part from plants on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). Samples represented with different letters (a, b, c and d) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. ■ **ALKMF**- Alkaloids extracts of *M. citrifolia* fruit, ■ **ALKAL**- Alkaloids extracts of *A. squamosa* leaves ■ **ALKAR**- Alkaloids extracts of *A. angustiloba* roots and ■ Control. All experiments were done in triplicates and values represent means ± SD.




A B C D

**Figure 4.15.** Inhibition zones of different concentration 100, 50 and 25 mg/ml of alkaloids extracts of *M. citrifolia* fruit on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* compared to positive control (10 mg/ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

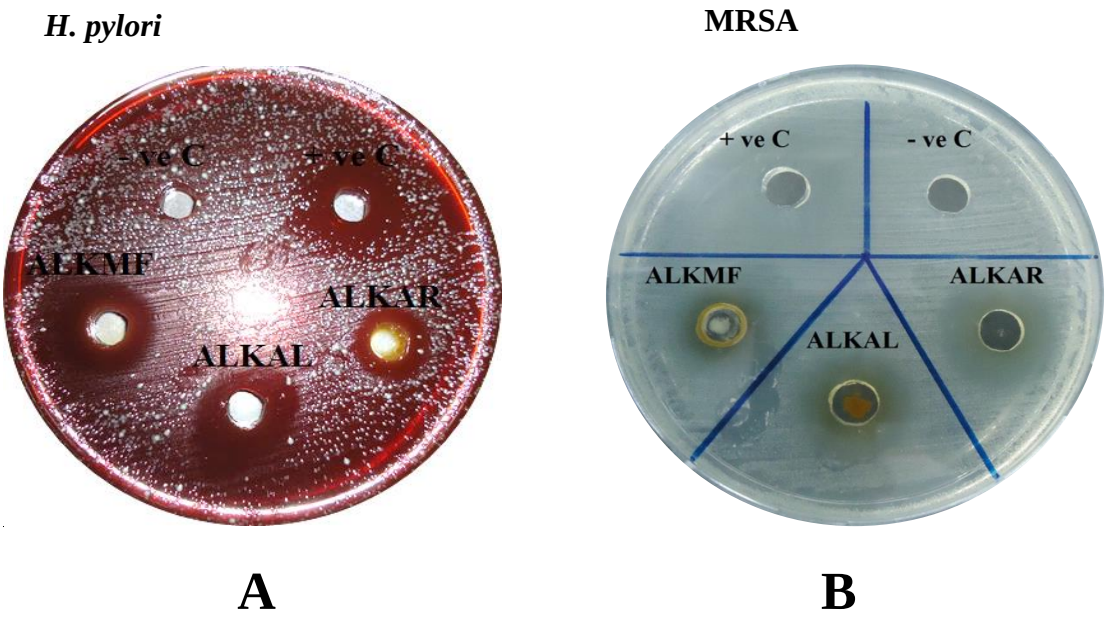
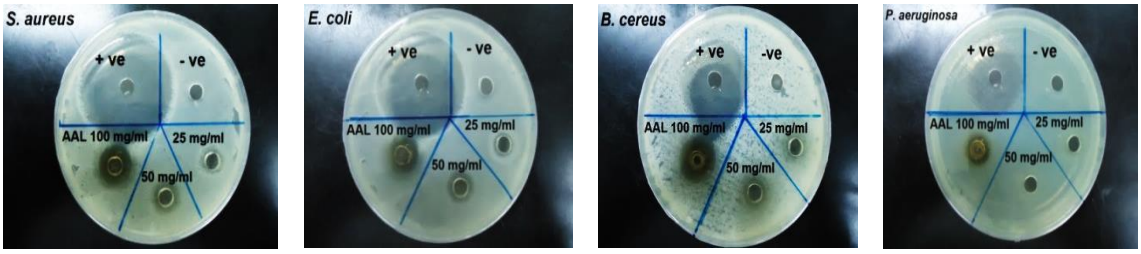


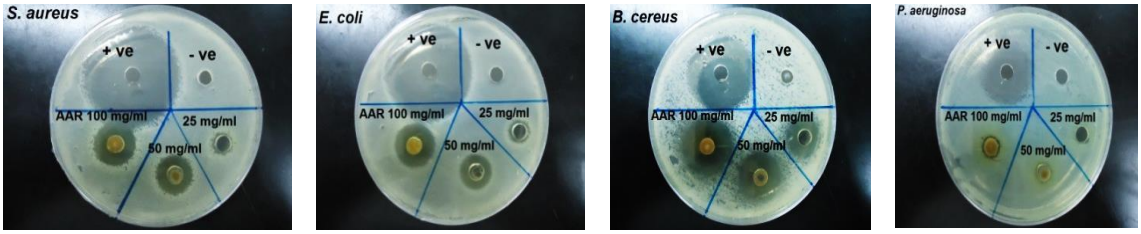

**Figure 4.16.** Inhibition zones of alkaloids extracts (100 mg/ml) of the different selected plants against (A) *H. pylori* and (B) MRSA. **ALKMF**- Alkaloids extracts of *M. citrifolia* fruit, **ALKAL**- Alkaloids extracts of *A. squamosa* leaves and **ALKAR**- Alkaloids extracts of *A. angustiloba* roots. Positive control (10 mg/ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

Figures 4.14, 4.15 and 4.17 define zones of inhibition for the alkaloid extract from the leaves of *A. squamosa* as 12.66 mm for *S. aureus*, 12.33 mm for *E. coli*, 14.66 mm for *B. cereus*, 9.66 mm for *P. aeruginosa*, 16.33 mm for MRSA and 16.66 mm for *H. pylori* at a high concentration of this extract (100 mg/ml). On the other hand, the alkaloid extract from the roots of *A. angustiloba* exhibited zones of inhibition against all the bacterial test strains (Figures 4.14, 4.15 and 4.18).




A B C D

**Figure 4.17.** Inhibition zones of different concentration 100, 50 and 25 mg/ml of alkaloids extracts of *A. squamosa* leaves on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* compared to positive control (10 mg/ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).




A B C D

**Figure 4.18.** Inhibition zones of different concentration 100, 50 and 25 mg/ml of alkaloids extracts of *A. angustiloba* roots on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* compared to positive control (10 mg/ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

Table 4.8 shows the values of MIC and MBC for alkaloids from the selected plants. Alkaloid extracted from the fruit of *M. citrifolia* had MIC/MBC at 50 mg/ml for *S. aureus*, *E. coli*, MRSA and *H. pylori* while *B. cereus* was inhibited at 25 mg/ml. No result was detected for *P. aeruginosa*. MIC and MBC values for alkaloid extract from the leaves of *A. squamosa* and the roots of *A. angustiloba* exhibited inhibition at 25 mg/ml for *S. aureus*, *E. coli*, MRSA and *H. pylori* while *B. cereus* was at 12.5 mg/ml. However, for *P. aeruginosa* was only achieved at 50 mg/ml.

**Table 4.8.** MIC and MBC of alkaloids extracts of the different selected plants on the microorganisms

| | **Plant extracts (mg/ml)** | | |
|---|---|---|---|
| **Bacteria** | **ALKMF** | **ALKAL** | **ALKAR** |
| ***S. aureus*** | 50 */ >50 | 25/ 25 ** | 25/ 25 |
| ***E. coli*** | 50/ > 50 | 25/ 25 | 25/ 25 |
| ***B. cereus*** | 25/ > 25 | 12.5/ >12.5 | 12.5/ 12.5 |
| ***P. aeruginosa*** | Na/ Na | 50 /50 | 50/50 |
| **MRSA** | 50/ 50 | >25/ 25 | 25/ 25 |
| ***H. pylori*** | 50/ 50 | < 25/ 25 | 25/ 25 |

**ALKMF**- Alkaloids extracts of *M. citrifolia* fruit, **ALKAL**- Alkaloids extracts of *A. squamosa* leaves, **ALKAR**- Alkaloids extracts of *A. angustiloba* roots and **Na**–non active at high concentration (100 mg/ml). * refers to MIC, **refers to MBC.

**C. Phenolic compounds and diterpenes**

Figures 4.19 and 4.20 show zones of inhibition for the phenolic compound extract from the Australian plant mixture as 8.33 mm for *S. aureus*, 9.33 mm for *E. coli*, 15.33 mm for MRSA and 11.33 mm for *B. cereus* at a high concentration of this extract (500 mg/ml). No inhibition was detected for *P. aeruginosa*. It is noteworthy that *B. cereus* was sensitive to the phenolic compound extract at a concentration of 50 mg/ ml.

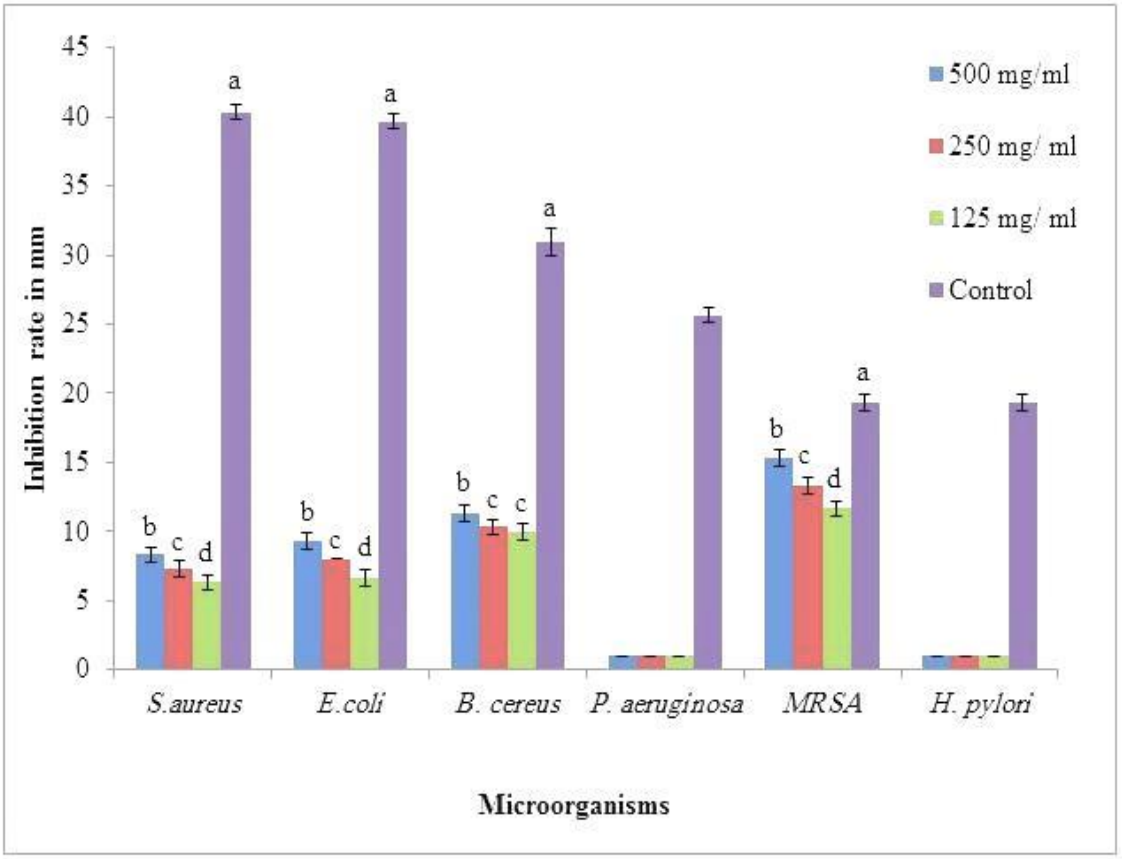
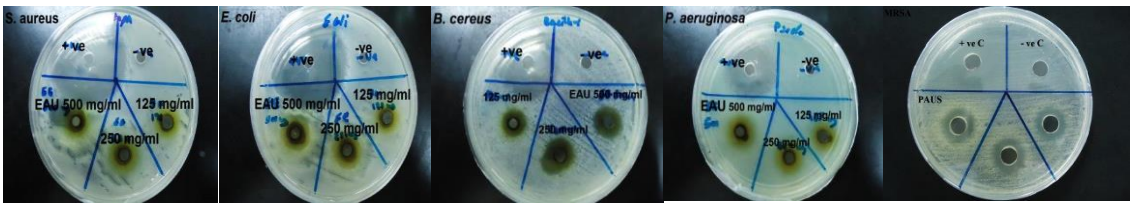



**Figure 4.19.** Inhibition zones of different concentration 500, 250 and 125 mg/ml of phenolic compounds extract of Australian plant mixture on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). ■ 500 mg/ml of the extract ■ 250 mg/ml of the extract ■ 125 mg/ml of the extract ■ Control. Samples represented with different letters (a, b, c and d) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All samples in three replicates ± SD.




A B C D E

**Figure 4.20.** Inhibition zones of different concentration 500, 250 and 125 mg/ml of phenolic compounds extracts of APM on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* (D) *P. aeruginosa* and (E) MRSA compared to positive control (10 mg/ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

However, the diterpene extract from the *A. squamosa* fruit displayed zones of inhibition against *S. aureus*, *E. coli*, *B. cereus*, MRSA and *P. aeruginosa* at a high concentration of 100 mg/ml (Figures 4.21 and 4.22).

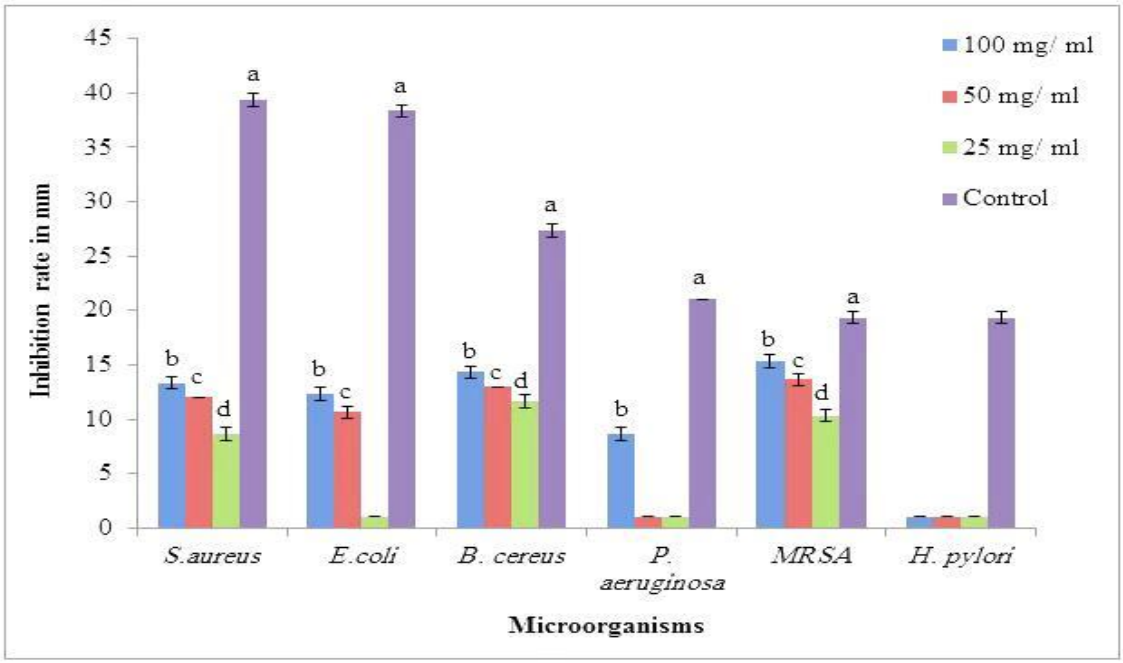
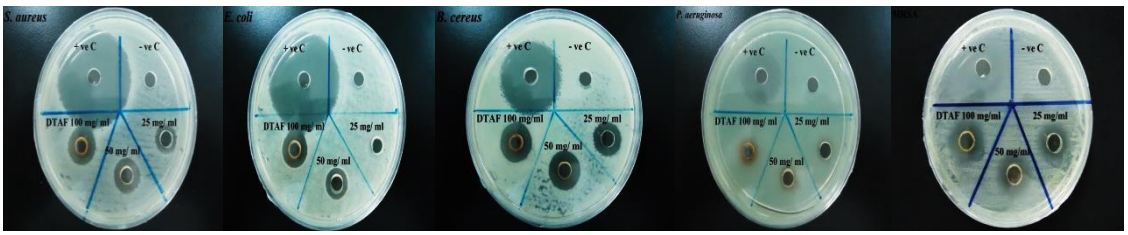



**Figure 4.21.** Inhibition zones of different concentration 100, 50 and 25 mg/ml of diterpenes extract from *A. squamosa* fruit on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). ■ 100 mg/ml of the extract ■ 50 mg/ml of the extract ■ 25 mg/ml of the extract ■ Control. Samples represented with different letters (a, b, c and d) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All samples in three replicates ± SD.




A B C D E

**Figure 4.22.** Inhibition zones of different concentration 100, 50 and 25 mg/ml of diterpenes extract from *A. squamosa* fruit on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* (D) *P. aeruginosa* and (E) MRSA compared to positive control (10 mg/ml of tetracycline) and negative control (5% tween 80). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

MIC and MBC values of phenolic compounds from APM showed at 125 mg/ml for *S. aureus*, *E. coli* and MRSA while *B. cereus* was at 31.25 mg/ml. No results were detected for *P. aeruginosa* and *H. pylori* (Table 4.9). However, MIC and MBC values for of the diterpenes extract from the *A. squamosa* fruit were at 25 mg/ml for *S. aureus*, *E. coli* and MRSA while for *B. cereus* it was at 12.5 mg/ ml. However, for *P. aeruginosa* was only achieved at 50 mg/ ml (Table 4.10).

**Table 4.9.** MIC and MBC of phenolic compounds extract from the APM on the microorganisms

| | **Plant extracts (mg/ml)** | |
|---|---|---|
| **Bacteria** | **MIC** | **MBC** |
| ***S. aureus*** | 125.00 | >125.00 |
| ***E. coli*** | > 125.00 | 125.00 |
| ***B. cereus*** | 31.25 | 31.25 |
| ***P. aeruginosa*** | Na | Na |
| **MRSA** | 125.00 | 125.00 |
| ***H. pylori*** | Na | Na |

**Na**- non active at the highest of concentration tested (500 mg/ml).

**Table 4.10.** MIC and MBC of diterpenes extract from *A. squamosa* fruit on the microorganisms

| | Plant extracts (mg/ml) | |
|---|---|---|
| **Bacteria** | **MIC** | **MBC** |
| ***S. aureus*** | 25.00 | 25.00 |
| ***E. coli*** | 25.00 | 25.00 |
| ***B. cereus*** | 12. 50 | 12.50 |
| ***P. aeruginosa*** | >50.00 | 50.00 |
| **MRSA** | 25.00 | 25.00 |
| ***H. pylori*** | Na | Na |

**Na** – non active at the highest of concentrations tested (100 mg/ml).

### 4.2.2. Antioxidant activity of bioactive extracts

### A. Anthraquinones

Figure 4.23 shows the DPPH radical scavenging activity of the anthraquinone extract from *M. citrifolia* fruit as 91.40% ($IC_{50}$ 5.48 mg/ml), leaves as 64.48% ($IC_{50}$ 7.76 mg/ml) and roots as 25.20% ($IC_{50}$ 19.85 mg/ml) compared to ascorbic acid as a positive control at 96.59% ($IC_{50}$ 5.18 mg/ml). The SOD-like activity of the anthraquinone extract from the fruit of *M. citrifolia* was 91.75% while the anthraquinone extract from its leaves was lower at 62.95%. The anthraquinone extract from the roots of this plant displayed the lowest antioxidant activity at 23.28% (Figure 4.24).

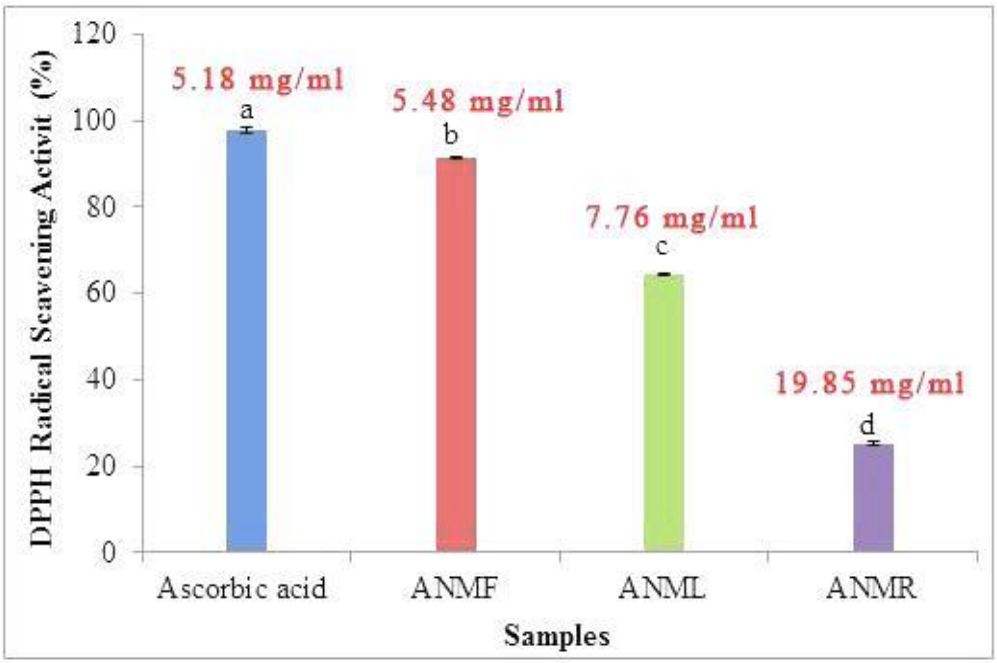



**Figure 4.23.** DPPH scavenging activity with $IC_{50}$ (in red) of anthraquinones extracts from *M. citrifolia.* The values are the average of three replicates ± SD. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b, c and d) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ ml of ascorbic acid. **ANMF**- Anthraquinones extracts of *M. citrifolia* fruit, **ANML**- Anthraquinones extracts of *M. citrifolia* leaves and **ANMR**- Anthraquinones extracts of *M. citrifolia* roots.

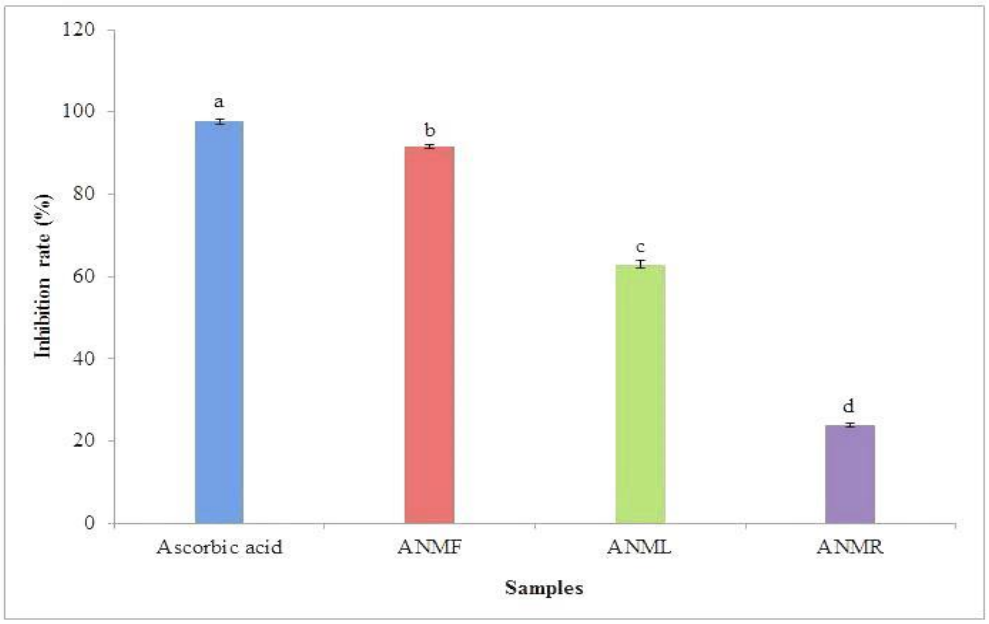



**Figure 4.24.** The rate of Inhibition of SOD-like activities of anthraquinones extracts from *M. citrifolia.* The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b, c and d) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ml of ascorbic acid. **ANMF**- Anthraquinones extracts of *M. citrifolia* fruit, **ANML**- Anthraquinones extracts of *M. citrifolia* leaves and **ANMR**- Anthraquinones extracts of *M. citrifolia* roots.

**B. Alkaloids**

The results to determine the DPPH radical scavenging activity of the alkaloid extract from *M. citrifolia* fruit showed 27.71% ($IC_{50}$ 18.05 mg/ml), from *A. squamosa* leaves 90.80% ($IC_{50}$ 5.51 mg/ml) and from *A. angustiloba* roots 49.85% ($IC_{50}$ 10.04 mg/ml) compared to ascorbic acid as a positive control at 96.59% ($IC_{50}$ 5.18 mg/ml) (Figure 4.25). While the inhibition rate of SOD-like activity of the alkaloid extract from *M. citrifolia* fruit was at 27.01%, it was at 90.69% for its leaves. For the alkaloid extract from the roots of *A. angustiloba*, antioxidant activity was observed at 49.74% (Figure 4.26).

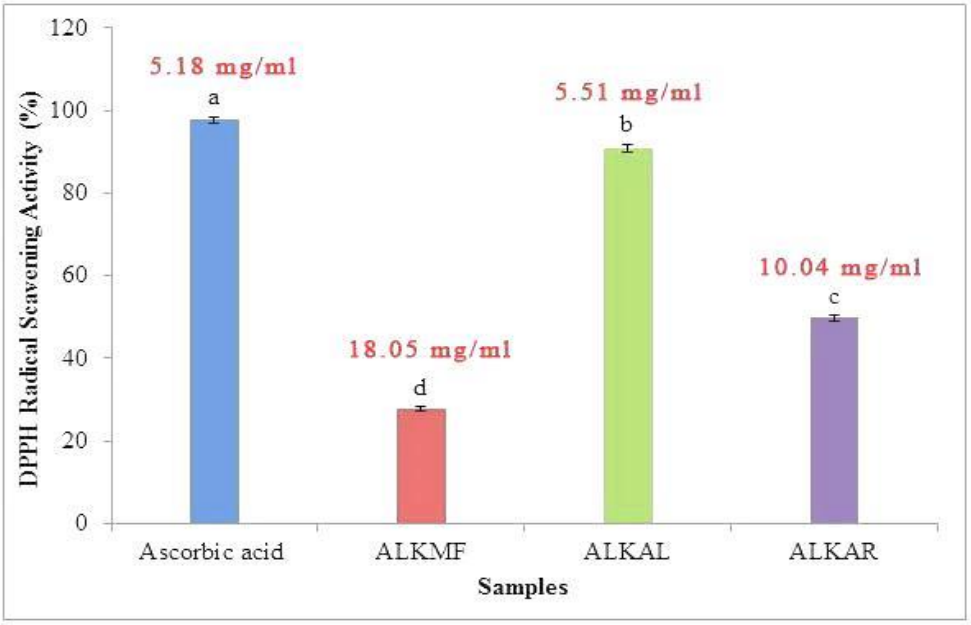



**Figure 4.25.** DPPH scavenging activity with $IC_{50}$ (in red) of alkaloids extracts from different plants. The values are the average of three replicates ± SD. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b and c) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ ml of ascorbic acid. **ALKMF**- Alkaloids extracts of *M. citrifolia* fruit, **ALKAL**- Alkaloids extracts of *A. squamosa* leaves and **ALKAR**- Alkaloids extracts of *A. angustiloba* roots.

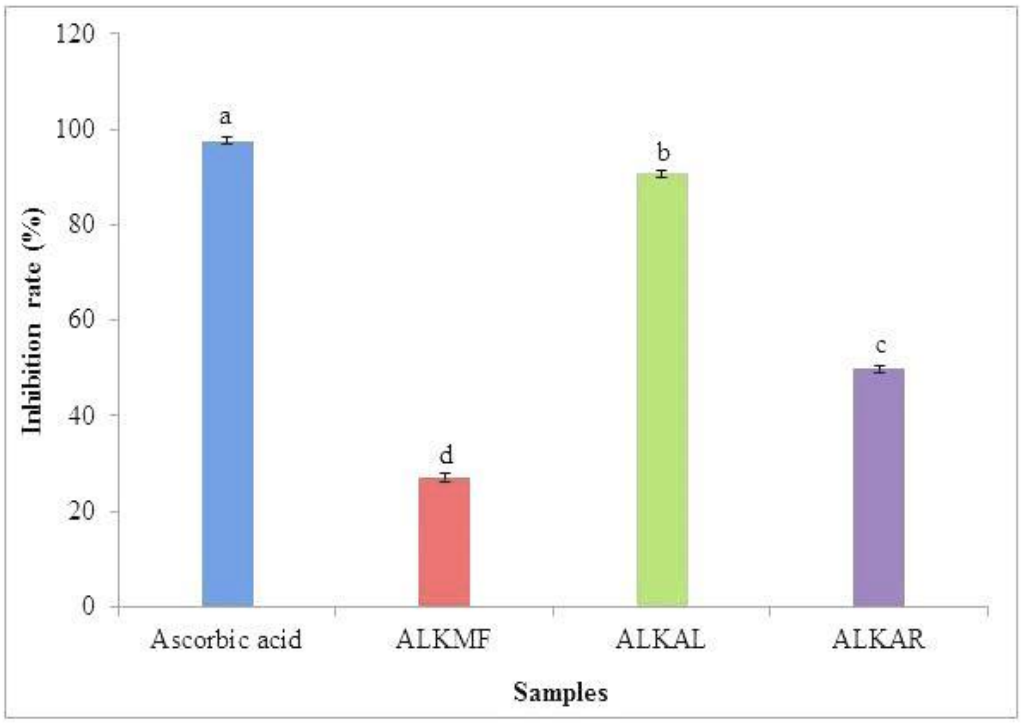



**Figure 4.26.** The rate of inhibition of SOD-like activities of alkaloids extracts from selected plants. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b and d) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ml of ascorbic acid. **ALKMF**- Alkaloids extracts of *M. citrifolia* fruit, **ALKAL**- Alkaloids extracts of *A. squamosa* leaves and **ALKAR**- Alkaloids extracts of *A. angustiloba* roots

**C. Phenolic compounds and diterpenes**

Figure 4.27 shows of DPPH radical scavenging activity of the phenolic compound extract from APM as 87.17% ($IC_{50}$ 5.74 mg/ml) and the diterpenes extract of *A. squamosa* fruit as 92.47% ($IC_{50}$ 5.40 mg/ml) compared to ascorbic acid as a positive control at 96.59% ($IC_{50}$ 5.18 mg/ml). The rate of inhibition SOD activity of the phenolic compound extract from APM was 87.87% while the diterpenes extract from the fruit of *A. squamosa* exhibited antioxidant activity at 92.74% (Figure 4.28).

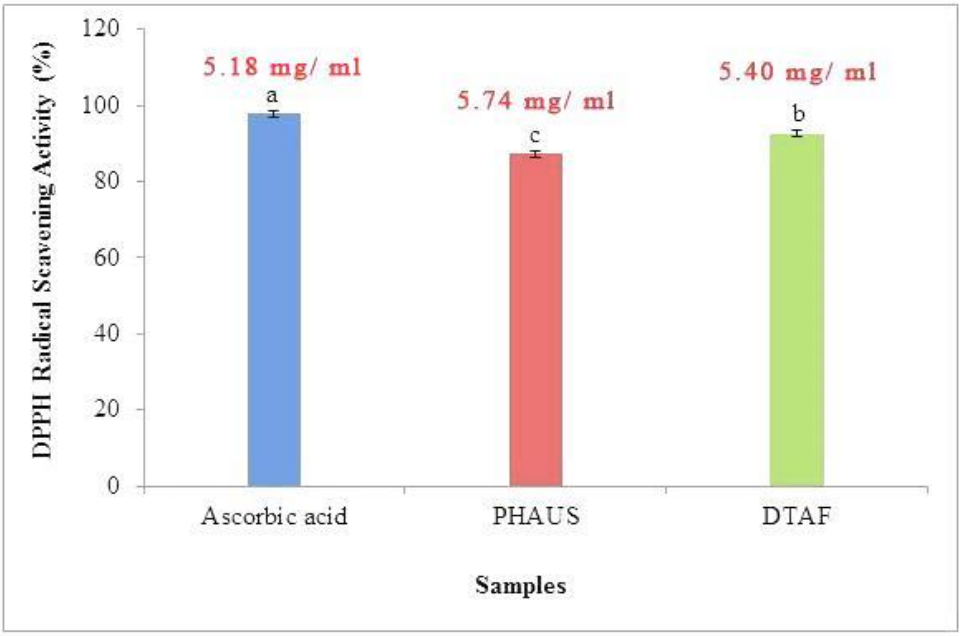



**Figure 4.27.** DPPH scavenging activity with $IC_{50}$ (in red) of phenolic compounds extract from APM and diterpenes extract from *A. squamosa* fruit. The values are the average of three replicates ± SD. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b and c) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ ml of ascorbic acid. **PHAUS** - phenolic compounds extract from APM and **DTAF**-diterpenes extract from *A. squamosa* fruit.

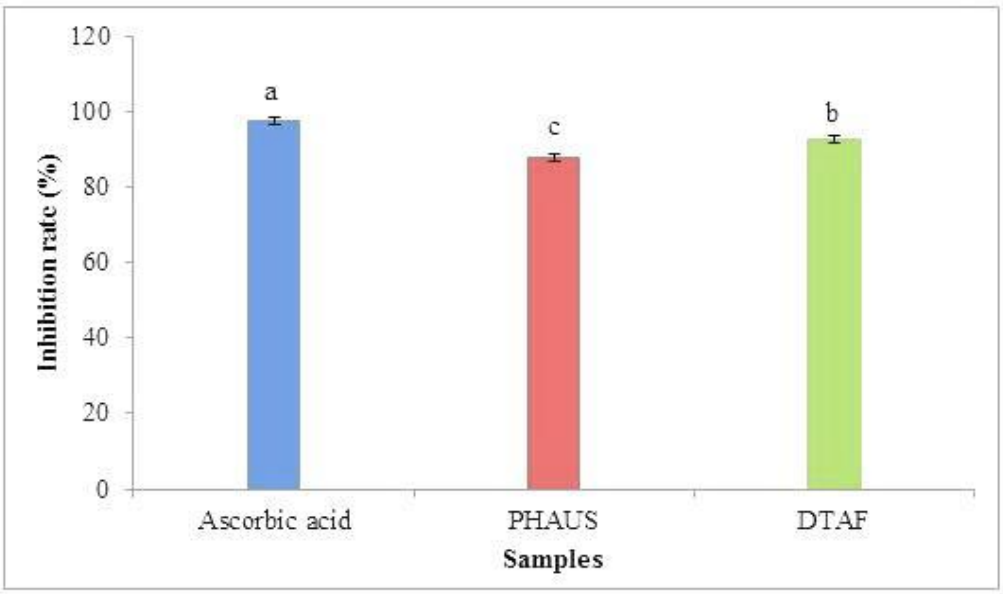



**Figure 4.28.** The rate of inhibition of SOD-like activities of phenolic compounds extract from APM and diterpenes extract from *A. squamosa* fruit. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b and c) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ml of ascorbic acid. **PHAUS** - phenolic compounds extract from APM and **DTAF**-diterpenes extract from *A. squamosa* fruit.

The results of total phenolic content in the APM revealed that the highest amount of phenolic compounds in the ethanolic extract was 427.70 mg GAE/g while for the hot aqueous extract it was 283.12 mg GAE/g in a higher concentration of the extract at 100 mg/ ml (Table 4.11).

**Table 4.11.** Total phenolic content of plant mixture extracts (APM)

| Concentration | Total phenolic content (mg GAE/g | |
|---|---|---|
| | **Ethanolic extract** | **Hot aqueous extract** |
| 100 mg/ml | 427.70 ± 2.60 [a] | 283.12 ± 1.25 [a] |
| 50 mg/ml | 248.53 ± 1.90 [b] | 169.37 ± 3.75 [b] |
| 10 mg/ml | 93.95 ± 0.75 [c] | 57.74 ± 1.40 [c] |

The values are the average of three replicates ± SD. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b and c) are significantly different ($p < 0.05$) from each other and control.

### 4.2.3. Thin layer chromatography (TLC) and IR spectrometry of bioactive extracts

TLC results of anthraquinone extract from *M. citrifolia* revealed six red bands for the fruit, five for the leaves and four for the roots under UV light at wavelength 245 nm and 356 nm. For alkaloids, five orange bands were observed for *M. citrifolia* fruit, six for *A. squamosa* and five bands for *A. angustiloba*. Also, phenolic compounds displayed nine blue bands and diterpene six under UV light at wavelength 245 nm and 356 nm. The results of the IR spectra (Appendix 5, 6 and 7) exhibited strong absorption bands at 3408.83, 3275.52 and 3308.88 $cm^{-1}$ for anthraquinone extract from the fruit, leaves and roots of *M. citrifolia* respectively identified by O-H stretching. C-H stretching groups were detected at the bands 2954.74 and 2857.38 $cm^{-1}$ (fruit), 2928.13 $cm^{-1}$ (leaf), 2922.31$cm^{-1}$ and 2852.95 $cm^{-1}$ (root). The C=O group of *M. citrifolia* fruit was detected at the band 1726.27 $cm^{-1}$, leaves at 1600.06 $cm^{-1}$ and roots at 1630.71 $cm^{-1}$.

The functional groups of IR spectra of alkaloids extract from the fruit of *M. citrifolia*, leaves of *A. squamosa*, and the roots of *A. angustiloba* exhibited a strong O-H at band 3334.89 from the fruit of *M. citrifolia* (Appendix 8, 9 and 10). C-H stretching groups are shown at bands 2925.74 $cm^{-1}$ and 2855.38 $cm^{-1}$ for the fruit of *M. citrifolia*, 2852.92 $cm^{-1}$ and 2922.89 $cm^{-1}$ for the leaves of *A. squamosa* as well as 2854.00$cm^{-1}$ and 2923.26 $cm^{-1}$ for the roots *A. angustiloba*. The N-H groups showed absorption bands at 3193.22 $cm^{-1}$ for the fruit of *M. citrifolia* and 3237.47 $cm^{-1}$ for the root *A. angustiloba*. The C=O bond at 1660 $cm^{-1}$ is attributed to the leaves of *A. squamosa* and 1723 $cm^{-1}$ to the roots of *A. angustiloba.* C-H group bonds were detected at absorption bands 1565.63 $cm^{-1}$ and 1512.64 $cm^{-1}$ for the fruit of *M. citrifolia*, 1456.56 $cm^{-1}$ for the leaves of *A. squamosa* and 1453.74 $cm^{-1}$ for the roots of *A. angustiloba*.

The functional groups of IR spectra of diterpene extract from *A. squamosa* fruit were observed at band 3268.62 $cm^{-1}$ by O-H stretching. C-H stretching groups were detected

at 2925.25 and 2853.14 $cm^{-1}$. The C=O functional group was detected at band 1716.57 $cm^{-1}$. C-H group bonds were detected at bands 1512.54, 1454.41 and 1325.53 $cm^{-1}$. The C-O functional group was detected at band 1233.33 $cm^{-1}$ (Appendix 11). The IR spectra of the phenolic compounds from the Australian plant mixture were detected at band 3298.16 $cm^{-1}$ for the O-H group (Appendix 12). C-H stretching groups were detected at bands 2926.37 and 2850.84 $cm^{-1}$. The C=O group was detected at band 1726.71 $cm^{-1}$. C-H group bonds were at absorption bands 1600.04, 1512.51, 1429.19, 1376.13 and 1342.13 $cm^{-1.}$

### 4.2.4. LC-MS analysis of bioactive extracts

### A. Anthraquinones

Figure 4.29 shows the LC chromatograms of the major compounds of anthraquinone extract from the fruit of *M. citrifolia.* As shown in Appendix 13, the peak at retention time 42.417 min exhibiting an $[M + H]^+$ at *m/z* 239.2117 as 1-hydroxy-2-methylanthraquinone was identified. The peak at retention time 42.798 min exhibiting an $[M + H]^+$ at *m/z* 329.3721 as 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl)anthraquinone was identified (Appendix 14). The peak at retention time 44.349 min exhibiting an $[M + H]^+$ at *m/z* 345.2424 as morindolin was identified (Appendix 15). The peak at retention time 45.256 min exhibiting an $[M + H]^+$ at *m/z* 315.1353 as 1,1-*O*i-O-methyl morindolin was identified (Appendix 16). The peak at retention time 46.353 min exhibiting an $[M + H]^+$ at *m/z* 241.221 as alizarin or 1,2-dihydroxyanthraquinone was identified (Appendix 17). The peak at retention time 50.732 min exhibiting an $[M + H]^+$ at *m/z* 287.2385 as 1,3-6 Trihydroxy-2-methoxyanthraquinone was identified (Appendix 18).

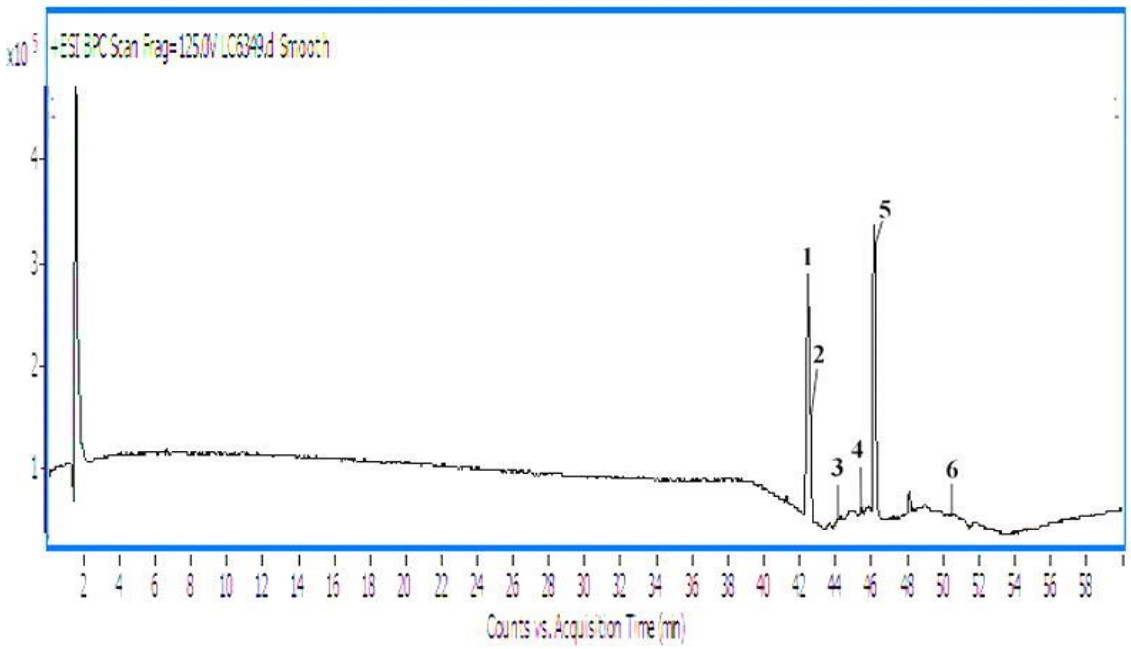



**Figure 4.29.** LC chromatograms of the major compounds of anthraquinones extracted from the fruit of *M. citrifolia* (1) 1-hydroxy-2-methylanthraquinones, (2) 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl)anthraquinones, (3) morindolin, (4) 1,1-*O*i-O-methyl morindol, (5) 1,2-dihydroxyanthraquinone and (6) 1,3-6-trihydroxy-2-methoxyanthraquinone. All compounds identified using natural products data base (NIST database library). 1: MS/MS spectrum of the peak in appendix 13, page 208; 2: MS/MS spectrum of the peak in appendix 14, page 208; 3: MS/MS spectrum of the peak in appendix 15, page 209; 4: MS/MS spectrum of the peak in appendix 16, page 209; 5: MS/MS spectrum of the peak at appendix 17, page 210; 6: MS/MS spectrum of the peak in appendix 18, page 210.

In the LC chromatograms, five major compounds of anthraquinone were extracted from *M. citrifolia* leaves (Figure 4.30). The peak at retention time 38.871 min exhibiting an $[M + H]^+$ at *m/z* 241.1434 as 1,2-dihydroxyanthraquinone was identified (Appendix 19). The peak at retention time 45.287 min exhibiting an $[M + H]^+$ at *m/z* 285.3341 as 1-hyroxy-2,3-methoxyanthraquinone was identified (Appendix 20). The peak at retention time 46.668 min exhibiting an $[M + H]^+$ at *m/z* 301.1413 as 2,6-diroxy-1,3-methoxyanthraquinone was identified (Appendix 21). The peak at retention time 47.001 min exhibiting an $[M + H]^+$ at *m/z* 255.2099 as 2-hydroxy-1-methoxyanthraquinone was identified (Appendix 22). The peak at retention time 47.322 min exhibiting an $[M + H]^+$ at *m/z* 329.2678 as 2-hydroxy-1, 5-dimethoxy-6-(methoxymethyl)anthraquinone was identified (Appendix 23).

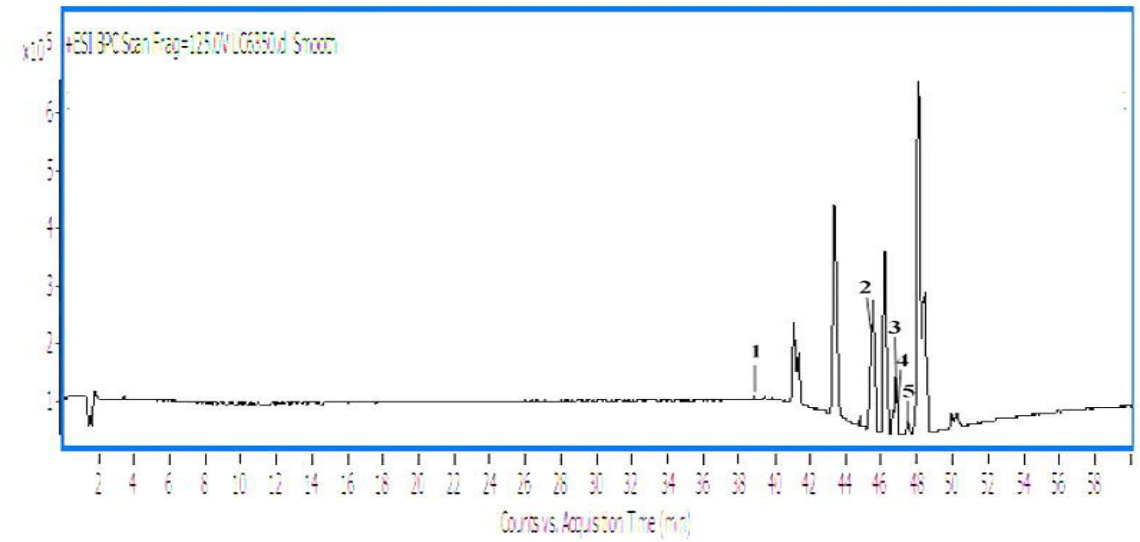



**Figure 4.30.** LC chromatograms of the major compounds of anthraquinones extracted from the leaves of *M. citrifolia* (1) 1,2-dihydroxyanthraquinone, (2) 1-hyroxy-2,3-methoxyanthraquinone, (3) 2,6-diroxy-1,3-methoxyanthraquinone, (4) 2-hydroxy-1-methoxyanthraquinone and (5) 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl) anthraquinones. All compounds identified using natural products data base (NIST database library). 1: MS/MS spectrum of the peak in appendix 19, page 211; 2: MS/MS spectrum of the peak in appendix 20, page 211; 3: MS/MS spectrum of the peak in appendix 21, page 212; 4: MS/MS spectrum of the peak in appendix 22, page 212; 5: MS/MS spectrum of the peak in appendix 23, page 213.

Figure 4.31 shows the LC chromatograms of the major compounds of anthraquinone extract from the roots of *M. citrifolia.* As shown in Appendix 24, the peak at retention time 1.746 min exhibiting an $[M + H]^+$ at *m/z* 285.0785 (MS/MS spectrum) as damnacanthal or 3-hyroxy-2-hydroxymethyl-1-methoxyanthraquinone was identified. The peak at retention time 45.657 min exhibiting an $[M + H]^+$ at *m/z* 299.3437 as 6-hydroxy-1,3-dimethoxy-1-7-methylanthraquinone was identified (Appendix 25). The peak at retention time 46.978 min exhibiting an $[M + H]^+$ at *m/z* 269.2453 as 2-ethoxy-1-hydroxyanthraquinone was identified (Appendix 26). The peak at 49.894 min retention time exhibiting an $[M + H]^+$ at *m/z* 283.2653 as damnacanthal or 3-hydroxy-1-methoxyanthraquinone-2-aldehyde was identified (Appendix 27).

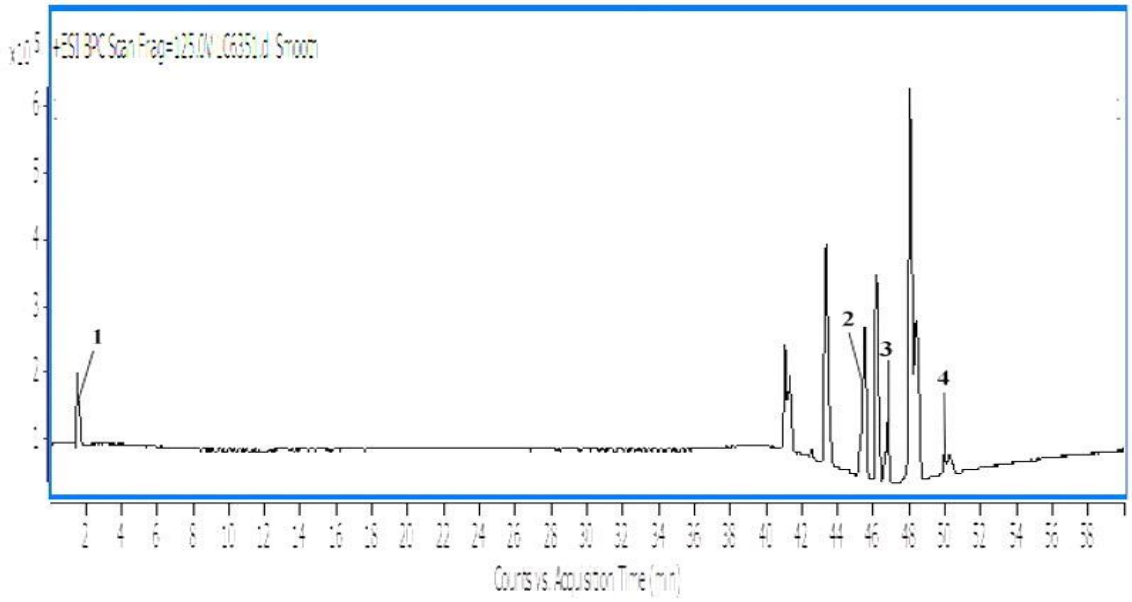



**Figure 4.31.** LC chromatograms of the major compounds of anthraquinones extracted from the roots of *M. citrifolia* (1) 3-hyroxy-2-hydroxymethyl-1-methoxyanthraquinone, (2) 6-hydroxy-1,3-dimethoxy-1-7-methylanthraquinone, (3) 2-ethoxy-1-hydroxyanthraquinone and (4) 3-hydroxy-1-methoxyanthraquinone-2-aldehyde. All compounds identified using natural products data base (NIST database library). 1: MS/MS spectrum of the peak in appendix 24, page 213; 2: MS/MS spectrum of the peak in appendix 25, page 214; 3: MS/MS spectrum of the peak in appendix 26, page 214; 4: MS/MS spectrum of the peak in appendix 27, page 215.

**B. Alkaloids**

LC-MS analysis of the five major compounds of alkaloid extract from the fruit of *M. citrifolia* is shown in Figure 4.32. As shown in Appendix 28, the peak at the retention time of 1.786 min exhibiting an $[M + H]^+$ at *m/z* 142.1201 (MS/MS spectrum) as pelletierine was identified. The peak at retention time 1.965 min exhibiting an $[M + H]^+$ at *m/z* 206.1101 as sedamine was identified (Appendix 29). The peak at retention time 2.203 min exhibiting an $[M + H]^+$ at *m/z* 207.1359 as pseudopelletierine was identified (Appendix 30). The peak at retention time 40.924 min exhibiting an $[M + H]^+$ at *m/z* 173.1413 as halosin was identified (Appendix 31). The peak at retention time 44.780 min exhibiting an $[M + H]^+$ at *m/z* 267.1764 as lycopodine was identified (Appendix 32).

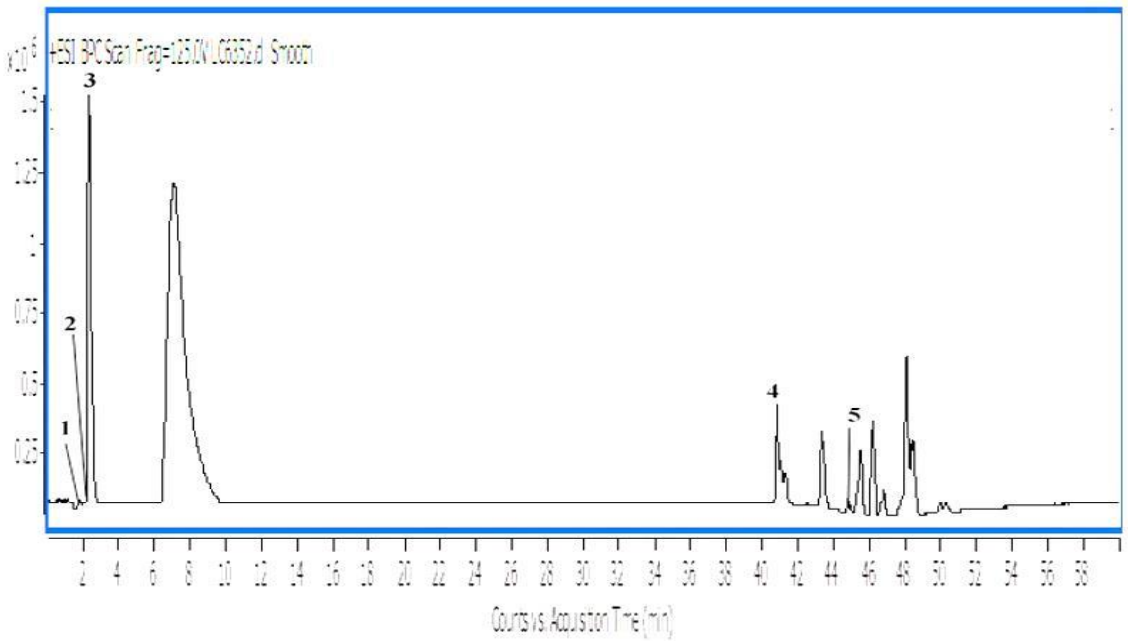



**Figure 4.32.** LC chromatograms of the major compounds of alkaloids extracted from the fruit of *M. citrifolia* (1) pelletierine, (2) sedamine, (3) pseudopelletierine, (4) halosine and (5) lycopodine. All compounds identified using natural products data base (NIST database library). 1: MS/MS spectrum of the peak in appendix 28, page 215; 2: MS/MS spectrum of the peak in appendix 29, page 216; 3: MS/MS spectrum of the peak in appendix 30, page 216; 4: MS/MS spectrum of the peak in appendix 31, page 217; 5: MS/MS spectrum of the peak in appendix 32, page 217.

Figure 4.33 shows the LC chromatograms of the major compounds of alkaloid extract from the leaves of *A. squamosa*. The peak at the retention time of 1.562 min exhibiting an $[M + H]^+$ at *m/z* 342.1366 (MS/MS spectrum) as corydine was identified (Appendix 33). The peak at retention time 1.741 min exhibiting an $[M + H]^+$ at *m/z* 328.1750 as sanjoinine was identified (Appendix 34). The peak at retention time 1.896 min exhibiting an $[M + H]^+$ at *m/z* 296.1270 as norlaureline was identified (Appendix 35). The peak at retention time 41.688 min exhibiting an $[M + H]^+$ at *m/z* 286.2870 as norcodeine was identified (Appendix 36). The peak at retention time 43.700 min exhibiting an $[M + H]^+$ at *m/z* 293.1047 as oxanalobine was identified (Appendix 37). The peak at retention time 45.569 min exhibiting an $[M + H]^+$ at *m/z* 236.0960 as aporphine was identified (Appendix 38).

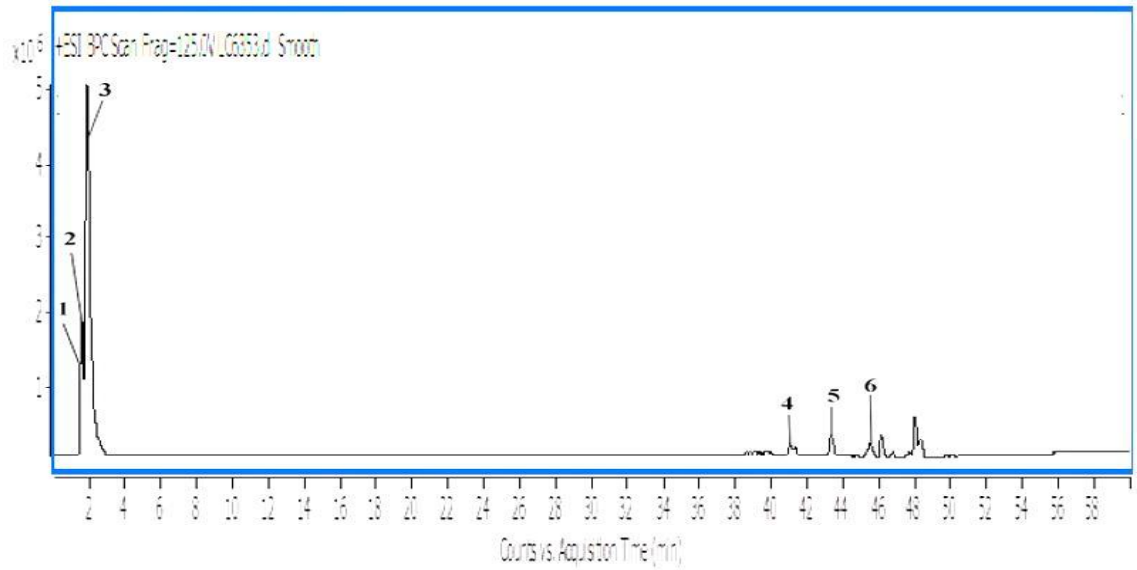



**Figure 4.33.** LC chromatograms of the major compounds of alkaloids extracted from the leaves of *A. squamosa* (1) corydine, (2) sanjoinine, (3) norlaureline, (4) norcodeine, (5) oxaanalobine and (6) aporphine. All compounds identified using natural products data base (NIST database library). 1: MS/MS spectrum of the peak in appendix 33, page 218; 2: MS/MS spectrum of the peak in appendix 34, page 218; 3: MS/MS spectrum of the peak in appendix 35, page 219; 4: MS/MS spectrum of the peak in appendix 36, page 219; 5: MS/MS spectrum of the peak in appendix 37, page 220; 6: MS/MS spectrum of the peak in appendix 38, page 220.

The five major compounds of alkaloid extract from *A. angustiloba* roots are shown in the LC chromatograms (Figure 4.34). The peak at retention time of 1.790 min exhibiting an $[M + H]^+$ at *m/z* 386.2201 as echitamine was identified (Appendix 39). The peak at retention time 43.308 min exhibiting an $[M + H]^+$ at *m/z* 257.3020 as 3-H-indole was identified (Appendix 40). The peak at retention time 44.832 min exhibiting an $[M + H]^+$ at *m/z* 243.1372 as 1-H-indole was identified (Appendix 41). The peak at retention time 45.010 min exhibiting an $[M + H]^+$ at *m/z* 343.1308 as alstilobanine B was identified (Appendix 42). The peak at retention time 48.462 min exhibiting an $[M + H]^+$ at *m/z* 357.2427 as alstilobanine E was identified (Appendix 43).

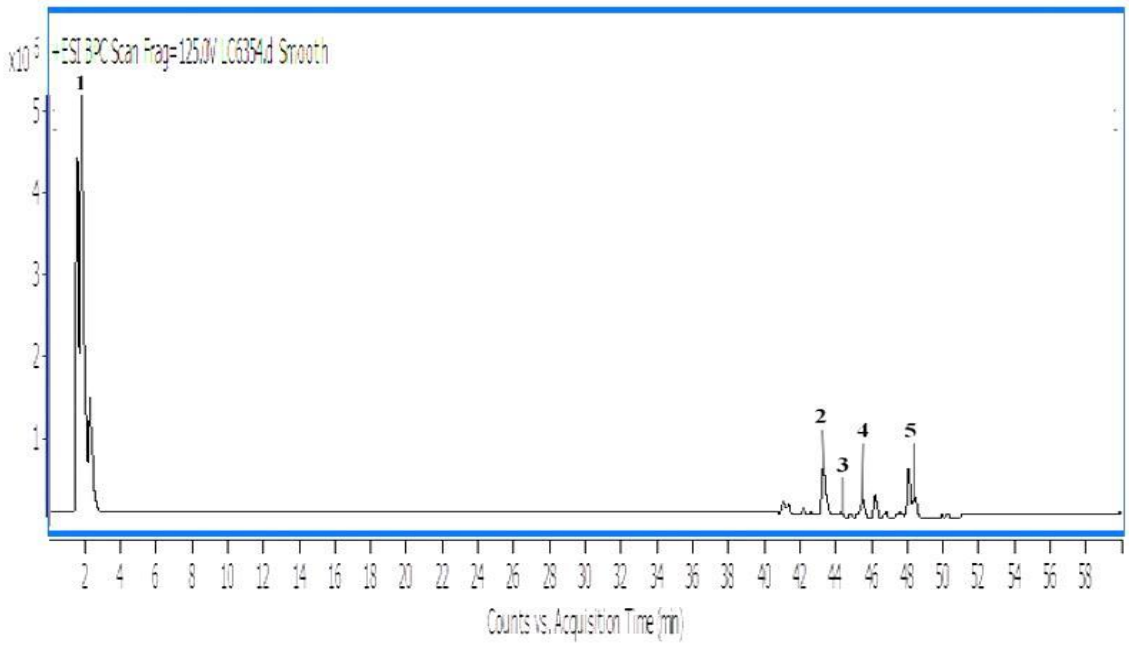



**Figure 4.34.** LC chromatograms of the major compounds of alkaloids extracted from the root *A. angustiloba* roots (1) echitamine, (2) 3-H-indole-3-one, (3) 1-H-indole, (4) alstilobanine B and (5) alstilobanine E. All compounds identified using natural products data base (NIST database library). 1: MS/MS spectrum of the peak in appendix 39, page 221; 2: MS/MS spectrum of the peak in appendix 40, page 221; 3: MS/MS spectrum of the peak in appendix 41, page 222; 4: MS/MS spectrum of the peak in appendix 42, page 222; 5: MS/MS spectrum of the peak in appendix 43, page 223.

**C. Phenolic compounds and diterpenes**

LC chromatograms exhibited the nine major phenolic compounds extracted from the Australian plant mixture (Figure 4.35). As shown in Appendix 44, the peak at retention time 1.473 min exhibiting an $[M + H]^+$ at *m/z* 300.1060 (MS/MS spectrum as hydroxybenzoic acid-hexoside) was identified. The peak at retention time 41.384 min exhibiting an $[M + H]^+$ at *m/z* 286.2754 as luteolin was identified (Appendix 45). The peak at retention time 43.217 min exhibiting an $[M + H]^+$ at *m/z* 316.3205 as isohamnetin was identified (Appendix 46). The peak at retention time 45.336 min an $[M + H]^+$ at *m/z* 578.4190 as apigenin-7-O-rutinoside exhibiting was identified (Appendix 47). The peak at retention time 46.169 min exhibiting an $[M + H]^+$ at *m/z* 302.1466 as quercetin was identified (Appendix 48). The peak at retention time 46.765 min exhibiting an $[M + H]^+$ at *m/z* 635.4841 as HHDP-gallogluco-pyranoside was identified (Appendix 49). The peak at retention time 47.181 min exhibiting an $[M + H]^+$ at *m/z* 516.3569 as dicaffeoyquinic acid was identified (Appendix 50). The peak at retention time 47.919 min exhibiting an $[M + H]^+$ at *m/z* 344.3171 as rosmadial was identified (Appendix 51). The peak at retention time 48.467 min exhibiting an $[M + H]^+$ at *m/z* 342.2722 as caffeic acid was identified (Appendix 52).

Figure 4.36 shows the LC chromatograms of the major compounds of the diterpenes extract from the fruit of *A. squamosa*. As shown in Appendix 53, the peak at retention time 44.685 min exhibiting an $[M + H]^+$ at *m/z* 347.3158 (MS/MS spectrum) as kuaran-18-al was identified. The peak at retention time 44.923 min exhibiting an $[M + H]^+$ at *m/z* 322.2053 as 16,17,19-kauranetriol was identified (Appendix 54). The peak at retention time 46.705 min exhibiting an $[M + H]^+$ at *m/z* 331.2253 as kauren-18-ol was identified (Appendix 55). The peak at retention time 47.494 min exhibiting an $[M + H]^+$ at *m/z* 273.1714 as kaur-16-ene was identified (Appendix 56). The peak at retention time 48.006 min exhibiting an $[M + H]^+$ at *m/z* 413.2656 as stigmasterol was identified

(Appendix 57). The peak at retention time 48.232 min exhibiting an $[M + H]^+$ at *m/z* 309.2027 as annosquamosin B was identified (Appendix 58).

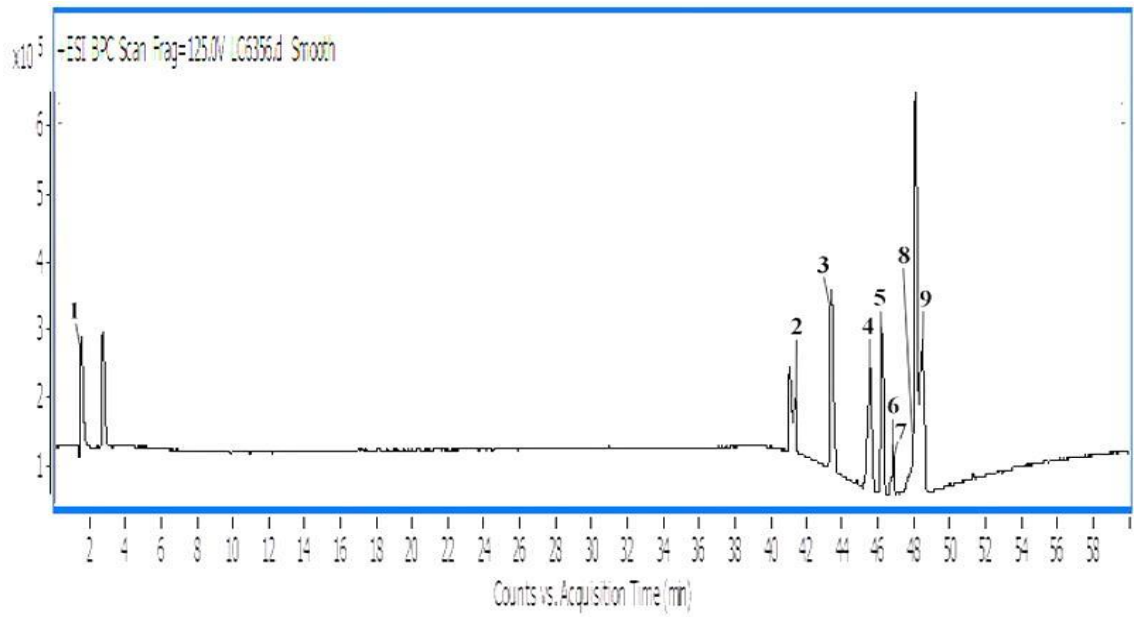



**Figure 4.35.** LC chromatograms of the major phenolic compounds extracted from the Australian plant mixture (1) hydroxybenzoic acid-hexoside, (2) luteolin, (3) isohamnetin, (4) apigenin-7-O-rutinoside, (5) quercetin, (6 HHDP-gallogluco-pyranoside, (7) dicaffeoyquinic acid, (8) rosmadial and (9) caffeic acid. All compounds identified using natural products data base (NIST database library). 1: MS/MS spectrum of the peak in appendix 44, page 223; 2: MS/MS spectrum of the peak in appendix 45, page 224; 3: MS/MS spectrum of the peak in appendix 46, page 224; 4: MS/MS spectrum of the peak in appendix 47, page 225; 5: MS/MS spectrum of the peak in appendix 48, page 225; 6: MS/MS spectrum of the peak in appendix 49, page 226; 7: MS/MS spectrum of the peak in appendix 50, page 226; 8: MS/MS spectrum of the peak in appendix 51, page 227; 9: MS/MS spectrum of the peak in appendix 52, page 227.

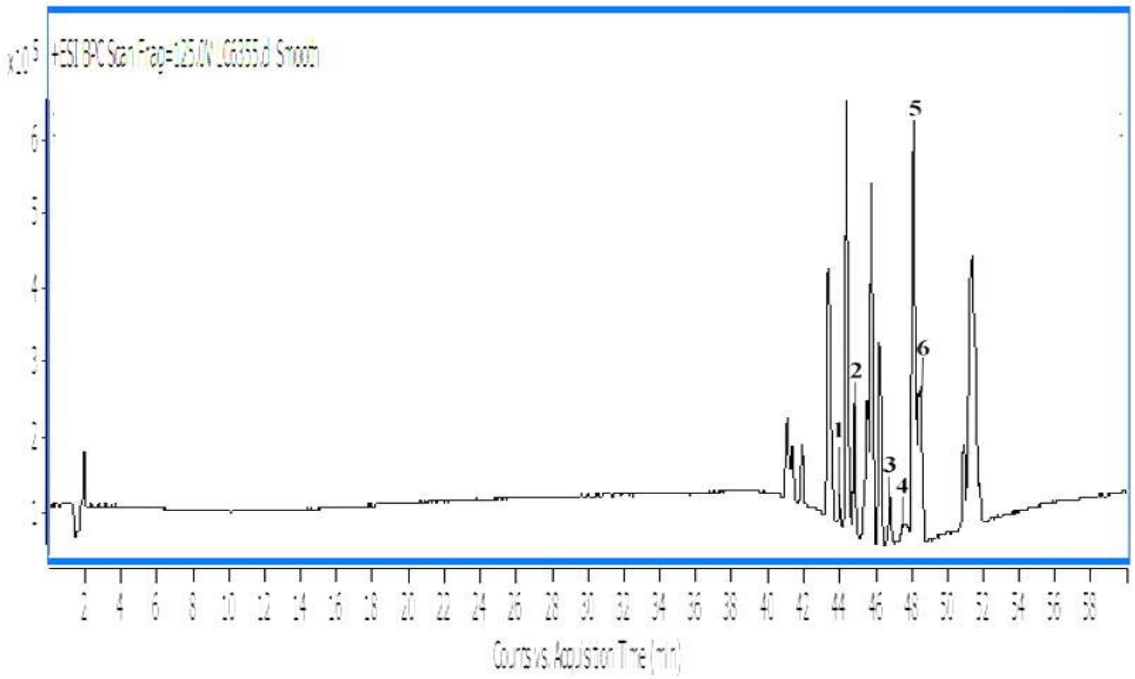



**Figure 4.36.** LC chromatograms of the major compounds of diterpenes extracted from the fruit of *A. squamosa* (1) kuaran-18-al, (2) 16,17,19-kauranetriol, (3) kauren-18-ol, (4) kaur-16-ene, (5) stigmasterol and (6) annosquamosin. All compounds identified using natural products data base (NIST database library). 1: MS/MS spectrum of the peak in appendix 53, page 228; 2: MS/MS spectrum of the peak in appendix 54, page 228; 3: MS/MS spectrum of the peak in appendix 55, page 229; 4: MS/MS spectrum of the peak in appendix 56, page 229; 5: MS/MS spectrum of the peak in appendix 57, page 230; 6: MS/MS spectrum of the peak in appendix 58, page 230.

### 4.2.5. Assessment of the lytic effect of bioactive extracts from selected plants with the utilization of a scanning electron microscope

#### A. Anthraquinones

Figure 4.37 shows the effect of anthraquinone extract from the fruit of *M. citrifolia* against *B. cereus.* It was observed that the bacterial cells treated with antibacterial anthraquinone underwent morphological changes that included disruption in the cell wall membrane, roughening of the cell surface, leakage of cytoplasmic contents and a build-up of cell debris. Untreated cells displayed a normal surface and the typical rod shape of *B. cereus*.

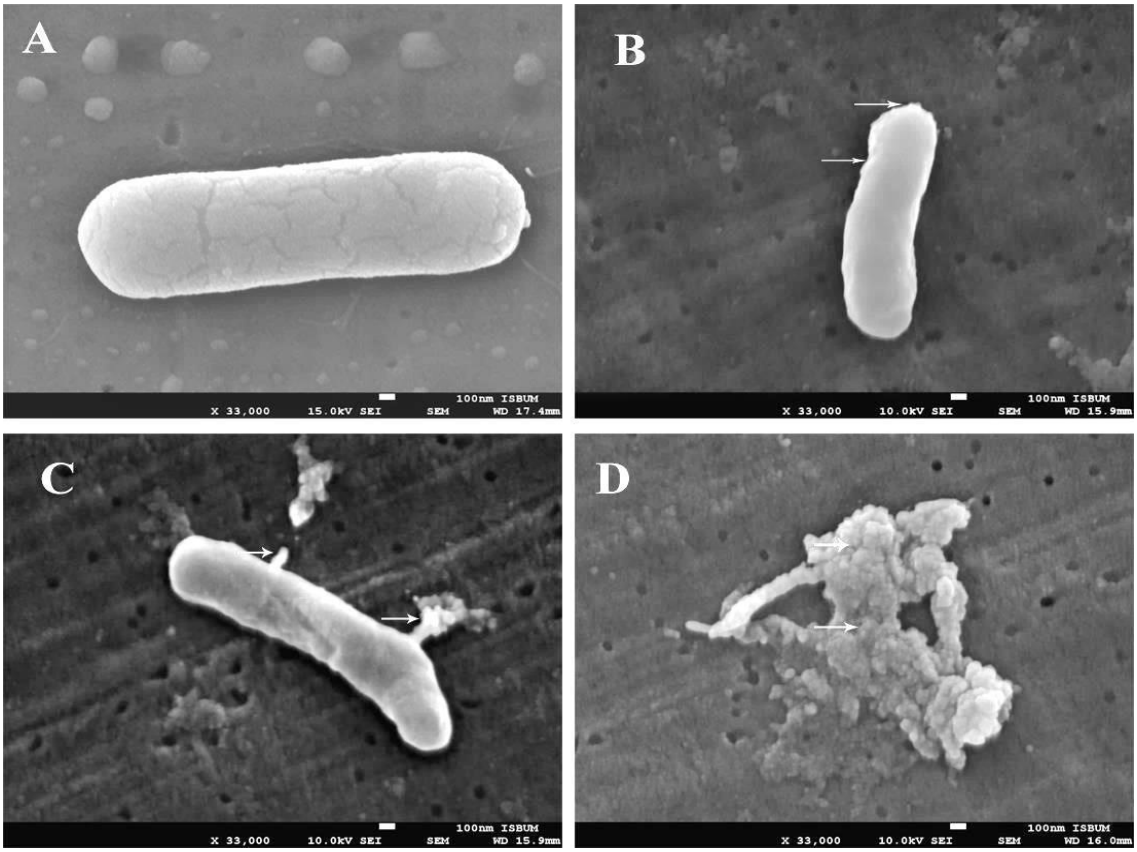



**Figure 4.37.** Effect of anthraquinones extracted from the fruit of *M. citrifolia* by scanning electron microscope. (A) Control: *B. cereus*. (B), (C) and (D) *B. cereus* treated with anthraquinones. (B) Arrows show changes in morphology with roughening of cell surface (C) Arrows show leakage of cytoplasmic content, (D) Arrows show buildup of cell debris.

**B. Alkaloids**

The effects on bacterial cells treated with alkaloid extract from the leaves of *A. squamosa* were observed with the utilization of a scanning electron microscope (Figure 4.38). The morphological changes included swelling, rupture in cell walls and cell lysis which eventually culminates in the death of the cell. Untreated cells exhibited an unchanged shape.

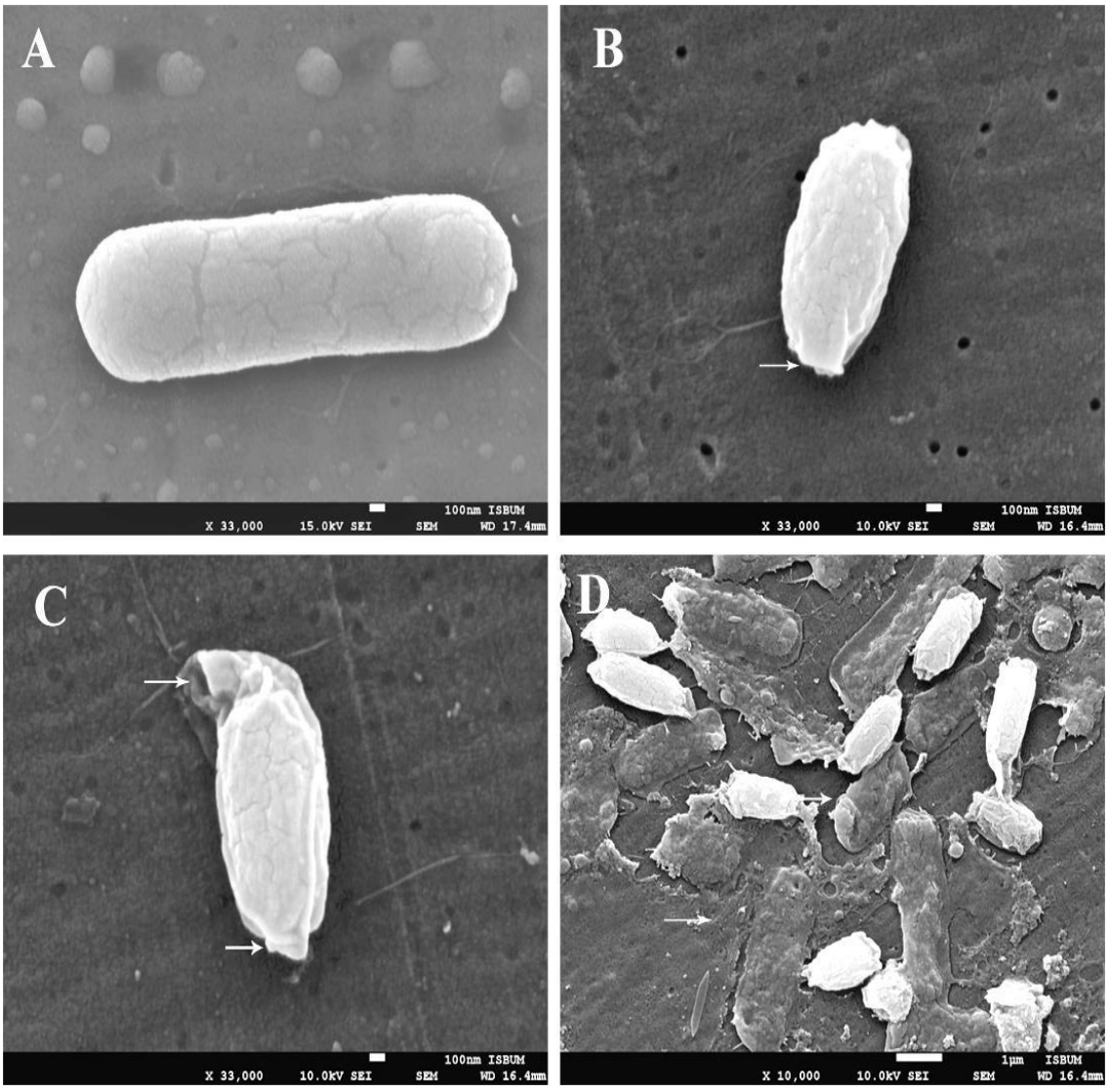



**Figure 4.38.** Effect of alkaloid extracted from the leaves of *A. squamosa* by scanning electron microscope. (A) Control: *B. cereus*. (B), (C) and (D) *B. cereus* treated with alkaloids. (B) Arrows show changes in morphology with swelling (C) Arrows show rupture in cell walls, (D) Arrows show buildup of cell debris and cell death.

**C. Diterpenes**

Figure 4.39 shows the effects of diterpenes extract from the fruit of *A. squamosa* against *B. cereus*. The morphological changes included swelling, rupture and collapse of cell walls as well as cell lysis with cell debris. Untreated bacterial cells exhibited a regular and smooth surface.

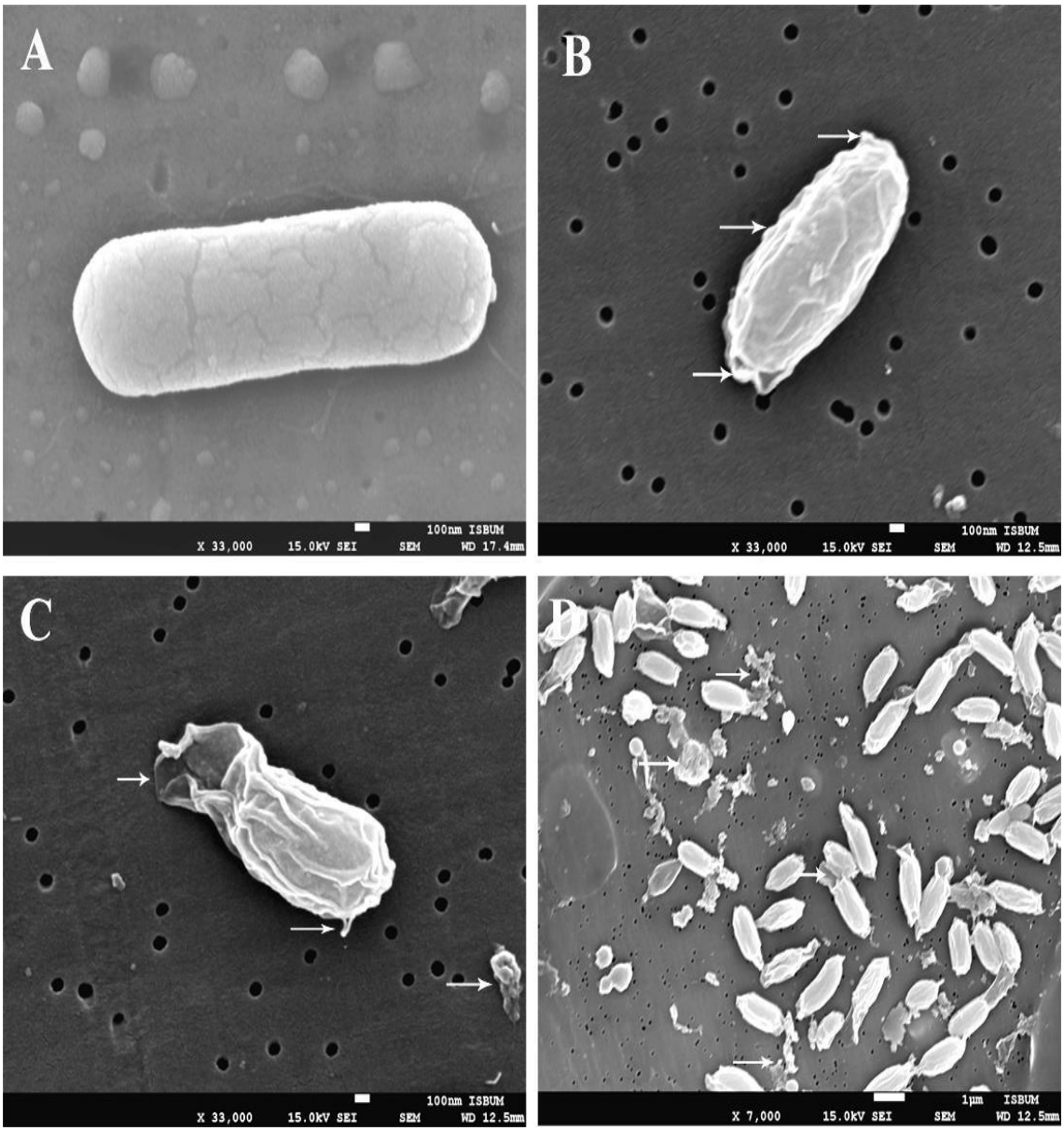



**Figure 4.39.** Effect of diterpenes extracted from the fruit of *A. squamosa* by scanning electron microscope. (A) Control: *B. cereus*. (B), (C) and (D) *B. cereus* treated with diterpenes. (B) Arrows show changes in morphology with swelling (C) Arrows show rupture and collapse of cell walls, (D) Arrows show cell lysis with cell debris.

### 4.3. Part 3: Peptides extraction

#### 4.3.1. Peptides extraction from lactic acid bacteria

##### 4.3.1.1. Isolation and identification of lactic acid bacteria

In this study, lactic acid bacteria from fermented dairy products (soy milk and cow milk) were isolated. Lactic acid bacteria were characterised as Gram positive, catalase and oxidase negative (Table 4.12). Figure 4.40 shows a comparison between PCR products of bacteria after amplification with primers (1492R: 5′-TACCTTGTTACGACTT, 27F: 5′-AGAGTTTGATCMTGGCTCAG) for the 16S rDNA gene and the control DNA ladder. After sequencing and blasting against the NCBI database, bacteria were at 1500 bp indicating the DNA sequences of *L. casei* BL 23 (Appendix 59), *L. paracasei* subsp. *paracasei* 25302 (Appendix 60) and *L. paracasei* subsp. *paracasei* 8700:2 (Appendix 61).

**Table 4.12.** Biochemical identification of lactic acid bacteria

| **Bacteria** | **Gram stain** | **Catalase test** | **Oxidase test** |
|---|---|---|---|
| *Lactobacillus casei* ATCC 11578 | + | - | - |
| *Lactobacillus casei* BL 23 | + | - | - |
| *Lactobacillus paracasei* subsp. *paracasei* 25302 | + | - | - |
| *Lactobacillus paracasei* subsp. *paracasei* 8700:2 | + | - | - |

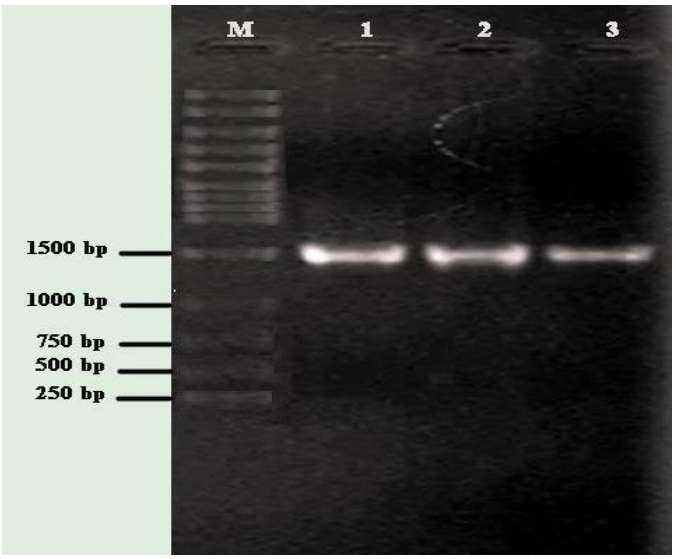



**Figure 4.40.** Agrose gel electrophoresis of PCR products of *lactobacillus* strains. Genomic DNA used was from: lane M was loaded with DNA ladder, lane 1, *Lactobacillus casei* BL 23; lane 2 *Lactobacillus paracasei* subsp. *paracasei* 25302 and lane 3 *Lactobacillus paracasei* subsp. *paracasei* 8700:2.

### 4.3.1.2. Antibacterial activity of peptide extracted from lactic acid bacteria

Peptide extracts from ammonium sulphate precipitate did not display any inhibition zones against all the test bacteria (*S. aureus, E. coli, B. cereus, P. aeruginosa* MRSA and *H. pylori*). Figures 4.41, 4.42 and 4.43 show the zones of inhibition attributed to peptide extracted from *L. casei* BL 23 by the acidic methanolic method. Zones of inhibition were 21.33 mm for *S. aureus*, 19.00 mm for *E. coli*, 22.33 mm for *B. cereus*, 19.66 mm for MRSA and 17.66 mm for *H. pylori* at a high concentration of 1.488 mg/ml of the peptide.

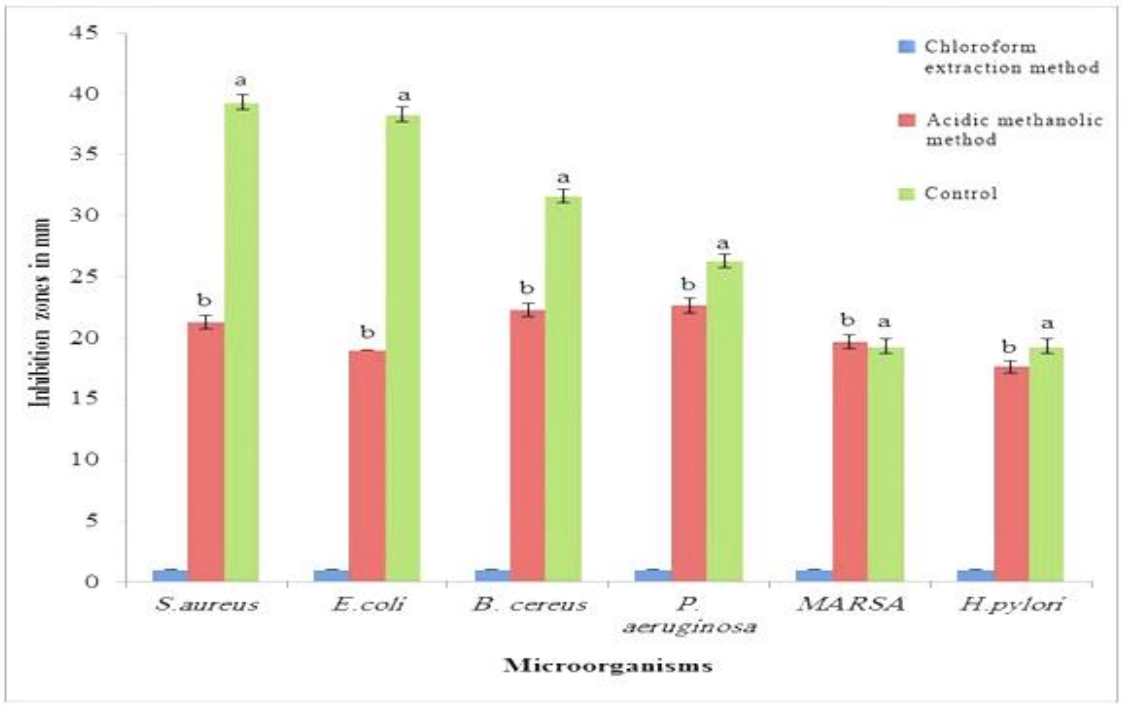
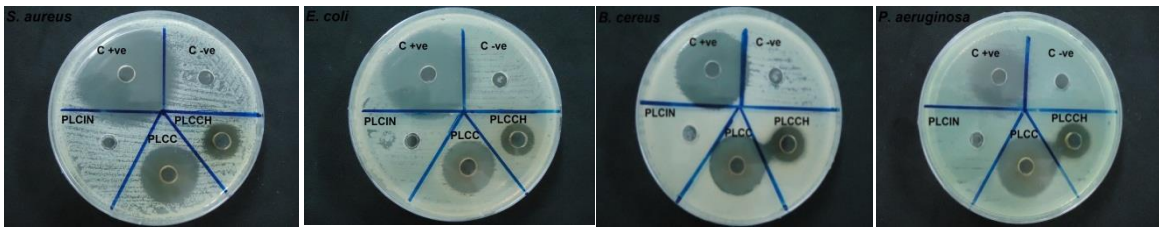
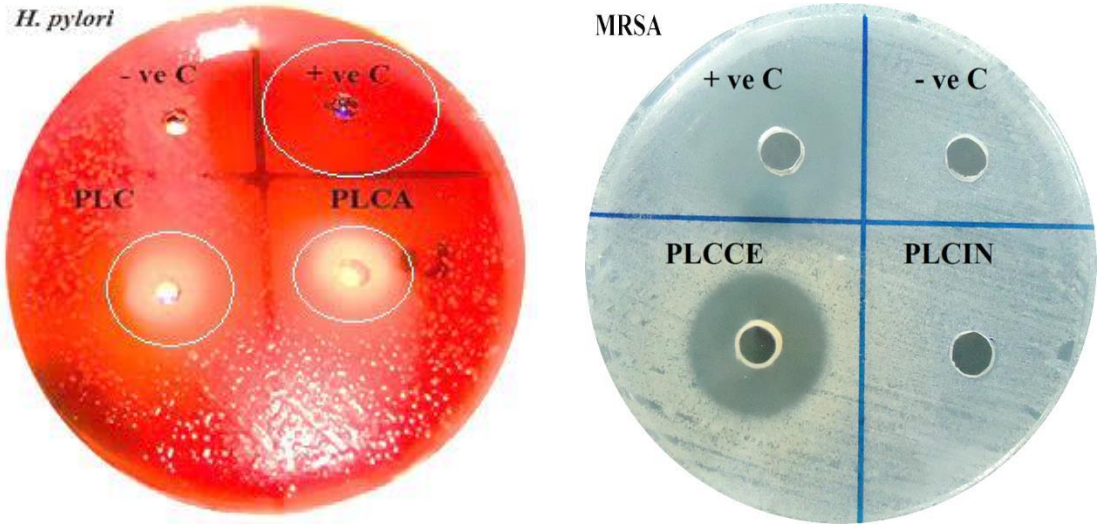



**Figure 4.41.** The inhibition zones of crude peptides extract (1.488 mg/ml) of *Lactobacillus casei* BL 23 on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). ■ Chloroform extraction method, ■ Acidic methanolic method and ■ Control. Samples represented with different letters (a, b) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All experiments were done in triplicates and values represent means ± SD.




A B C D

**Figure 4.42.** Inhibition zones of crude peptides extract of *Lactobacillus casei* BL 23 on the test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* with (clockwise from top left) positive control (10 mg/ml of tetracycline), negative control (distilled water), low concentration (0.744 mg/ml), high concentration (1.488 mg/ml) of peptide, chloroform extraction method (refer to methods section). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).




**A** **B**

**Figure 4.43.** Inhibition zones of crude peptide extract (1.488 mg/ml) of *Lactobacillus casei* BL 23 against (A) *H. pylori* and (B) MRSA. Fig. A shows (clockwise from top left) negative control (distilled water), positive control (10 mg/ml of tetracycline), peptide extract of *Lactobacillus casei* ATCC 11578 (PLCA) and peptide extract of *Lactobacillus casei* BL 23 by acidic methanolic method (PLCCE). Fig. B shows (clockwise from top left) positive control (10 mg/ml of tetracycline), negative control (distilled water), chloroform extraction method of *Lactobacillus casei* BL 23 (PLCIN) and peptide extract of *Lactobacillus casei* BL 23 by acidic methanolic method (PLCCE). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

Peptide extracted from *L. casei* ATCC 11578 by the acidic methanolic method inhibited all test bacteria. Means of inhibition zones ranged from 20.33 mm to 12.50 mm compared to the positive control (tetracycline 10 mg/ml) with the zones of inhibition varying from 39.44 mm to 19.33 mm (Figures 4.44, 4.45, 4.46 and 4.43A).

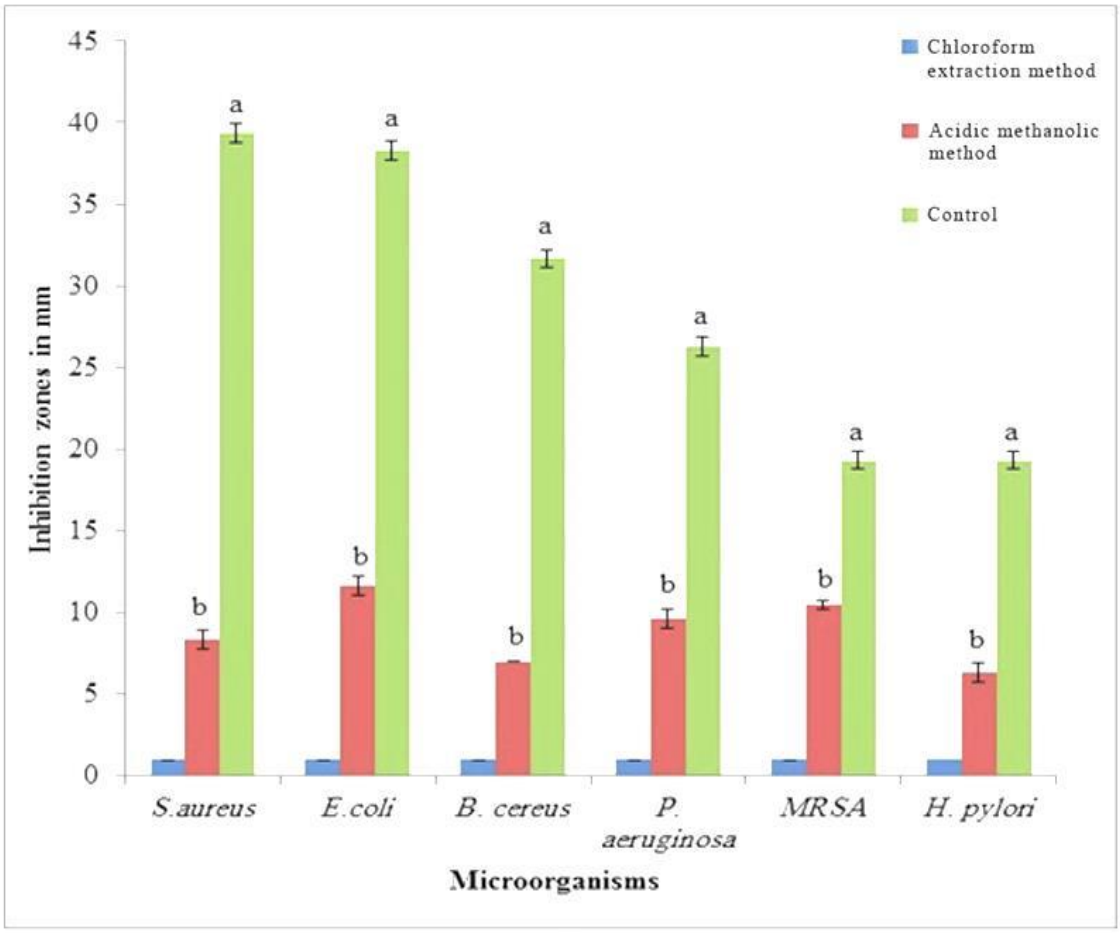
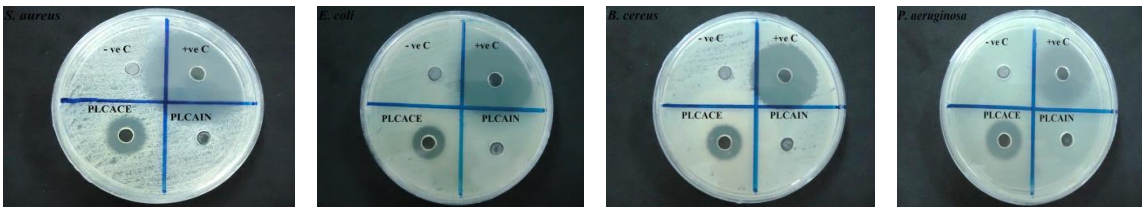



**Figure 4.44.** The inhibition zones of crude peptides extract (1.978 mg/ml) of *Lactobacillus casei* ATCC 11578 on the test microorganisms. Extracts (50 µl) were dispensed into each well (diameter 6 mm). ■ Chloroform extraction method, ■ acidic methanolic method ■ Control. Samples represented with different letters (a, b) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All experiments were done in triplicates and values represent means ± SD.




A B C D

**Figure 4.45.** Inhibition zones of crude peptide extract (1.978 mg/ml) of *Lactobacillus casei* ATCC 11578 on selected test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* with (clockwise from top left) negative control (distilled water), positive control (10 mg/ml of tetracycline), peptide extract of *Lactobacillus casei* ATCC 11578 (PLCAIN) by chloroform extraction method and peptide extract of *Lactobacillus casei* ATCC 11578 by acidic methanolic method (PLCACE). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

**MRSA**

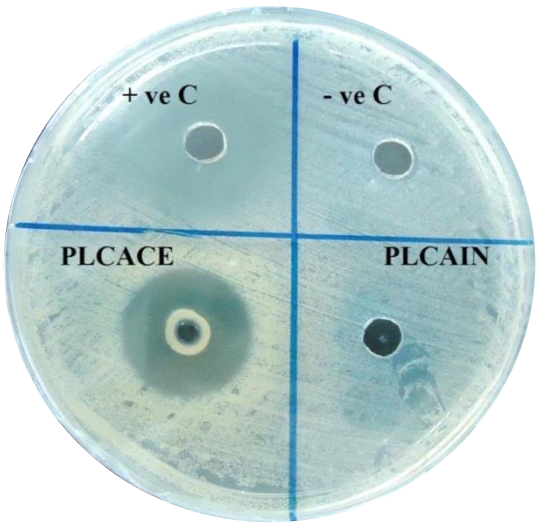



**Figure 4.46.** Inhibition zones of crude peptides extracts (1.978 mg/ml) of *Lactobacillus casei* ATCC 11578 against MRSA with (clockwise from top left) positive control (10 mg/ml of tetracycline), negative control (distilled water), peptide extract of *Lactobacillus casei* ATCC 11578 (PLCAIN) by chloroform extraction method and peptide extract of *Lactobacillus casei* ATCC 11578 by acidic methanolic method (PLCACE). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

The peptide from *L. paracasei* subsp. *paracasei* 25302 exhibited inhibition zones ranging from 11.66 mm to 7.00 mm against the test bacteria (Figures 4.47, 4.48 and 4.49).

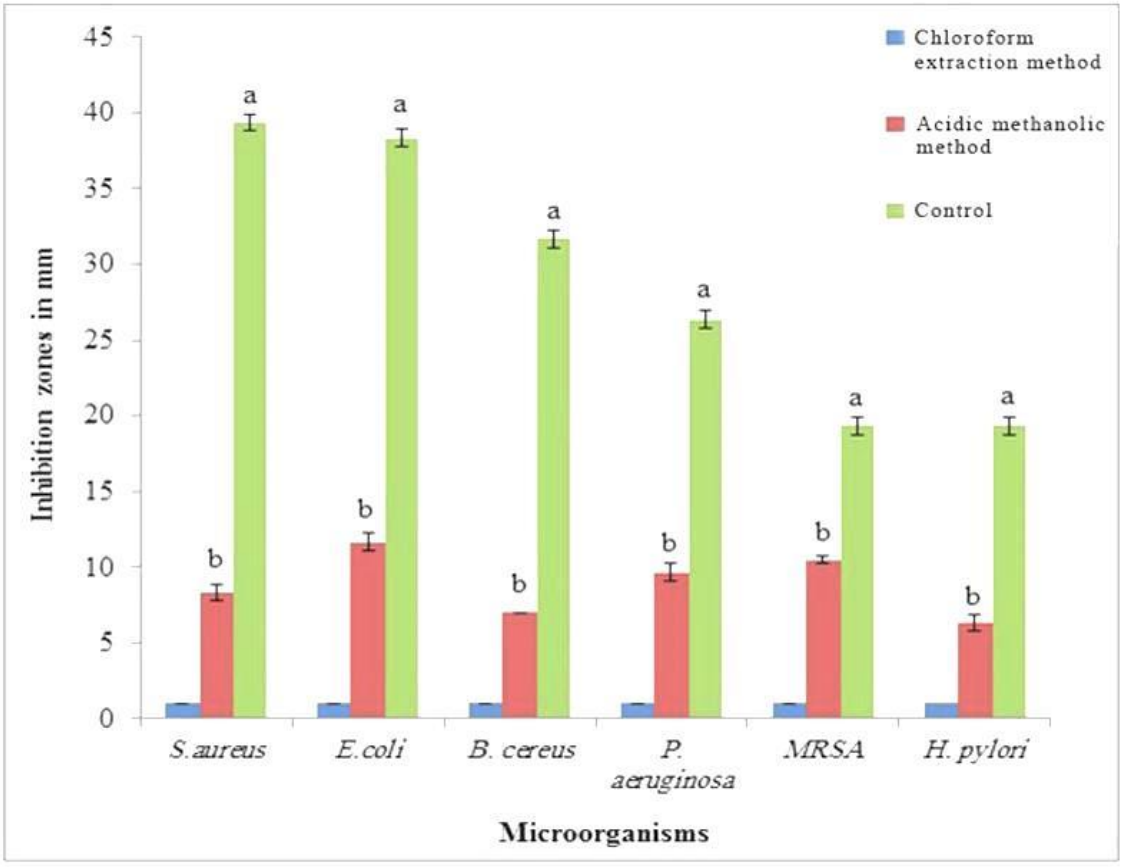
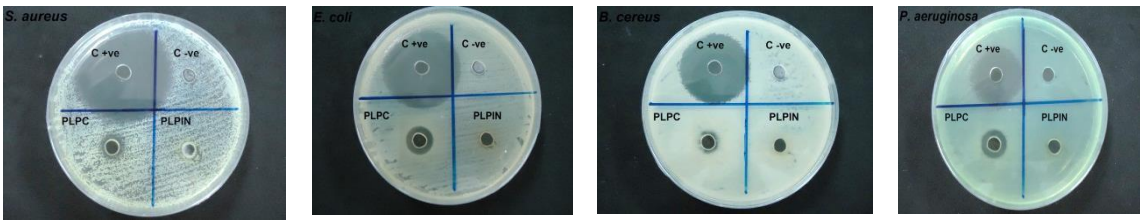
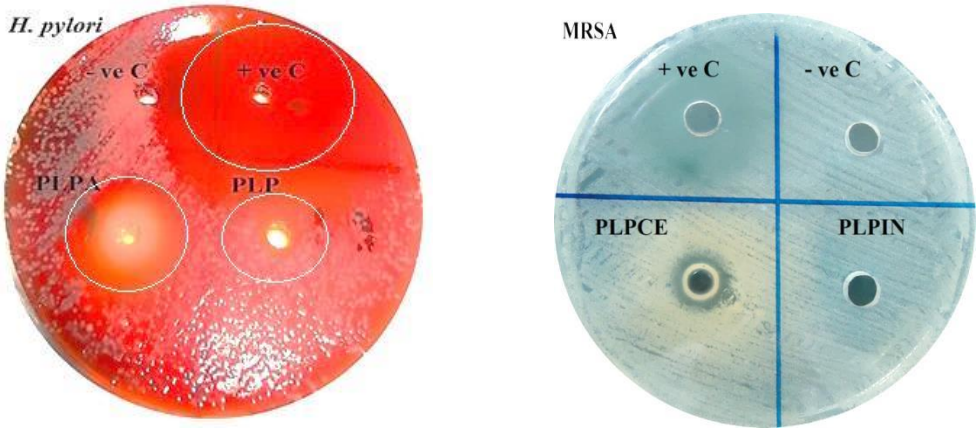



**Figure 4.47.** The inhibition zones of crude peptides extract (1.387 mg/ml) of *Lactobacillus paracasei* subsp. *paracasei* 25302 on the test microorganisms. ■ Chloroform extraction method, ■ acidic methanolic method and ■ Control. Samples represented with different letters (a, b) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All experiments were done in triplicates and values represent means ± SD.




A B C D

**Figure 4.48.** Inhibition zones of crude peptides extracts (1.387 mg/ml) of *Lactobacillus paracasei* subsp. *paracasei* 25302 on selected test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* with (clockwise from top left) positive control (10 mg/ml of tetracycline), negative control(distilled water), peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 25302 by chloroform extraction method (PLPIN) and peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 25302 by acidic methanolic method (PLPC). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).




A B

**Figure 4.49.** Inhibition zones of crude peptides extracts (1.387 mg/ml) of *Lactobacillus paracasei* subsp. *paracasei* 25302 against (A) *H. pylori* and (B) MRSA. Fig. A shows negative control (distilled water), positive control (10 mg/ml of tetracycline), peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 25302 and peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 8700:2. Fig. B shows (clockwise from top left) positive control (10 mg/ml of tetracycline), negative control (distilled water), peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 25302 by chloroform extraction (PLPIN) method and peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 25302 by acidic methanolic method (PLPCE). Peptide extracts of *Lactobacillus paracasei* subsp. *paracasei* 25302 (PLP) and peptide extracts of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 (PLPA). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

The zones of inhibition attributed to the peptide extract by acidic methanolic method of *L. paracasei* subsp. *paracasei* 8700:2 varied from 20.66 mm for *S. aureus*, 16.66 mm for *E. coli*, 19.00 mm for *B. cereus*, 21.33 mm for *P. aeruginosa* and 19.83 mm for MRSA in a high concentration of 2.502 mg/ml. For *H. pylori*, the inhibition zone was observed at 20.66 mm (Figures 4.50, 4.51, 4.52 and 4.49A).

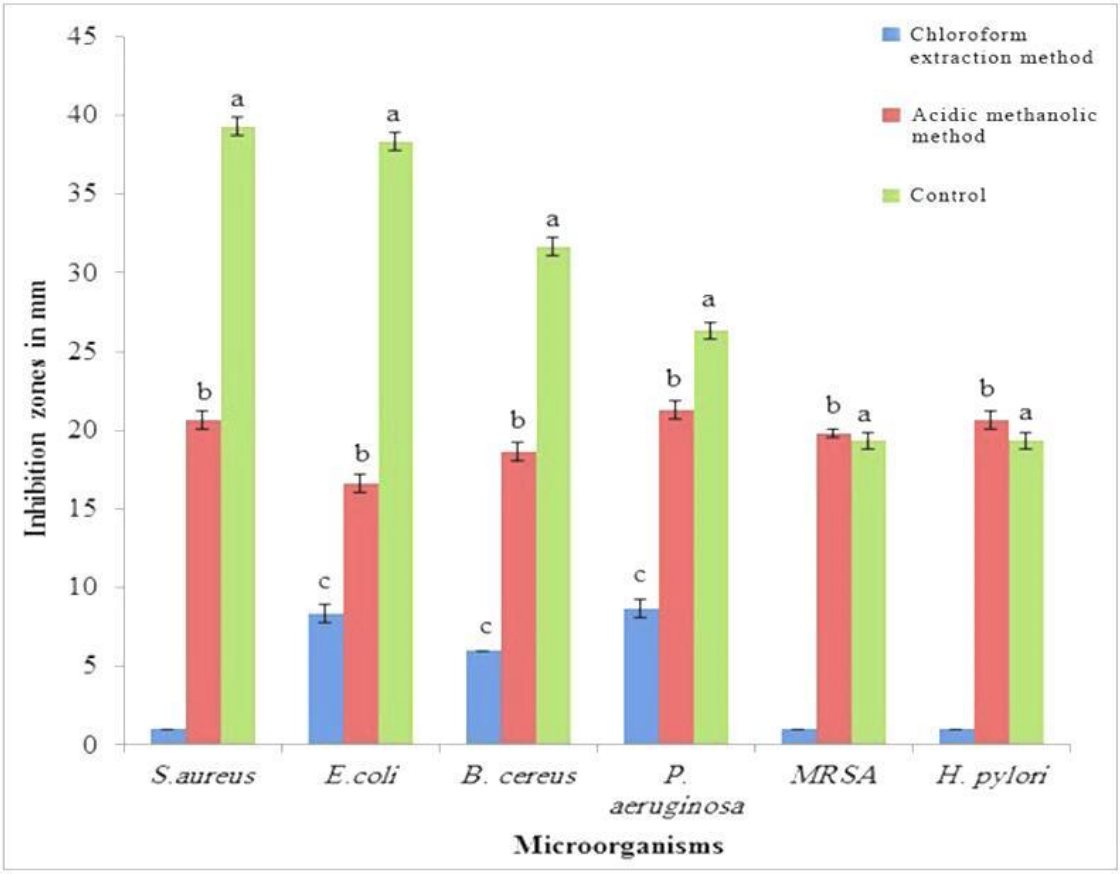
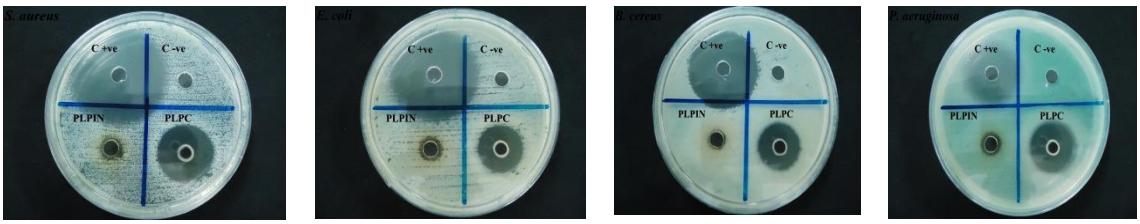



**Figure 4.50.** The inhibition zones of crude peptides extracts (2.502 mg/ml) of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 on selected test microorganisms. ■ Chloroform extraction method, ■ Acidic methanolic method and ■ Control. Samples represented with different letters (a, b) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. All experiments were done in triplicates and values represent means ± SD. Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).




A B C D

**Figure 4.51.** Inhibition zones of crude peptides extract (2.502 mg/ml) of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 on selected test microorganisms (A) *S. aureus* (B) *E. coli* (C) *B. cereus* and (D) *P. aeruginosa* with (clockwise from top left) positive control (10 mg/ml of tetracycline), negative control (distilled water), peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by acidic methanolic method (PLPC) and peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by chloroform extraction method (PLPIN). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

**MRSA**

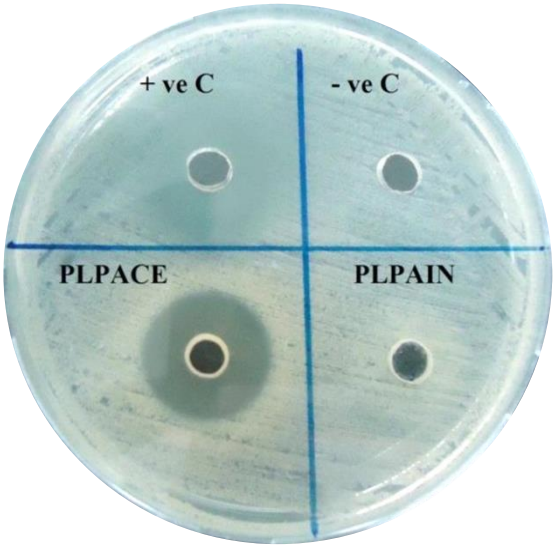



**Figure 4.52.** Inhibition zones of crude peptides extracts (2.502 mg/ml) of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 against MRSA with (clockwise from top left) positive control (10 mg/ml of tetracycline), negative control (distilled water), peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by chloroform extraction method (PLPAIN) and peptide extract of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by acidic methanolic method (PLPACE). Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

MIC and MBC values for peptide extracts of lactic acid bacteria acquired through the acidic methanolic method displayed results for all the test bacteria, but chloroform extract of lactic acid bacteria (*L. casei* ATCC 11578, *L. casei* BL 23 and *L. paracasei* subsp. *paracasei* 2530) did not show any results for MIC and MBC. However, the chloroform extraction method used for *L. paracasei* subsp. *paracasei* 8700:2 obtained MIC values at 2.091 mg/ml for *E. coli* and *P. aeruginosa*, while for *B. cereus* it was at 0.697 mg/ml (Tables 4.13 and 4.14).

**Table 4.13.** MIC of peptides extracts of lactic acid bacteria on the test microorganisms

| | **Peptide extracts (mg/ml)** | | | | |
|---|---|---|---|---|---|
| **Bacteria** | **PLCACE** | **PLCCE** | **PLPCE** | **PLPACE** | **PLPAIN** |
| ***S. aureus*** | 1.978 | 1.488 | 1.387 | >1.251 | Na |
| ***E. coli*** | >1.978 | 1.488 | 1.387 | >1.251 | 2.091 |
| ***B. cereus*** | < 0.989 | 0.496 | 1.387 | > 0.417 | 0.697 |
| ***P. aeruginosa*** | 1.978 | >1.488 | 1.387 | < 2.502 | 2.091 |
| **MRSA** | 1.978 | > 1.488 | 1.387 | 1.251 | Na |
| ***H. pylori*** | > 1.987 | 1.488 | 1.387 | 1.251 | Na |

(**PLCACE**) peptide extracted by acidic methanolic method of *Lactobacillus casei* ATCC 11578, (**PLCCE)** of *Lactobacillus casei* BL 23, (**PLPCE**) of *Lactobacillus paracasei* subsp. *paracasei* 25302, **(PLPACE**) of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 **(PLPAIN**) Peptide extract of chloroform extraction method of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 and **Na**-non active at high concentration (2.502 mg/ml).

**Table 4.14.** MBC of peptides extracts of lactic acid bacteria on the test microorganisms

| Bacteria | Peptide extracts (mg/ml) | | | | |
|---|---|---|---|---|---|
| | **PLCACE** | **PLCCE** | **PLPCE** | **PLPACE** | **PLPAIN** |
| ***S. aureus*** | 1.978 | 1.488 | >1.387 | 1.251 | Na |
| ***E. coli*** | 0.978 | 1.488 | >1.387 | 1.251 | >2.091 |
| ***B. cereus*** | 0.989 | 0.496 | >1.387 | 0.417 | <0.697 |
| ***P. aeruginosa*** | >1.978 | 1.488 | >1.387 | 2.502 | >2.091 |
| **MRSA** | >1.978 | < 1.488 | 1.387 | 1.251 | Na |
| ***H. pylori*** | 1.987 | 1.488 | 1.387 | 1.251 | Na |

(**PLCACE**) peptide extracted by acidic methanolic method *Lactobacillus casei* ATCC 11578, (**PLCCE**) of *Lactobacillus casei* BL 23, (**PLPCE**) of *Lactobacillus paracasei* subsp. *paracasei* 25302, (**PLPACE**) of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 and (**PLPAIN)** Peptide extract of chloroform extraction method of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 and Na-non active at high concentration (2.502 mg/ml).

Since this extract gave significant inhibition against all bacteria attempt was made to purify the peptide. The chloroform extracts of *L. casei* ATCC 11578, *L. casei* BL 23 and *L. paracasei* subsp. *paracasei* 25302 did not exhibit inhibition against all test bacteria. However with, the chloroform extracts of *L. paracasei* subsp. *paracasei* 8700:2 displayed inhibitions against the test bacteria except MRSA and *H. pylori*. Figure 4.53 shows the inhibition zone of fraction 2 of purified peptide obtained from *L. paracasei* subsp. *paracasei* 8700:2 through gel filtration against *B. cereus*.

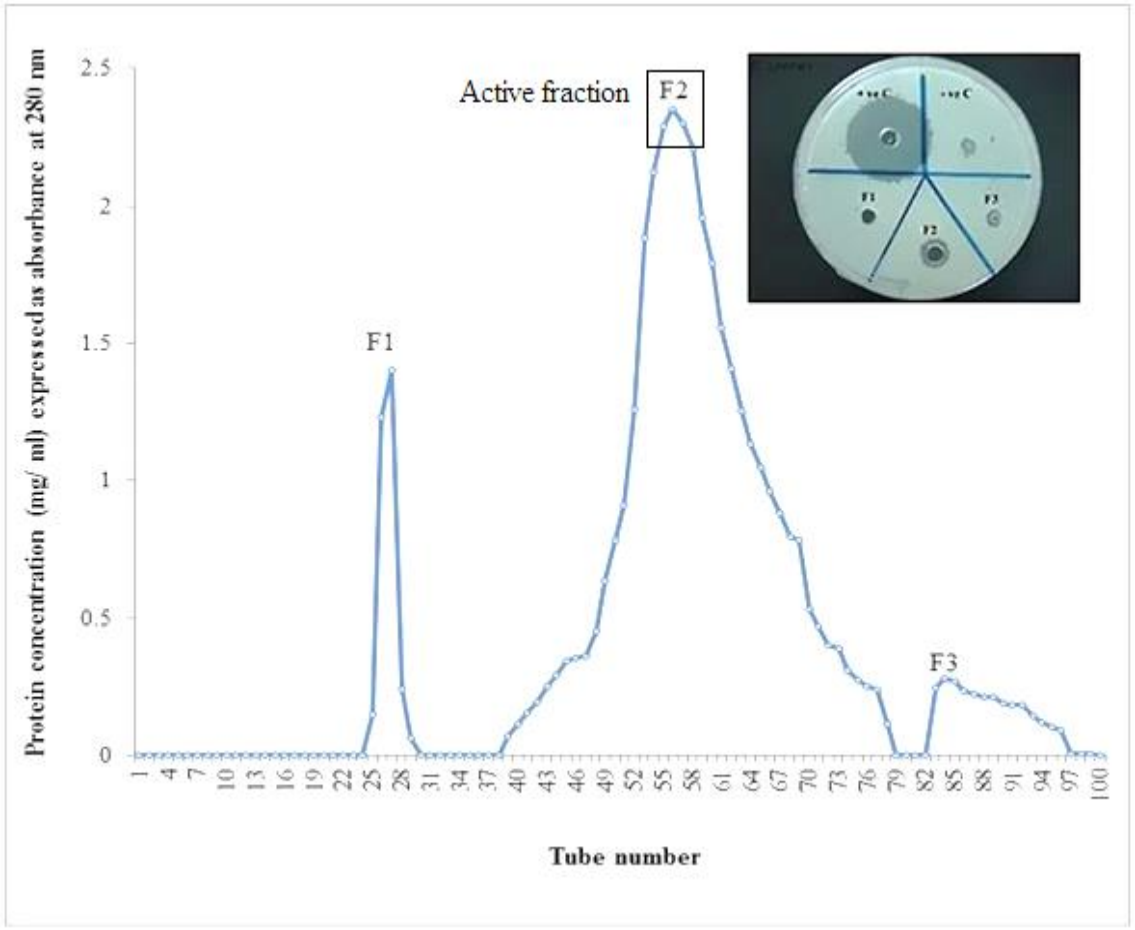



**Figure 4.53.** Fractionations of peptides extracted from *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by Sephadex G-25 gel filtration. The zone of inhibition of fraction 2 (F2) was at 12 mm.

The bound purified fraction (F3) from *L. paracasei* subsp. *paracasei* 8700:2 by FPLC shows inhibition zones against *B. cereus* compared to positive control (nisin 50 mg/ml) (Figure 4.54).

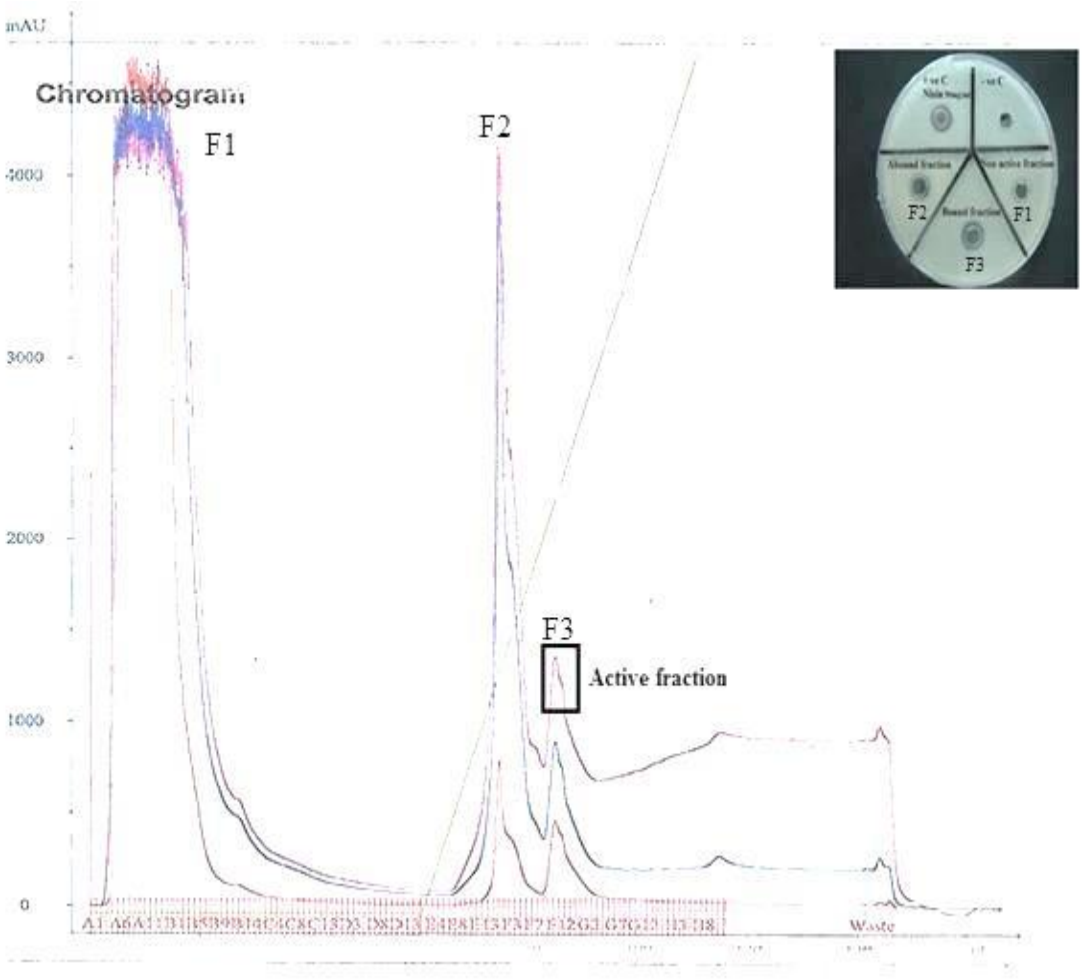



**Figure 4.54.** Fractionation of peptides extracted from *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by FPLC. The zone of inhibition of F3 was 12 mm. (—) shows absorbance at 280 nm, ( —) absorbance at 214 nm ( —) NaCl concentration and ( — ) absorbance at 207 nm.

Figure 4.55 shows the LC chromatograms of the different retention time of peptide purified from *L. paracasei* subsp. *paracasei* 8700:2 by FPLC. The peak at retention time 6.6 min an $[M + H]^+$ at *m/z* 1124.6143 as novel bacteriocin-like peptide exhibiting was identified. The results of the IR spectra (Figure 4.56) exhibited strong absorption bands of 3273.46 $cm^{-1}$ for peptide extracted from *L. paracasei* subsp. *paracasei* 8700:2 to identify O-H stretching. C-H stretching groups were detected at the bands 2929.90 $cm^{-1}$. For the C=N group it was detected at the band 1583.75 $cm^{-1}$ and for the C-C group it was detected at the band 1452.91 $cm^{-1}$.

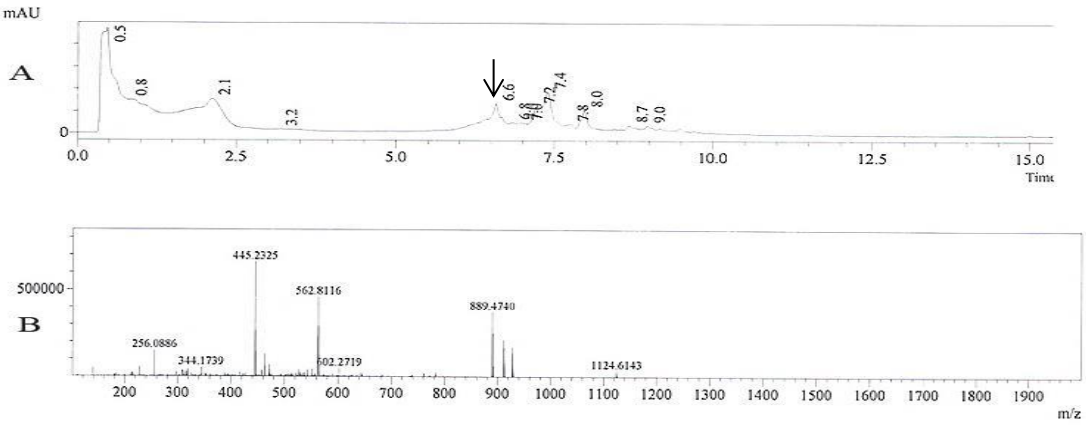



**Figure 4.55**. (A) LC chromatograms of the peptide purified fraction from *L. paracasei* subsp. *paracasei* 8700:2 by FPLC (B) MS/MS spectrum of the active peptide at $[M + H]^+$ at *m/z* 1124.6143. (⟶) shows the retention time of the active peptide at 6.6 min.

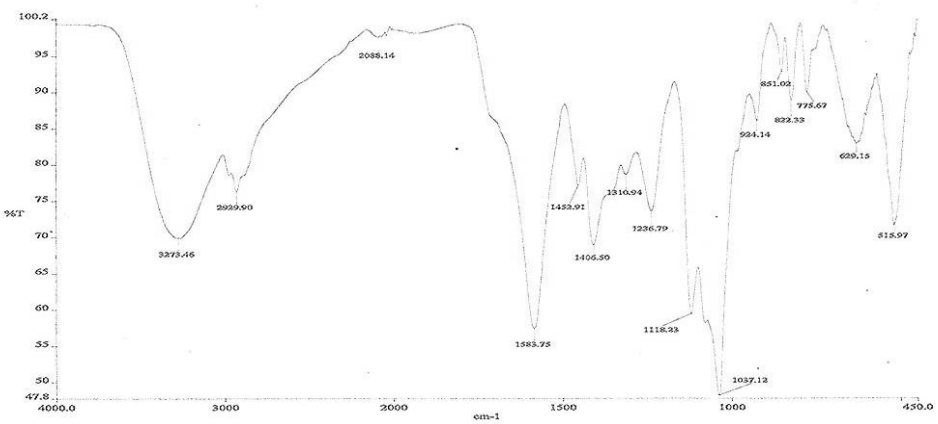



**Figure 4.56.** IR spectra of active peptide extracted from *L. paracasei* subsp. *paracasei* 8700:2.

### 4.3.1.3. Antioxidant activity of peptide extracted from lactic acid bacteria

Figure 4.57 shows the DPPH radical scavenging activity of peptides extracted by different methods. The peptide extracted from *L. casei* ATCC 11578 using the acidic methanolic method showed DPPH scavenging activity at 81.07% ($IC_{50}$ 6.17 mg/ml) while peptide extracted from *L. casei* BL 23 showed 83.10 % ($IC_{50}$ 6.21 mg/ml) in a DPPH assay. Peptide extracts of *L. paracasei* subsp. *paracasei* 25302 showed 86.43% ($IC_{50}$ 5.78 mg/ml) DPPH value while *L. paracasei* subsp. *paracasei* 8700:2 exhibited a value of 75.70% ($IC_{50}$ 6.60 mg/ml). With the exception of *L. paracasei* subsp. *paracasei* 8700:2, the peptides extracted by chloroform precipitation showed a high level of DPPH activity at 88.28% ($IC_{50}$ 5.66 mg/ml).

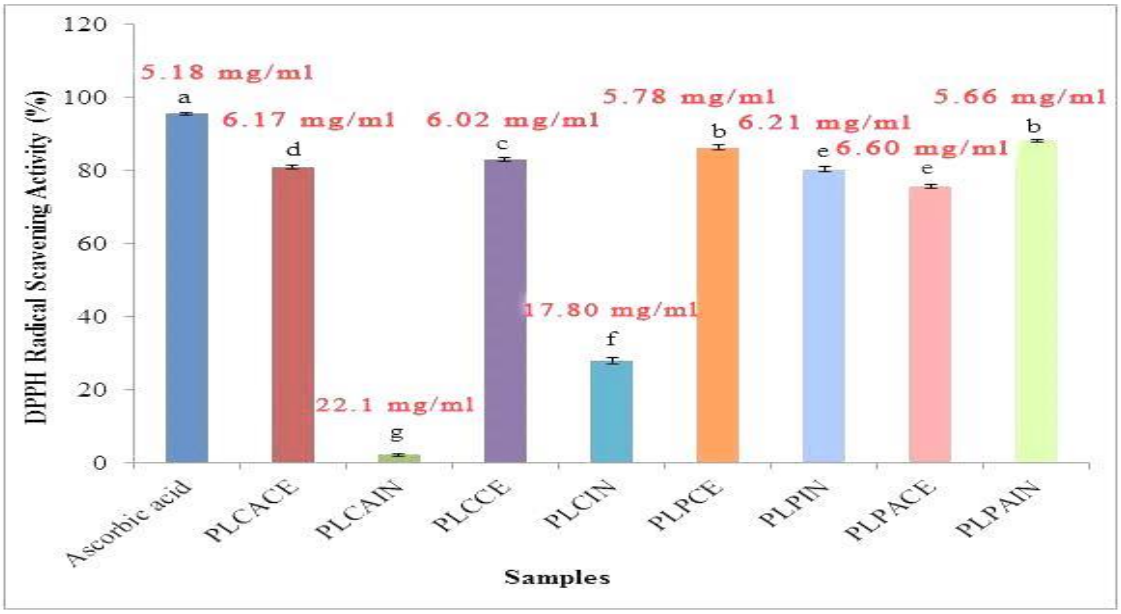



**Figure 4.57.** DPPH scavenging activity $IC_{50}$ values (in red) of peptides extracts of lactic acid bacteria. The values are the average of three replicate ± SD. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test Samples represented with different letters (a, b, c, d, e, f and g) are significantly different ($p < 0.05$) from each other and control. Positive control indicates to 10 mg/ml of ascorbic acid. (**PLCACE**) peptides extracts from acidic methanolic method of *Lactobacillus casei* ATCC 11578, (**PLCCE**) of *Lactobacillus casei* BL 23, (**PLPCE**) of *Lactobacillus paracasei* subsp. *paracasei* 25302, (**PLPACE**) of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 and (**PLCAIN-PLCIN-PLPIN-PLPAIN**) peptides extracts of chloroform extraction method of all bacteria.

The of SOD-like activity for peptides extracted by the acidic methanolic method on all strains of lactic acid bacteria presented good antioxidant results while chloroform extraction method exhibited a high level of antioxidant activity of 88.55% for all strains except *L. paracasei* subsp. *paracasei* 8700:2 (Figure 4.58).

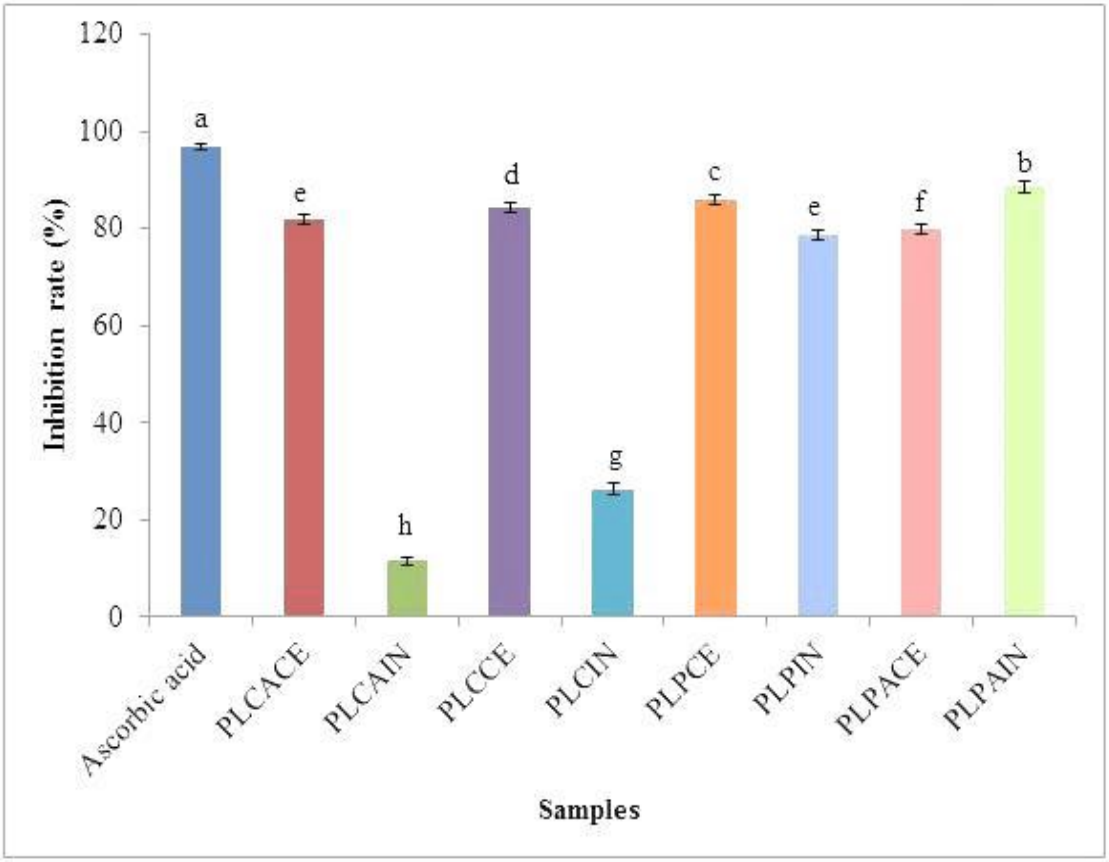



**Figure 4.58.** The rate Inhibition of SOD-like activities of peptides extracts of lactic acid bacteria. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b, c, d, e, f, g and h) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ ml of ascorbic acid. (**PLCACE**) peptides extracts from cells of *Lactobacillus casei* ATCC 11578, (**PLCCE**) of *Lactobacillus casei* BL 23, (**PLPCE**) of *Lactobacillus paracasei* subsp. *paracasei* 25302, (**PLPACE**) of *Lactobacillus paracasei* subsp. *paracasei* 8700:2 and (**PLCAIN-PLCIN-PLPIN-PLPAIN**) peptides extracts of chloroform extraction method of all bacteria.

### 4.3.1.4. Assessment of the lytic effect of peptide extracts from lactic acid bacteria with the utilization of a scanning electron microscope

Figures 4.59, 4.60 and Appendices 62, 63 show the effect of peptide from *L. paracasei* subsp. *paracasei* 8700:2 against *B. cereus* and MRSA. It was noted that test bacteria treated with antibacterial peptide (fraction 2) displayed significant alterations in cell morphology and shape. These alterations include roughening of the cell surface, the formation of numerous blebs and lysis accompanied with an accumulation of cell debris.

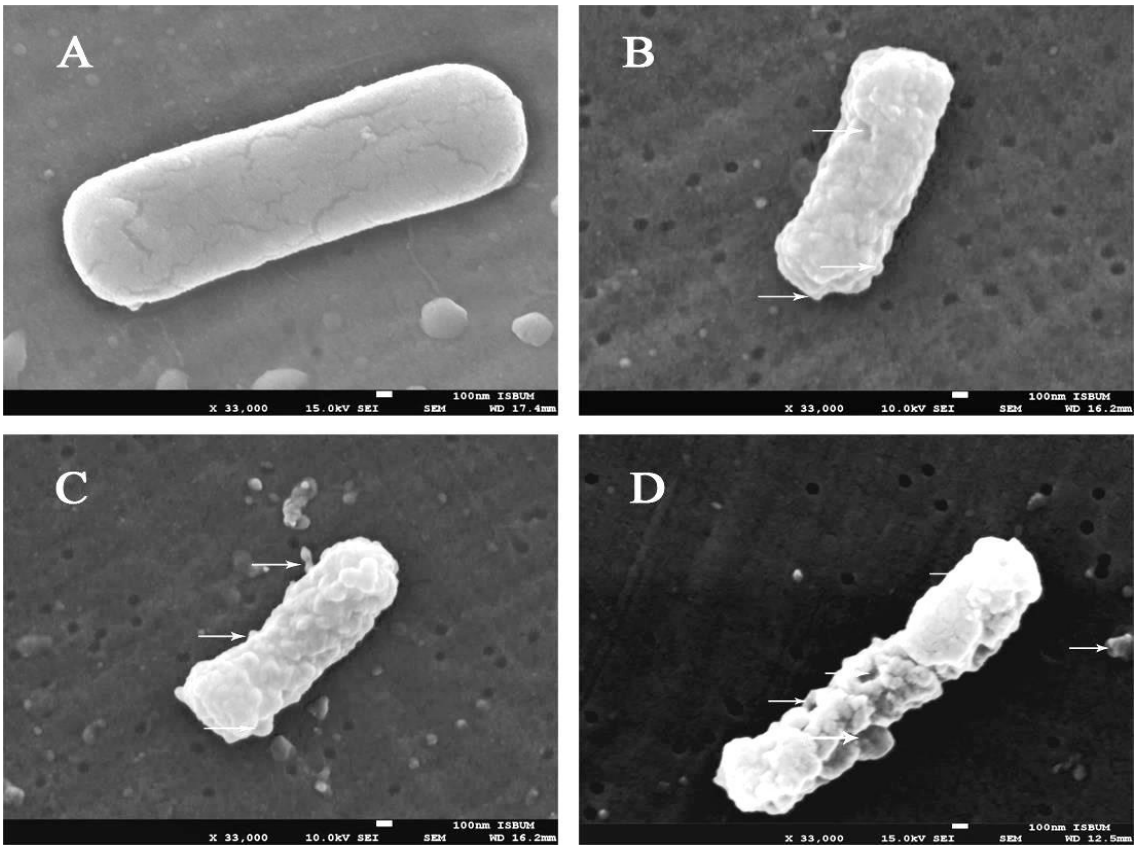



**Figure 4.59.** Effect of active peptide extracted (50 µg/ml) from *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by scanning electron microscope. (A) Control: *B. cereus*. (B), (C) and (D) *B. cereus* treated with peptide. (B) Arrows show significant alterations in cell morphology and shape include roughening of the cell surface (C) Arrows show the formation of numerous blebs, (D) Arrows show lysis accompanied with an accumulation of cell debris.

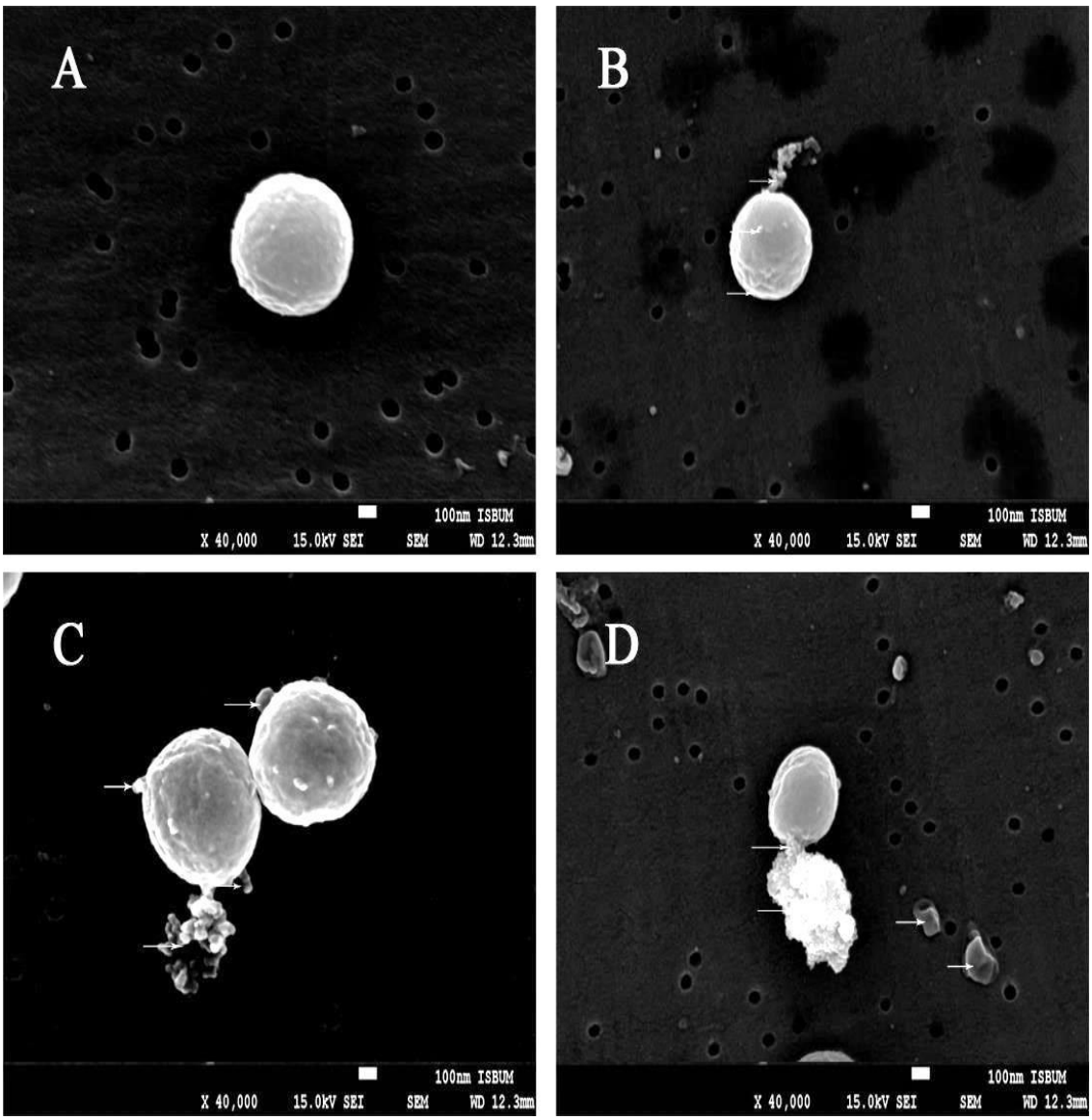



**Figure 4.60.** Effect of active peptide extracted (50 µg/ml) from *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by scanning electron microscope. (A) Control: MRSA. (B), (C) and (D) MRSA treated with peptide. (B) Arrows show significant alterations in cell morphology and shape include roughening of the cell surface (C) Arrows show the formation of numerous blebs, (D) Arrows show lysis accompanied with an accumulation of cell debris.

The effect of active peptide from *L. paracasei* subsp. *paracasei* 8700:2 against *H. pylori* observed with the use of a SEM is displayed in Figure 4.61. The morphological changes noted in bacterial cells treated with peptide extracted from *L. paracasei* subsp. *paracasei* 8700:2 include the formation of blebs and pores.

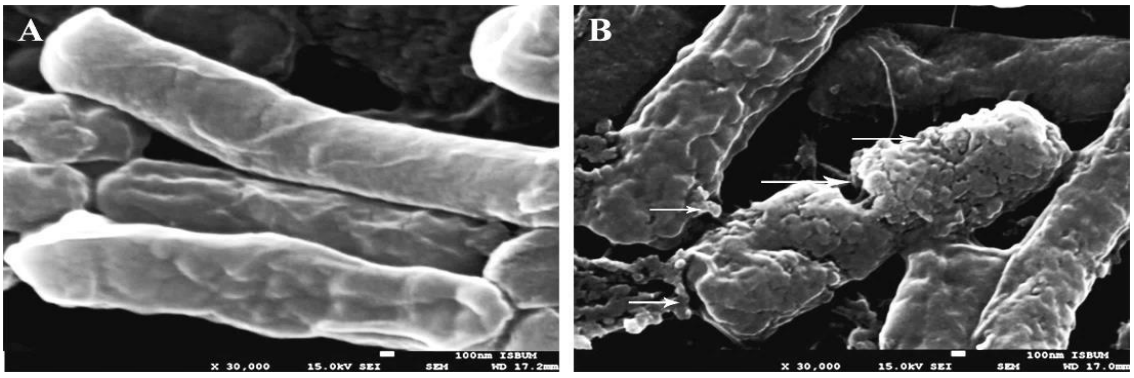



**Figure 4.61.** Effect of active peptide extracted (50 µg/ml) from *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by scanning electron microscope. (A) Control: *H. pylori*. (B) *H. pylori* treated with peptide (B) Arrows show the formation of blebs and pores.

Figure 4.62 shows the morphological changes in cells treated with this active peptide observed through transmission electron microscopy. These changes included leakage of cellular contents, damage to outer membrane attributed to pore formation, membrane sloughing and leakage of cytoplasm.

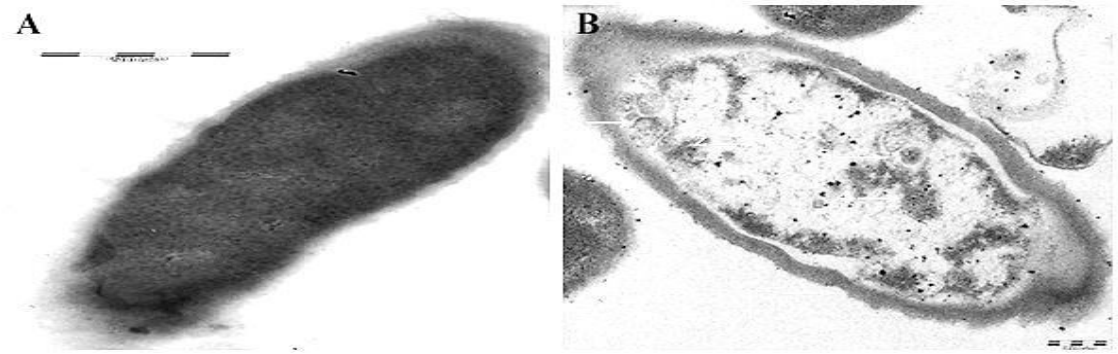



**Figure 4.62.** Effect of active peptide extracted (50 µg/ml) from *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by transmission electron microscope. (A) Control: *H. pylori*. (B) *H. pylori* treated with peptide. (A) Arrows show intact bacteria while (B) Arrows show (1, 2) leakage of cellular contents, arrows show (3,4) leakage of cytoplasm and arrows show (5) pore formation, membrane sloughing.

### 4.3.2. Peptide extracted from medicinal plants

#### 4.3.2.1. Antibacterial activity of peptide extracted from medicinal plants

In a well diffusion assay, peptide extracts from selected plants (*M. citrifolia*, *A. squamosa*, and Australian plant mixture) showed inhibition zones against selected test microorganisms (*S. aureus*, *E. coli*, *B cereus*, *P. aeruginosa*, MRSA and *H. pylori* (Figures 4.63, 4.64 and 4.65). Peptides from *M. citrifolia* leaves (PML) and peptide of APM (PAUS) showed inhibition zones against most test bacteria with the exceptions of *P. aeruginosa* and *H. pylori*. The peptides from *M. citrifolia* seeds (PMS) showed inhibition zones against bacterial strains except for *H. pylori*. However, the peptides from *M. citrifolia* fruit (PMF) showed antibacterial activity against bacterial strains with the exception of *E. coli* and *H. pylori*. Peptides from *A. squamosa* fruit (PAF) and seeds displayed antibacterial activity against *P. aeruginosa*, *S. aureus*, MRSA and *B. cereus* but not against *H. pylori* and *E. coli*.

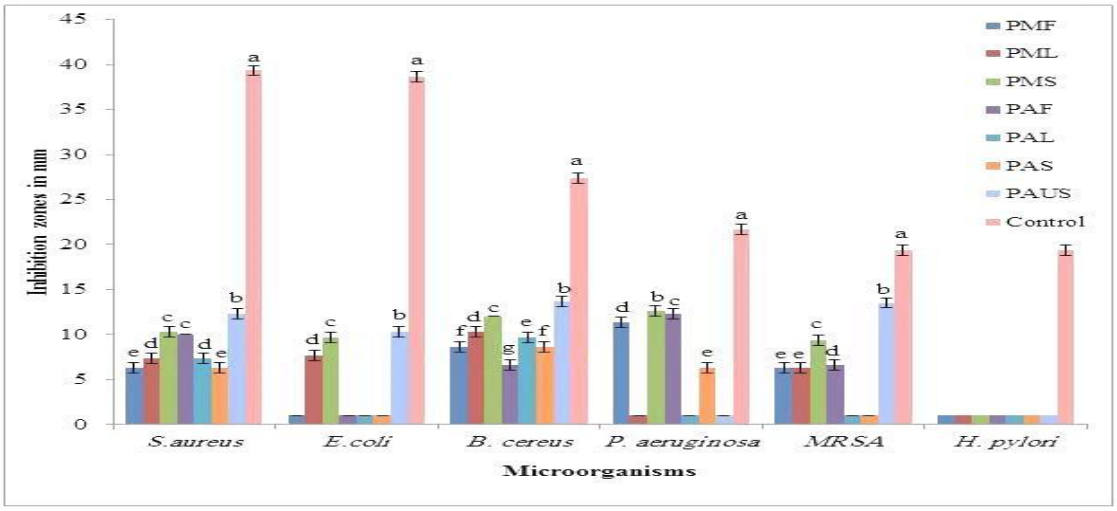



**Figure 4.63.** Inhibition zones of crude peptides extract of selected plants on the test microorganisms. Samples represented with different letters (a, b, c, d, e and f) are significantly different ($p < 0.05$) from each other and control (10 mg/ml of tetracycline). Results were analysed by using one-way ANOVA followed by Duncan's multiple comparison test. ■ **PMF**- peptides of *M. citrifolia* fruit, ■ **PML**-, peptides of *M. citrifolia* leaves , ■ **PMS**- peptides of *M. citrifolia* seeds , ■ **PAF**- peptides of *A. squamosa* fruit , ■ **PAL** – peptides of *A. squamosa* leaves, ■ **PAS** peptides of *A. squamosa* seeds, ■ **PAUS**- peptides of Australian plant mixture and ■ Control. All experiments were done in triplicates and values represent means ± SD. Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

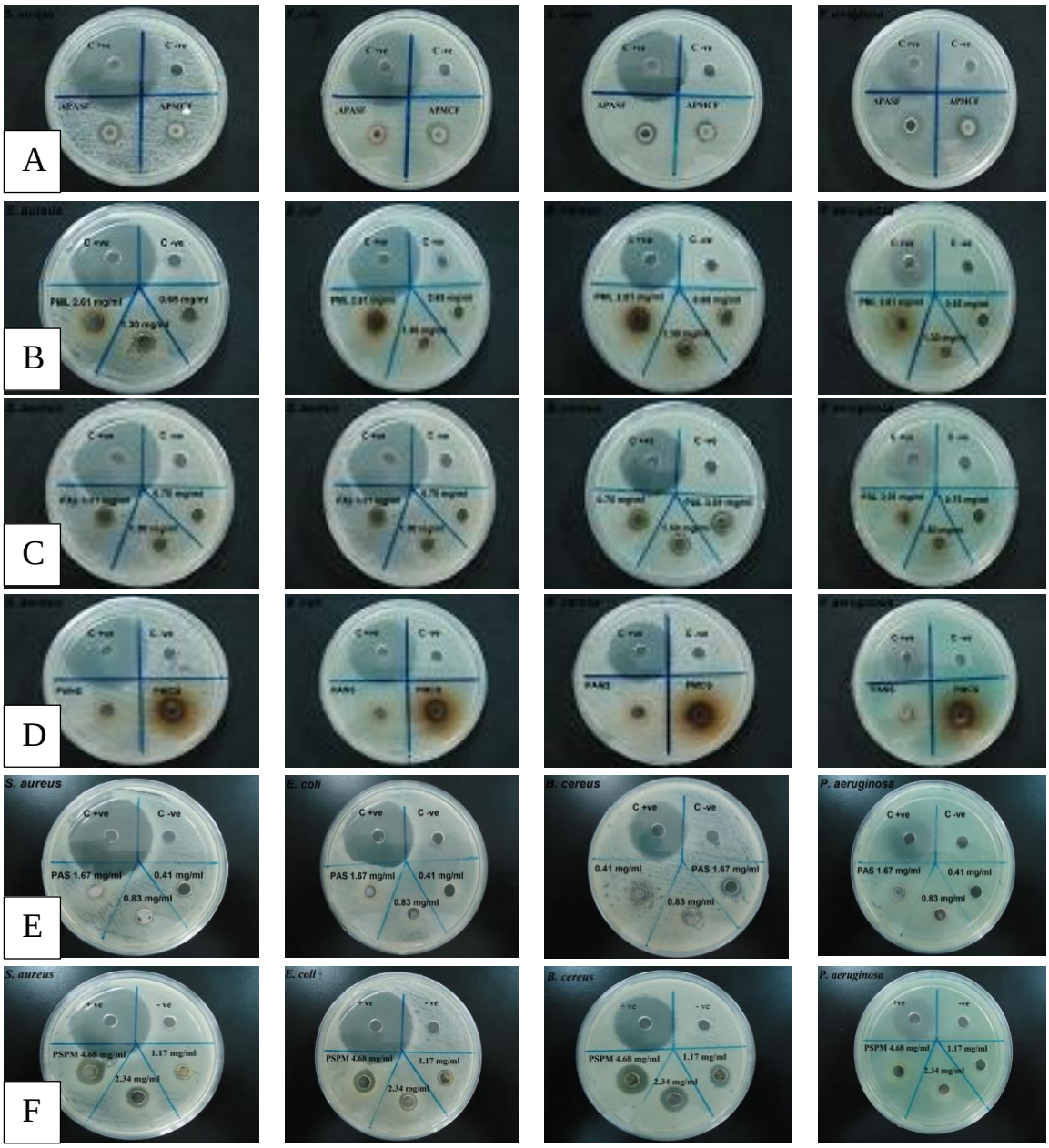



**Figure 4.64.** Effect of (A) peptide (PMF) extracts of *M. citrifolia* and *A. squamosa* fruits, (B) peptide (PML) extracts of *M. citrifolia* leaves, (C) peptide (PAL) extracts of *A. squamosa* leaves (D) peptide (PMS) extracts of *M. citrifolia* seeds, (E) peptide (PAS) extracts of *A. squamosa* seeds and (F) peptide (PAUS) of APM against microorganisms compared to positive control (10 mg/ml of tetracycline) and negative control (distilled water). Each plate shows (clockwise from top left) positive control, negative control and peptide extracts from plants. Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

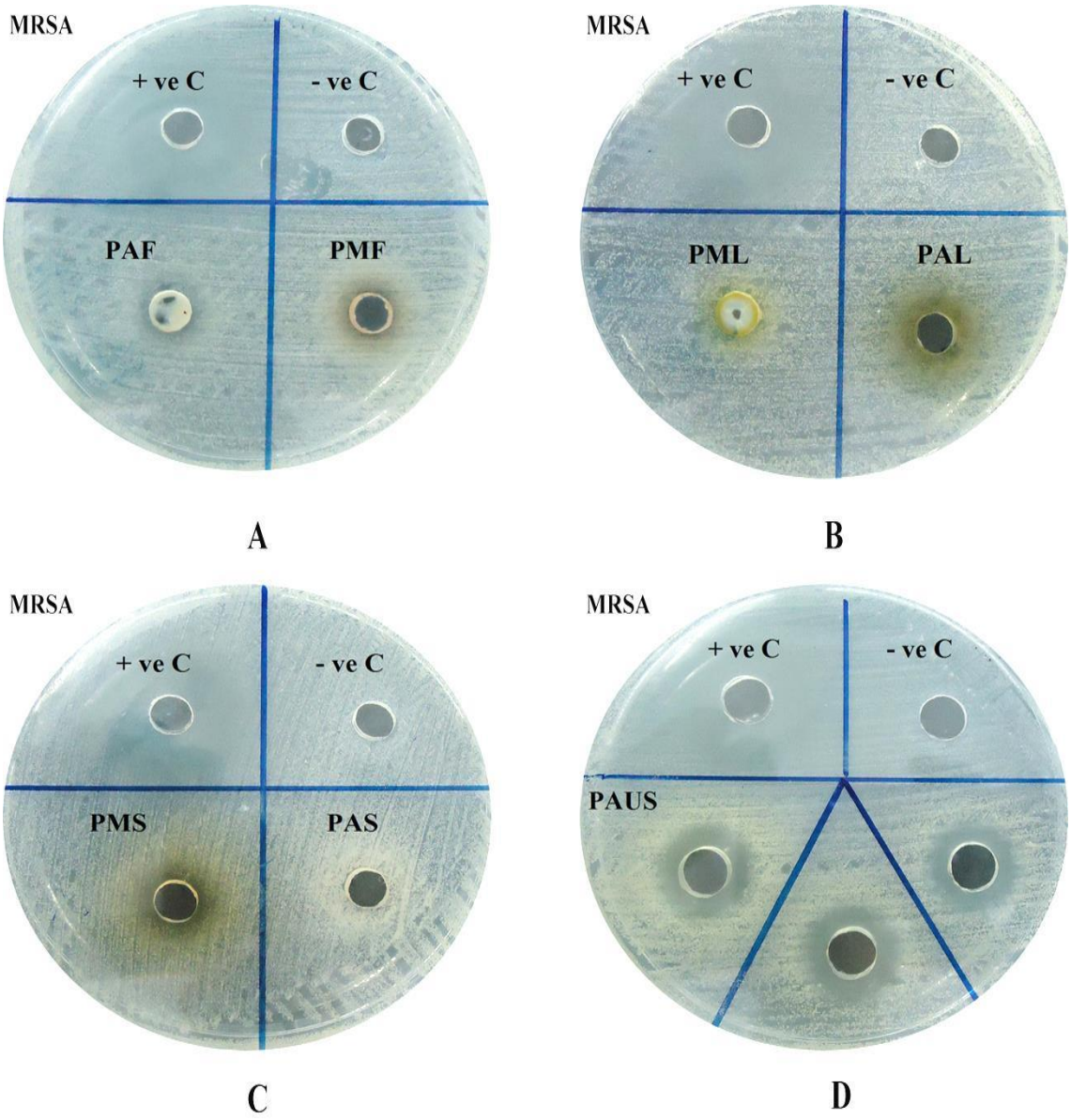



**Figure 4.65.** Effect of (A) peptide extracts of *M. citrifolia* (PMF) and *A. squamosa* fruits (PAF), (B) peptide extracts of *M. citrifolia* leaves (PML) and *A. squamosa* leaves (PAL) (C) peptide extracts of *M. citrifolia* seeds (PMS) and *A. squamosa* seeds (PAS) (D) peptide of APM (PAUS) against MARSA compared to positive control (10 mg/ml of tetracycline) and negative control (distilled water).. Each plate shows (clockwise from top left) positive control, negative control and peptide extracts from plants. Extracts, positive control and negative control (50 µl) were dispensed into each well (diameter 6 mm).

MIC and MBC values for peptide extracts from *M. citrifolia* fruit and seeds as well as *A. squamosa* fruit showed inhibition against all the test bacteria while no result was detected for *H. pylori*. The peptide from *M. citrifolia* leaves showed MIC/MBC against most of the test bacteria but not *P. aeruginosa* and *H. pylori*, while peptide from *A. squamosa* leaves exhibited MIC/MBC at 1.70 mg/ml against *S. aureus* and 0.85 mg/ml against *E. coli*. Peptide extracted from *A. squamosa* seeds at a concentration of 1.67 mg/ml displayed inhibition against *S. aureus* and *B. cereus* (Tables 4.15 and 4.16). MIC and MBC values for crude peptide extract of the mixture were noted as 1.17 mg/ml for *S. aureus*, MRSA and *E. coli*, while *B. cereus* was inhibited at a lower concentration of 0.58 mg/ml (Tables 4.15 and 4.16).

**Table 4.15.** MIC of peptides extracts of selected plants against the test microorganisms

| | **Peptides extracts (mg/ml)** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bacteria** | **PMF** | **PML** | **PMS** | **PAF** | **PAL** | **PAS** | **PAUS** |
| ***S. aureus*** | 1.275 | 1.30 | 2.02 | 3.13 | 1.70 | 1.67 | 1.17 |
| ***E. coli*** | Na | 1.30 | 2.02 | Na | Na | Na | 1.17 |
| ***B. cereus*** | 0.63 | 0.65 | 1.01 | 0.78 | 0.85 | 0.83 | 0.58 |
| ***P. aeruginosa*** | >2.55 | Na | >2.02 | 3.13 | Na | 1.67 | Na |
| **MRSA** | 1.275 | 1.30 | 2.02 | 3.13 | 1.70 | 0.00 | >1.17 |
| ***H. pylori*** | Na | Na | Na | Na | Na | Na | Na |

**PMF**- peptides of *M. citrifolia* fruit, **PML**- peptides of *M. citrifolia* leaves, **PMS**- peptides of *M. citrifolia* seeds, **PAF**- peptides of *A. squamosa* fruit, **PAL** – peptides of *A. squamosa* leaves, **PAS**- peptides of *A. squamosa* seeds **PAUS**-peptide of APM and **Na**- Non active at high concentration (2.55 mg/ml).

**Table 4.16.** MBC of peptides extracts of selected plants against the test microorganisms

| | Peptides extracts (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bacteria** | **PMF** | **PML** | **PMS** | **PAF** | **PAL** | **PAS** | **PAUS** |
| ***S. aureus*** | 1.275 | 1.30 | 2.02 | 3.13 | 1.70 | 1.67 | 1.17 |
| ***E. coli*** | Na | >1.30 | >2.02 | 3.13 | Na | Na | 1.17 |
| ***B. cereus*** | >0.63 | 0.65 | 1.01 | 0.78 | 0.85 | <0.83 | < 0.58 |
| ***P. aeruginosa*** | 2.55 | Na | 2.02 | 3.13 | Na | 1.67 | 0.00 |
| **MRSA** | 1.275 | 1.30 | 2.02 | 3.13 | >1.70 | Na | >1.17 |
| ***H. pylori*** | Na | Na | Na | Na | Na | Na | Na |

**PMF**- peptides of *M. citrifolia* fruit, **PML**- peptides of *M. citrifolia* leaves, **PMS**- peptides of *M. citrifolia* seeds, **PAF**- peptides of *A. squamosa* fruit, **PAL** – peptides of *A. squamosa* leaves, **PAS**- peptides of *A. squamosa* seeds **PAUS**-peptide of APM and **Na**- Non active at high concentration (2.55 mg/ml).

### 4.3.2.2. Antioxidant activity of peptide extracted from selected plants

Figure 4.66 presents the DPPH radical scavenging activity of the peptide extracted from *M. citrifolia* fruit as 26.45% ($IC_{50}$ 18.90 mg/ml) , from *M. citrifolia* leaves as 13.92% ($IC_{50}$ 35.91 mg/ml), from *M. citrifolia* seeds as 88.29% ($IC_{50}$ 5.67 mg/ml), from *A. squamosa* fruit as 80.36% ($IC_{50}$ 6.23 mg/ml), from *A. squamosa* leaves as 37.41% ($IC_{50}$ 13.37 mg/ml), from *A. squamosa* seeds as 49.62% ($IC_{50}$ 10.08 mg/ml) and from APM as 88.66% ($IC_{50}$ 5.66 mg/ml) compared to ascorbic acid as a positive control at 96.59% ($IC_{50}$ 5.18 mg/ml).

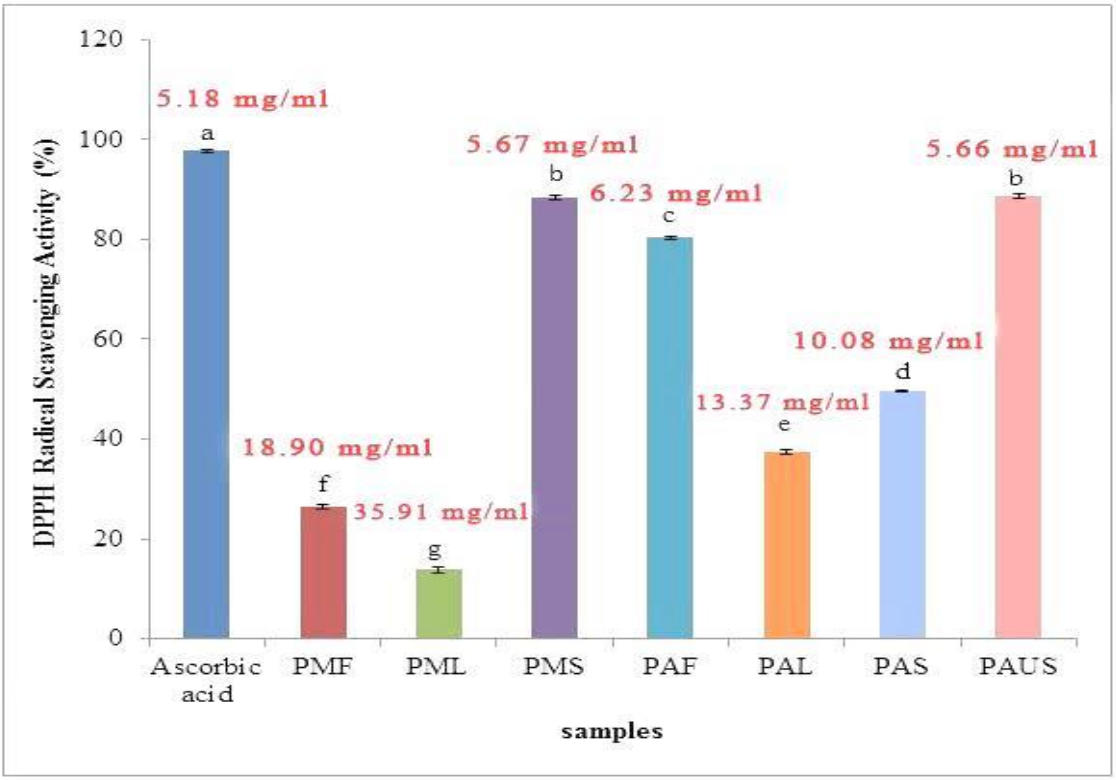



**Figure 4.66.** DPPH scavenging activity with IC $_{50}$ values (in red) of peptides extracts of selected plants. The values are the average of three replicate ± SD. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b, c, d, e, f and g) are significantly different ($p < 0.05$) from each other and control. Positive control indicates to 10 mg/ ml of ascorbic acid. **PMF**- peptides of *M. citrifolia* fruit, **PML**- peptides of *M. citrifolia* leaves, **PMS**- peptides of *M. citrifolia* seeds, **PAF**- peptides of *A. squamosa* fruit, **PAL** – peptides of *A. squamosa* leaves, **PAS**- peptides of *A. squamosa* seeds and **PAUS**- peptide of APM.

Figure 4.67 show the rate of inhibition of SOD-like activity for peptides of *M. citrifolia* seeds, APM and *A. squamosa* fruit. Peptide extracts of *A. squamosa* leaves and seeds have moderate antioxidant activity while *M. citrifolia* fruit and leaves have low antioxidant activity.

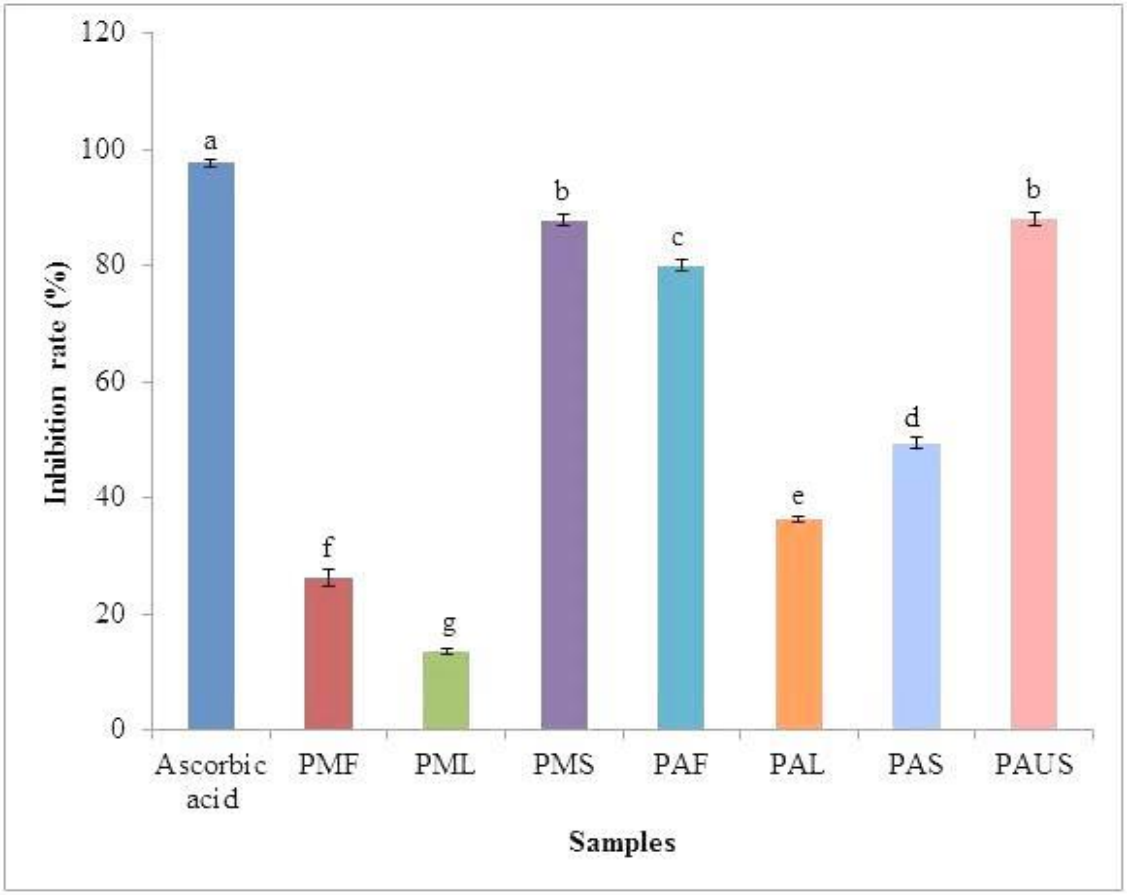



**Figure 4.67.** The rate of inhibition of SOD–like activities of peptides extracts of selected plants. The results were analysed by one-way ANOVA followed by Duncan's multiple comparison test. Samples represented with different letters (a, b, c, d, e, f and g) are significantly different ($p < 0.05$) from each other and control. Positive control was 10 mg/ ml of ascorbic acid. **PMF**- peptides of *M. citrifolia* fruit, **PML**- peptides of *M. citrifolia* leaves, **PMS**- peptides of *M. citrifolia* seeds, **PAF**- peptides of *A. squamosa* fruit, **PAL** – peptides of *A. squamosa* leaves, **PAS**- peptides of *A. squamosa* seeds and **PAUS**-peptide of APM.

### 4.3.2.3. Bioactivity Guided Purification

Fraction 1 of peptide extracted from APM by gel filtration displayed higher antibacterial activity against *B. cereus* as an indicator organism (Figure 68).

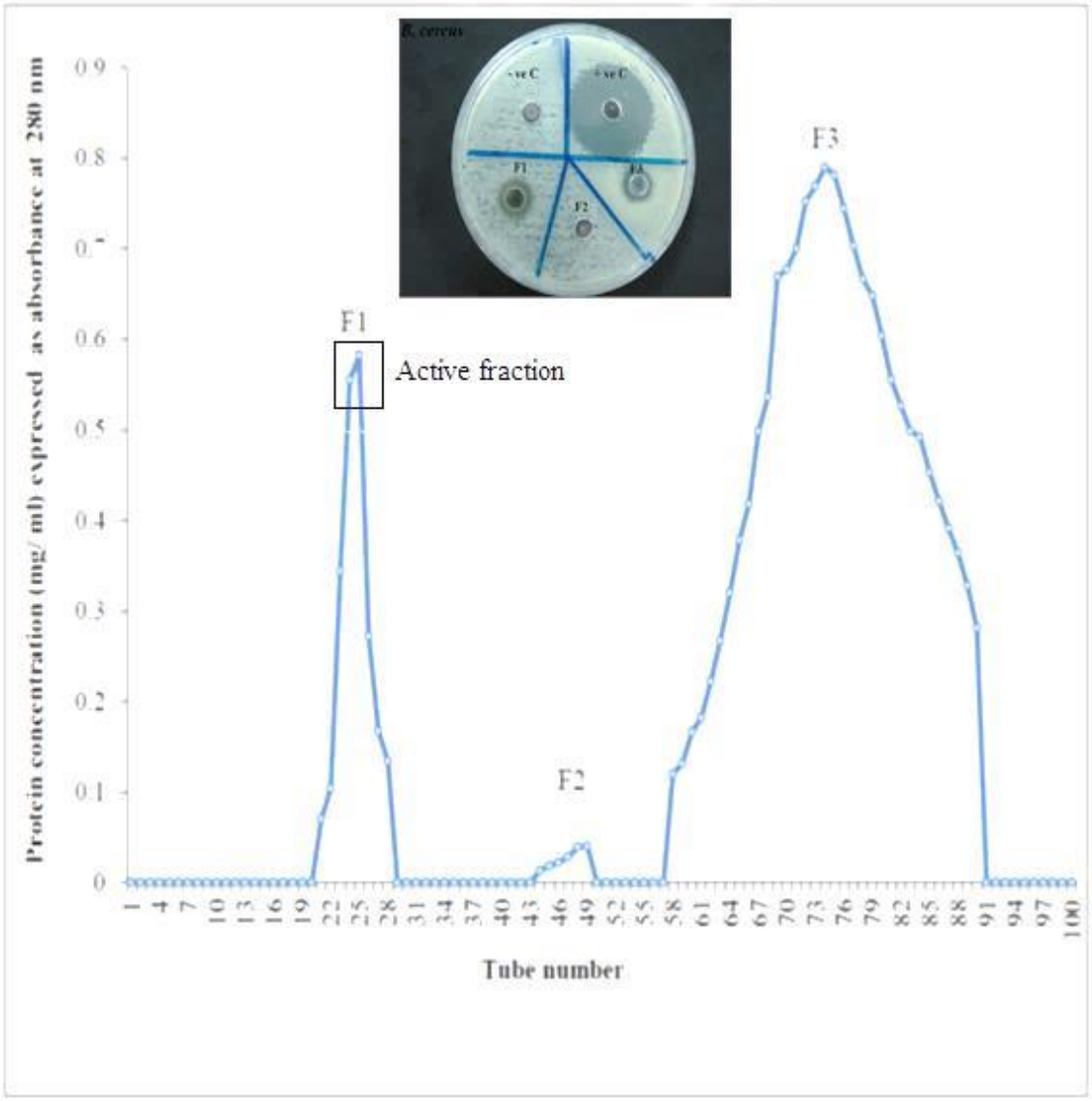



**Figure 4.68.** Fractionation of peptides extracted from selected plant mixture by Sephadex G-75 gel filtration. Active fraction (F1) gave a zone of inhibition against *B. cereus* at 13 mm.

### 4.3.2.4. LC- MS/MS analysis and HPLC results of peptide extracted from APM

Figure 4.69 shows the peaks obtained on HPLC analysis of the peptides in F1 of APM. The peaks at retention time of 4.181 min showed inhibition of B. cereus. Other peptides were observed at varying retention times of 3.048, 3.327 and 3.504 min of HPLC chromatogram but these had no antibacterial activity (Figure 4.69).

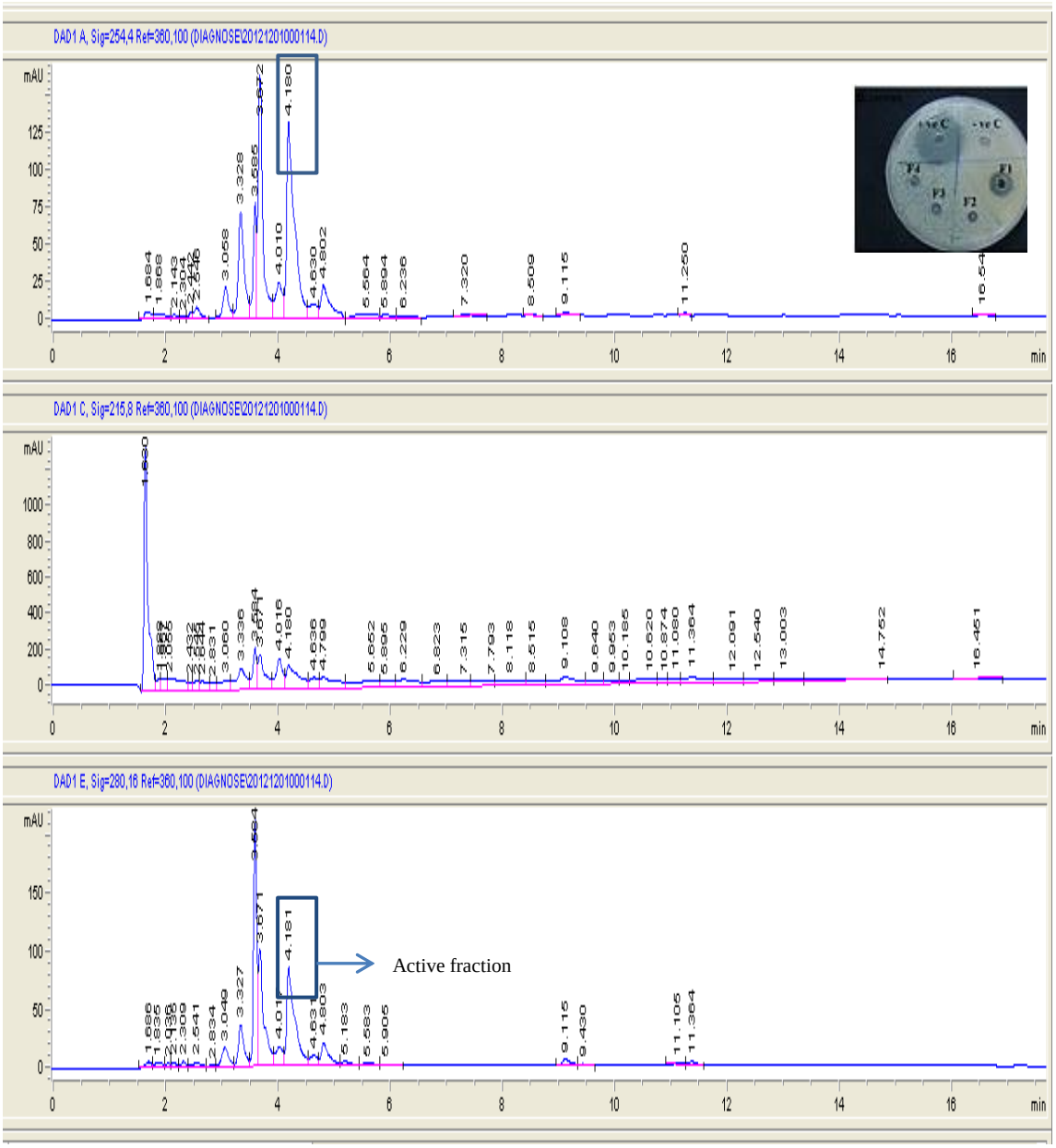
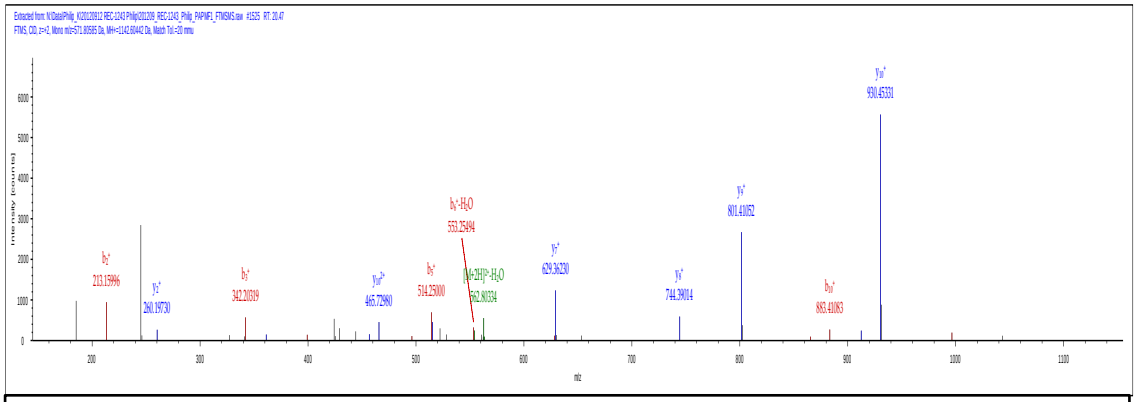



**Figure 4.69.** HPLC chromatogram of active fraction (F1) of APM (A) show absorbance at 254 nm, (B) show absorbance at 215 and (C) show absorbance at 280 nm respectively. Active fraction peak was at retention time of 4.181 min.

LC-MS/MS analysis of the active fraction of APM showed the amino acid sequences (IVEGNGGPGTIK) after referring to Uniprot-Swissprot software for the verification (Figure 4.70). The peptide was identified as Pathogenesis-related protein 2 OS=*Phaseolus vulgaris* (Figure 4.71).



| Peptide accession | Sequence | Score | $MH^+$ [Da] | Charge |
|---|---|---|---|---|
| P25986 | IVEGNGGPGTIK | 48 | 1142.60442 | 2 |

**Figure 4.70.** MS/MS spectrum of active fraction of APM with amino acid sequences. The spectrum was deduced to being part of pathogenesis-related protein 2 of *Phaseolus vulgaris* with sequence of (IVEGNGGPGTIK), peptide accession P25986, score 48, $MH^+$ at *m/z* 1142.60442 and charge 2. Enlarged diagram in appendix 65, page 237.

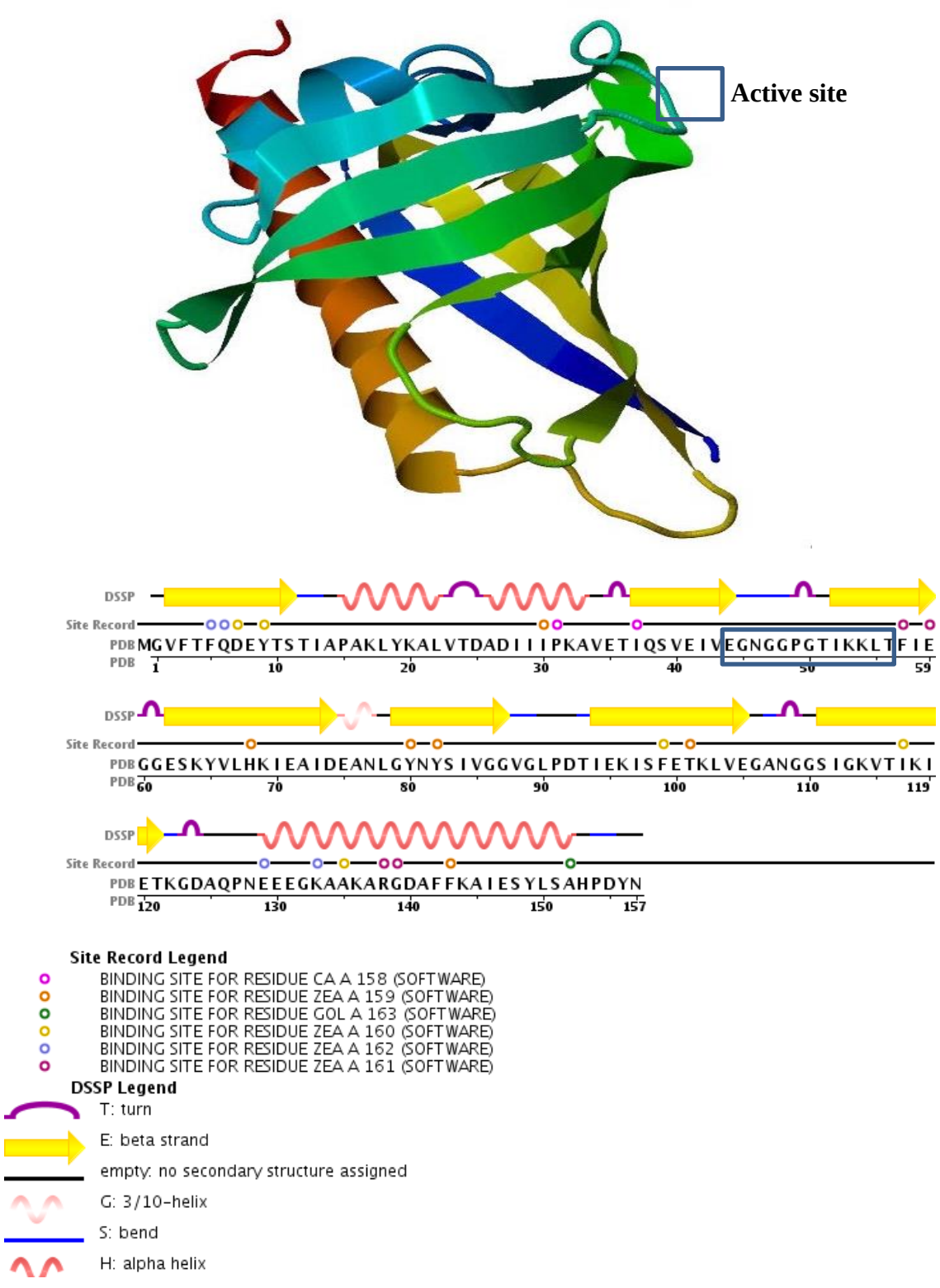
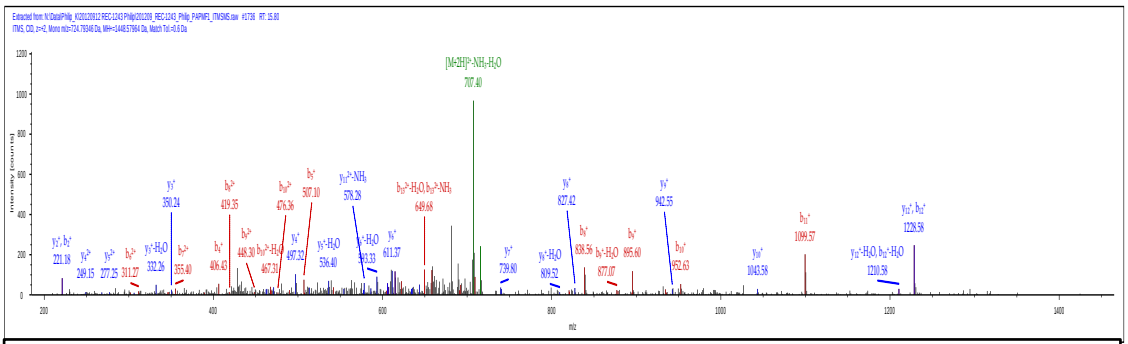



**Figure 4.71**. 3D model of active fraction (pathogenesis-related protein 2 of *Phaseolus vulgaris*) of APM. (Uniprot-Swissprot software).

Other inactive peptides were observed at varying retention times of 3.048, 3.327 and 3.504 min. LC- MS/ MS analysis of these peptides revealed the amino acid sequences (GGKTDSEGGFESD)**,** (EMIREADVDGDGQIN**)** and (AEEPTAAAPAEPAPA ADEKPE) respectively after referring to Uniprot-Swissprot software for verification (Figure 4.72-77). These peptides are oxygen-evolving enhancer protein 2, calmodulin OS=*Fagus sylvatica* and Photosystem I reaction centre subunit IV, and chloroplastic protein respectively.



| Peptide accession | Sequence | Score | $MH^+$ [Da] | Charge |
|---|---|---|---|---|
| Q00434 | GGKTDSEGGFESD | 71 | 1285.51738 | |

**Figure 4.72.** MS/MS spectrum of fraction 2 of APM with amino acid sequences. The spectrum was deduced to being part of oxygen-evolving enhancer protein 2 from APM with sequence of (GGKTDSEGGFESD), peptide accession Q00434, score 40, $MH^+$ at *m/z* 1285.51738 and charge 2. Enlarged diagram in appendix 66, page 238.

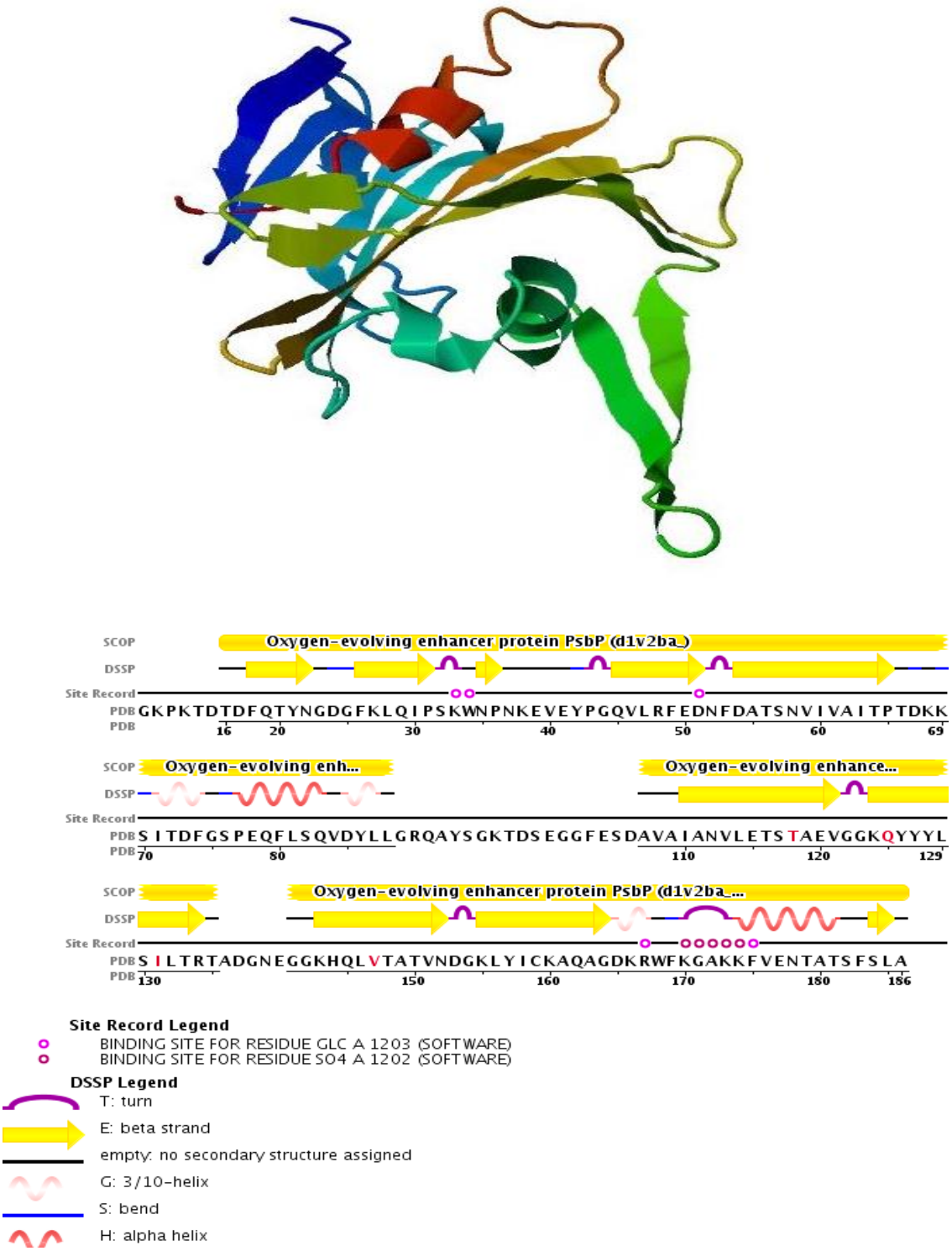
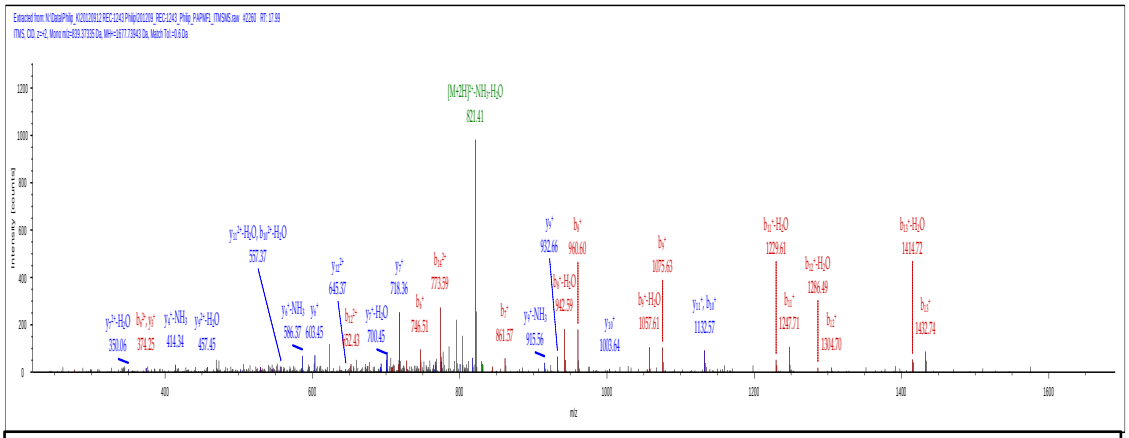



**Figure 4.73.** 3D model of fraction 2 (oxygen-evolving enhancer protein 2 chloroplastic OS=*Triticum aestivum*) of APM. (Uniprot-Swissprot software).



| Peptide accession | Sequence | Score | $MH^+$ [Da] |
|---|---|---|---|
| Charge | Q39752 | EMIREADVDGDGQIN | 44 |

**Figure 4.74.** MS/MS spectrum of fraction 3 of APM with amino acid sequences. The Spectrum was deduced to being part of calmodulin of *Fagus sylvatica* from APM with sequence of (EMIREADVDGDGQIN), peptide accession Q39752, score 44, $MH^+$ at *m/z* 1677.73943 and charge 2. Enlarged diagram in appendix 67, page 239.

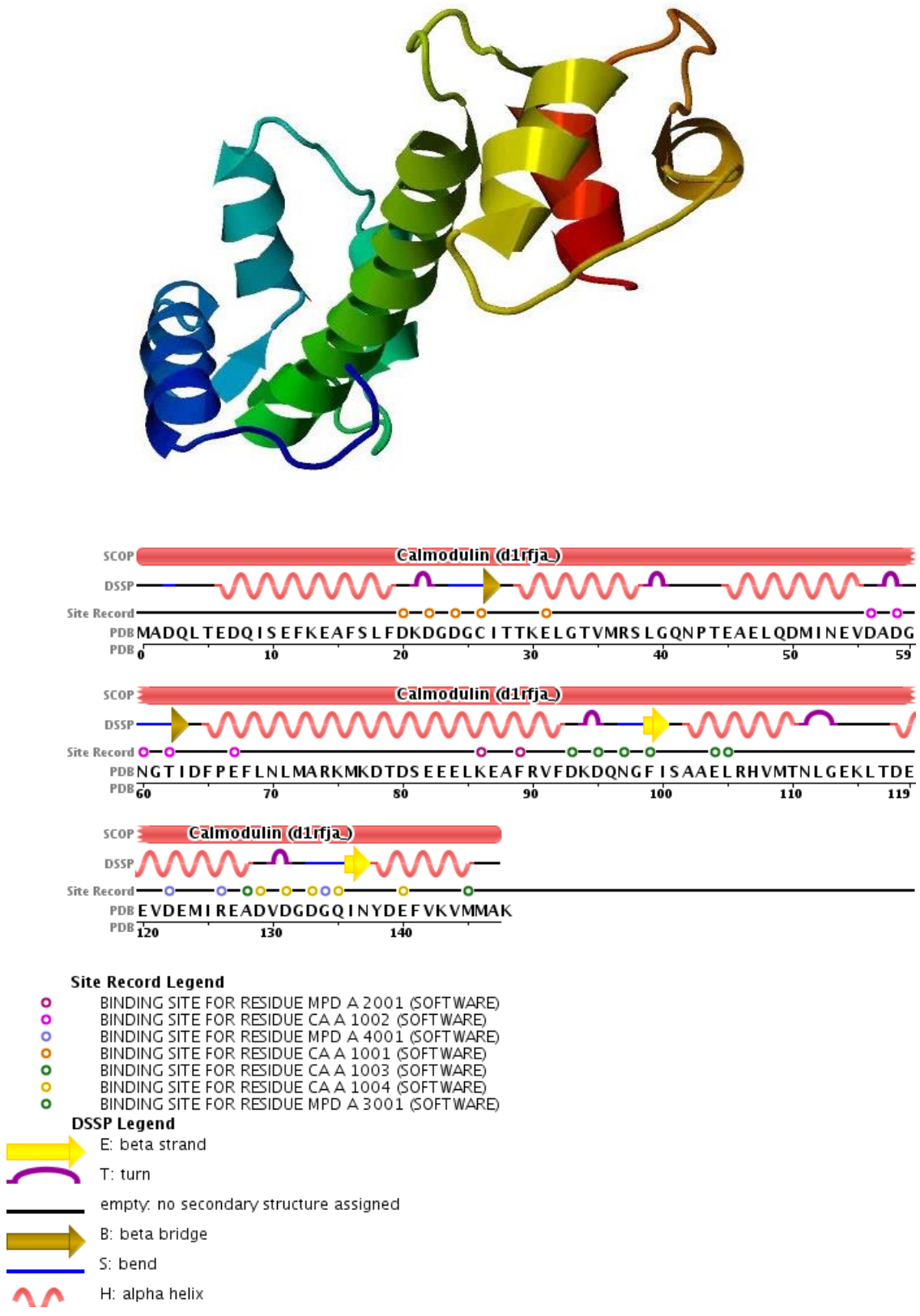
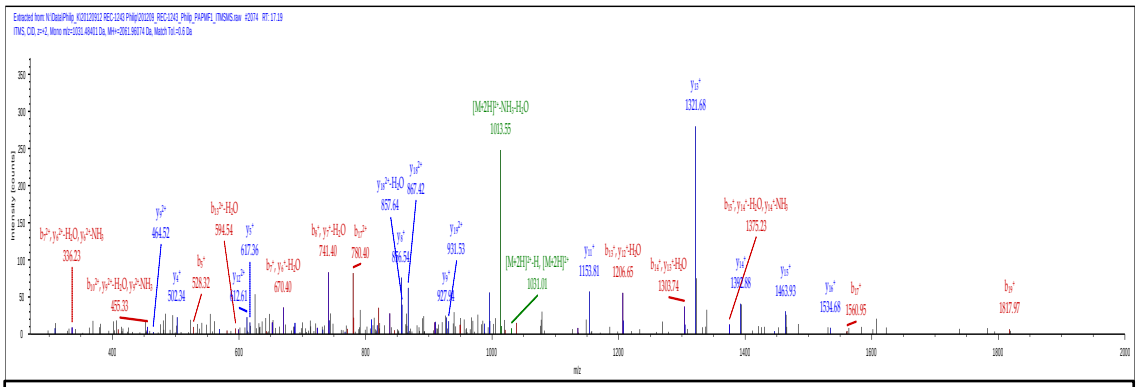



**Figure 4.75.** 3D model of fraction 3 (calmodulin OS=*Fagus sylvatica*) of APM. (Uniprot-Swissprot software).



| Peptide accession | Sequence | Score | $MH^+$ [Da] | Charge |
|---|---|---|---|---|
| P13194 | AEEPTAAAPAEPAPAADEKPE | 32 | 2061.9607 | 2 |

**Figure 4.76.** MS/MS spectrum of fraction 4 of APM with amino acid sequences. The spectrum was deduced to being part of photosystem I reaction center subunit IV, chloroplastic protein**)** of APM with sequence of (AEEPTAAAPAEPAPAADEKPE), peptide accession P13194, score 32, $MH^+$ at *m/z* 2061.96074 and charge 2. Enlarged diagram in appendix 68, page 240.

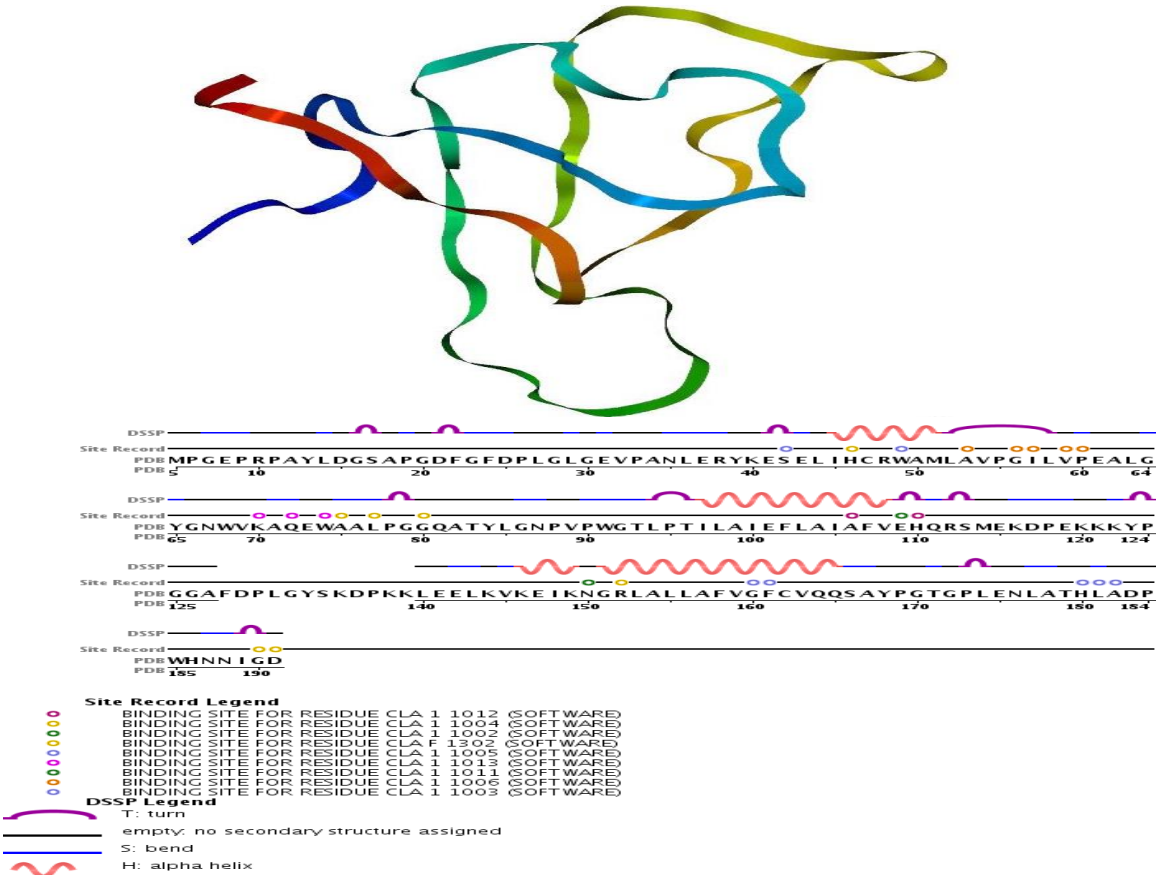



**Figure 4.77.** 3D model of fraction 4 (photosystem I reaction center subunit IV, chloroplastic protein**)** of APM. (Uniprot-Swissprot software).

### 4.3.2.5. Attachment of the lytic peptide extracts of AMP to *B. cereus* and MRSA cells as examined under SEM

Figures 4.78, 4.79 and Appendix 64 show the effects of peptides from APM against *B. cereus* and MRSA. It was observed that the bacterial cells treated with antibacterial peptide (Fraction 1) of the mixture displayed significant alterations in morphology and shape. These alterations included roughening of cell surface, the formation of numerous blebs and lysis of bacterial cells accompanied by the accumulation of cell debris.

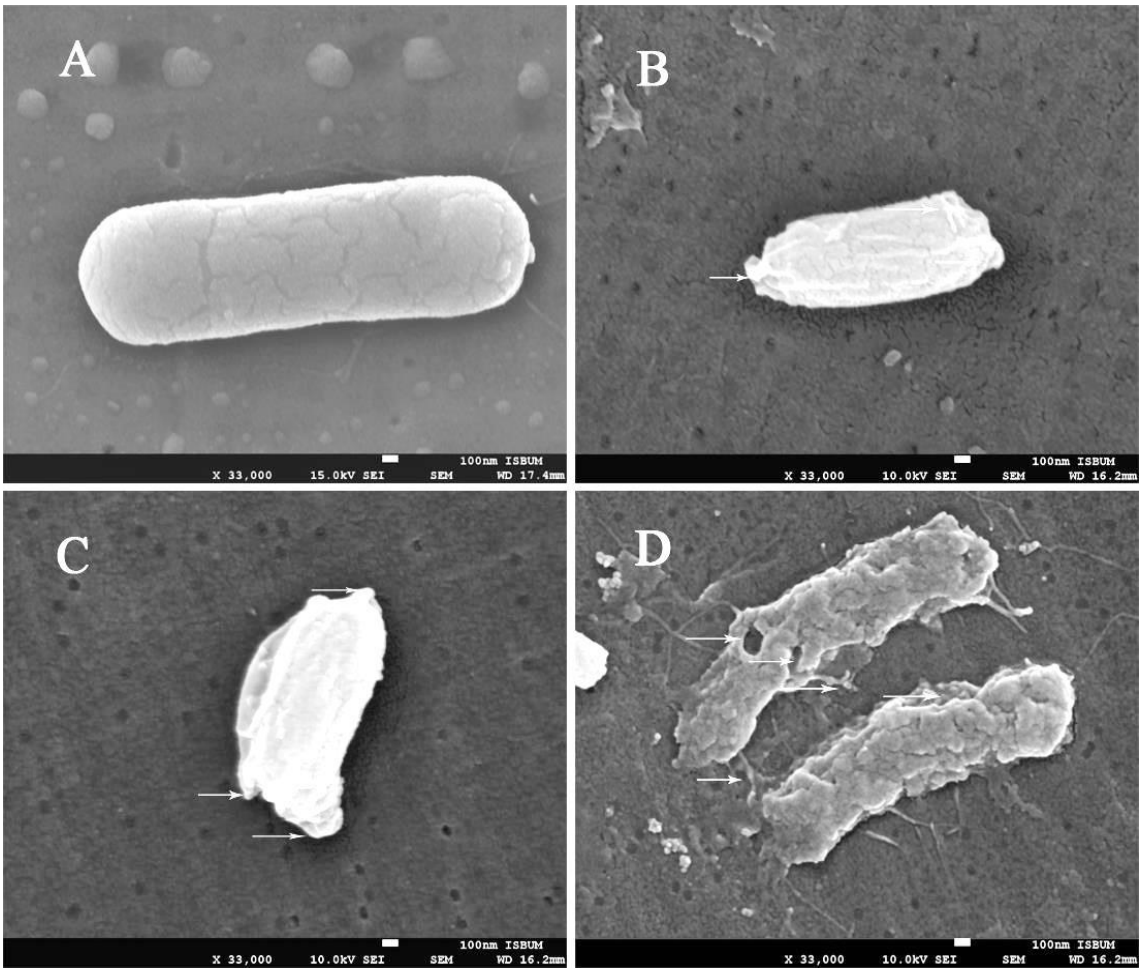



**Figure 4.78.** Effect of F1 fraction of peptide extracted (50 µg/ml) from APM by scanning electron microscope. (A) Control: *B. cereus*. (B), (C) and (D) *B. cereus* treated with peptide. (B) Arrows show changes in morphology with roughening of cell surface (C) Arrows show the formation of numerous blebs, (D) Arrows show lysis of bacterial cells accompanied by the accumulation of cell debris.

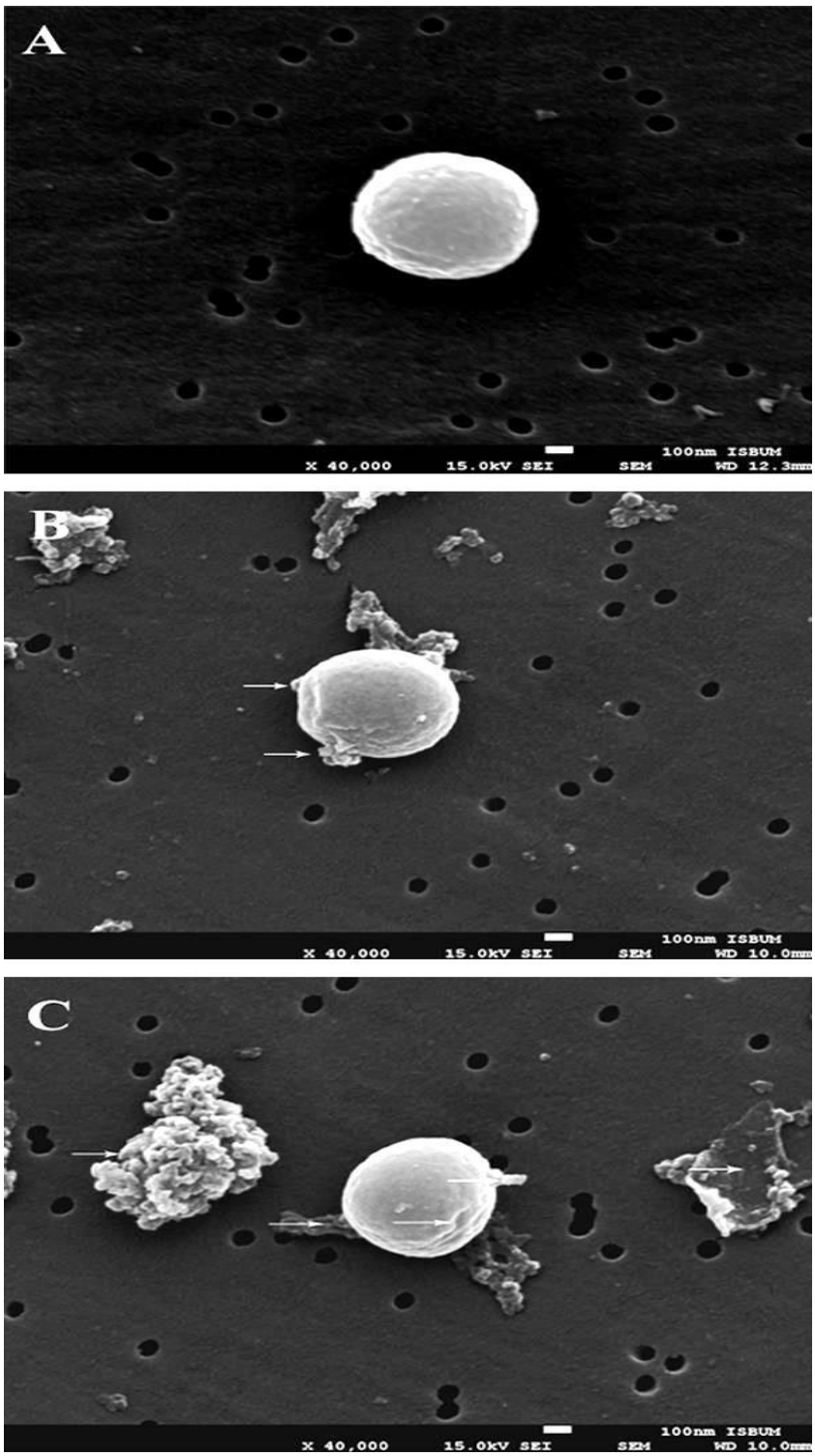



**Figure 4.79.** Effect of F1 fraction of peptide extracted (50 µg/ml) from APM by scanning electron microscope. (A) Control: MRSA. (B), and (C) MRSA treated with active peptide. (B) Arrows show changes in morphology with roughening of cell surface (C) Arrows show the formation of numerous blebs, (D) Arrows show lysis of bacterial cells accompanied by the accumulation of cell debris

# 5. DISCUSSION

## 5.1. Crude extracts

### 5.1.1. Antibacterial activity

#### A. Hot and cold aqueous extracts

Hot and cold aqueous extracts of *M. citrifolia* fruit and leaves, *A. angustiloba* roots and an Australian plant mixture (APM) showed no inhibition against all strains of test bacteria. This result was in contrast with that of Rivera *et al.* (2011) who reported that the juice extract of *M. citrifolia* inhibited the growth of *Mycoplasma pneumonia*, *M. penetrans,* and *M. fermentas*. The test microorganisms in their study were different from the test strains used in the current study. Dittmar (1993) reported that the ripe fruit extract of *M. citrifolia* inhibited the growth of *P. aeruginosa, M. pyrogenes, E. coli, Salmonella typhosa, Salmonella ontevideo, Salmonella schottmuelleri* and *Shigella paradys*. The noni juice has been shown to have antibacterial properties against aerobic bacterial species isolated from gingival pouches such as *Klebsiella oxytoca*, *P. aeruginosa*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Glang *et al.*, 2013). No reports have cited antibacterial activity against any microorganisms using hot and cold aqueous extracts *A. angustiloba* roots or Australian plant mixture (APM).

Hot and cold aqueous extracts of *A. squamosa* fruit showed antibacterial activity against S. *aureus*, *E. coli*, *B. cereus* and MRSA. However, hot and cold aqueous extracts of *A. squamosa* leaves showed no inhibition against all test bacteria. Jagtap and Bapat (2012) reported that silver nanoparticles of aqueous extract from the leaves of sweet apple exhibited antibacterial activity against *B. cereus* (NCIM 2703), *B. subtilis* (NCIM 2635), *Salmonella typhimurium* (NCIM 2501), *S. aureus* (NCIM 2654), *P. aeruginosa* (NCIM 5032) and *Proteus vulgricus* (NCIM 2813). Previous studies on the ethanol and aqueous extracts from the same part of this plant showed it had significant

antibacterial activity against the Gram positive bacteria, *Staphylococcus epidermidis* and *Streptococcus agalactiae* and the Gram negative bacteria, *E. coli* and *P. aeruginosa* (Sailaja *et al.*, 2009). This is the first study to demonstrate antibacterial activity of hot and cold aqueous extracts of *A. squamosa* fruit against MRSA and *H. pylori*.

Hot and cold aqueous extracts of *M. citrifolia* fruit and leaves, *A. angustiloba* roots and an Australian plant mixture showed no values of MIC and MBC against all strains of bacteria. However, hot and cold aqueous extracts of *A. squamosa* showed good results for MIC and MBC values. This is the first study to test antibacterial activity of hot, cold aqueous extracts of *A. squamosa* fruit against pathogenic bacteria including MRSA and *H. pylori*.

**B. Methanolic extracts**

Methanolic extracts of *M. citrifolia* leaves, *A. angustiloba* roots and APM showed no inhibition against all strains of test bacteria. However, the methanolic extracts of *M. citrifolia* fruit showed inhibition zones against all test bacteria including MRSA and *H. pylori*. This result was in agreement with the work of Natheer *et al.* (2012) who reported that methanolic extract of the fruit of *M. citrifolia* have antibacterial activity against *E. coli*, *S. aureus*, *Proteus vulgaris, P. aeruginosa, Klebsiella pneumonia*, *Streptococcus sp*., *Shigella flexneri*, *Proteus mirabilis*, *P. diminuta*, *P. fluorescens*, *S. aureus* ATCC 6538, *E. coli* ATCC 25922 and *Enterobacter cloacae*. There was good correlation between the zones of inhibition and the concentration of the extract, the highest concentration of this extract revealing a higher inhibition zone against microorganisms. In addition, *M. citrifolia* is known to contain alkaloids, phenolic compounds, terpenoids, and anthraquinones which possess good antibacterial properties against a variety of microorganisms (Potterat and Hamburger, 2007).

The methanolic extract from the fruit and leaves of *A. squamosa* showed inhibition zones with all test bacteria except *H. pylori*. This study was in agreement with the work of Hassan *et al.* (2003) who reported the methanolic extract of *A. squamosa* fruit had antibacterial effect against both clinical and ATCC strains of *S. aureus* and *S. pneumonia.* The fruit of this plant was shown to contain 16α-hydroxy-(-) kuaran-19-oic acid. This compound can display antibacterial activity against *S. aureus* and *S. pneumonia*. Leaf extracts for different solvents have been reported antibacterial activity against all strains of *Neisseria gonorrhoeae* from patients infected with acute gonococcal urethritis (Shokeen *et al.*, 2005). Aher *et al.* (2012) reported that the methanolic, petroleum ether and chloroform extracts from the fruit of nona have antibacterial properties against *S. aureus, E. coli, P. aeruginosa, B. subtilis* and *Klebsiella pneumonia*. Their methanolic extract exhibited high antibacterial activity against *S. aureus* compared to other bacterial strains. Whereas, the methanolic extract of the same morphological part of the plant showed high antimicrobial activity against *Klebsiella pneumonia* while *B. subtilis* was lowest. The authors reported that the chloroform extract of the fruit of this plant had significant antibacterial activity against *E. coli* but *B. subtilis* was lowest.

The MIC/MBC of the methanolic extracts of *M. citrifolia* leaves, *A. angustiloba* roots and an Australian plant mixture against all strains of test bacteria could not be determined since they showed no antibacterial activity. However, MIC and MBC values of methanolic extracts of *M. citrifolia* fruit showed that the extract is very potent against all test bacteria with *B. cereus* being most sensitive compared to the other test strains. Natheer *et al.* (2012) documented that MIC values of methanolic, ethanolic and ethyl acetate extracts of the fruit of *M. citrifolia* was lower (12.5 mg/ml) for *E. coli* and *S. aureus* ATCC 6538 compared to the other organism. However, with *Shigella flexneri*, *P. mirabilis*, *P. diminuta* and *Enterobacter cloacae* the MIC and MBC was higher at 25

mg/ml. In general, the methanolic extract of *A. squamosa* showed low values MIC and MBC for Gram positive bacteria. These results were in agreement with the work of Padhi *et al.* (2011) who reported that the values of MIC and MBC of methanolic extract of *A. squamosa* leaves were lower against *S. aureus*, *Staphylococcus epidermidis* and *B. subtilis* compared to Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *Vibrio cholerae* and *V. alginolyticus*).

### 5.1.2. Antioxidant activity

#### A. Hot and cold aqueous extracts

Hot and cold aqueous extracts of *A. squamosa* fruit had significant antioxidant activities with $IC_{50}$ values comparable to ascorbic acid as a positive control. *A. squamosa* extracts are known to contain bioactive compounds (Kris-Etherton *et al.*, 2002). The current study was in agreement with the work of Jagtap and Bapat (2012) who reported the aqueous extracts from the fruit pulp of nona had high antioxidant activity using N.N. dimethyl-p-phenylendiamine (DMPD) radical scavenging assay while the aceton extract gave high antioxidant level using TPC and DPPH methods. The hydroalcoholic extract of the leaves of this plant had antioxidant activity depend on the concentration of this extract using ferric reducing power assay. Hydroalcoholic extracts have phenolic compounds which are responsible for antioxidant activity (Tomar, 2013).

**B. Methanolic extracts**

Methanolic extracts of *A. squamosa* fruit and leaves had significant antioxidant activities with $IC_{50}$ comparable to ascorbic acid as a positive control because these extracts are known to contain bioactive compounds (Kris-Etherton *et al.*, 2002). Nandhakumar and Indumathi (2013) demonstrated that the methanolic extract of the fruit pulp of *A. squamosa* had high level antioxidant activity using DPPH, lipid peroxidation, nitric oxide, superoxide anion ($O_2^-$), hydroxyl radical (OH), reducing power and total antioxidant assays. Mariod *et al.* (2012) reported that the methanolic extract of the leaves, roots, bark and cake of *A. squamosa* exhibited good antioxidant activity using oxygen radical absorbance capacity and MTT assays because these parts of this plant have a high levels of phenolic compounds. These plants had high total phenolic content and contained compounds such as quercetin, syringic acid, hydroxybenzoic acid, gallic acid, vanillin, p-coumaric and p-coumaric.

The methanolic extracts of *M. citrifolia* fruit also have significant antioxidant activities with an IC50 value of 5.19 mg/ml compare to ascorbic acid which was had an $IC_{50}$ of 5.18 mg/ml. The fruit juice of this plant has been known to show three times the antioxidant activity compared to vitamin C indicating that noni juice is more effective at reducing or scavenging reactive oxygen free radicals (Wang and Su, 2001). This work was in agreement with the work of Krishnaiah *et al.* (2013) who reported that methanolic extract of *M. citrifolia* fruit prepared by membrane separation has antioxidant activity using DPPH assay because this extract has phenolic compounds. The phenolic compounds of the methanolic extracts of *M. citrifolia* fruit have been related with antioxidant activity. Zin *et al.* (2002) documented the methanolic extract of *M. citrifolia* fruit have antioxidant activity measured by FTC and TBA methods because methanolic extracts have phenolic and hydroxyl-phenolic compounds with other compounds which are responsible for the antioxidant activity of the morphological part

of this plant. Further research reported that the phenolic compounds of the fruit of this plant have significant antioxidant compared to BHT or α- tocopherol using FTC and TBA methods (Zin *et al.*, 2007).

### 5.1.3. GC-MS analysis of crude extracts

#### A. Aqueous extracts

The major compounds in hot and cold aqueous extracts of *A. squamosa* fruit are the diterpenes ent-spathulenol, kauran-18-al, kaur-16-ene, kauren-18-ol and trihydroxycholan-24-oic acid. They were found in an aqueous extract because these compounds are soluble in water (Tiwari *et al.*, 2011). Cuevas-Glory *et al.* (2013) found 14 monoterpene hydrocarbons, 17 oxygenated monoterpenes, 14 sesquiterpene hydrocarbons and 11 oxygenated sesquiterpenes in *A. squamosa* fruit by utilizing a gas chromatography–flame ionization detector and employing gas chromatography–mass spectrometry. Isolation was achieved by SDE with dichloromethane. These results are in contrast to this current study due to disparities in the origin of the plant, the GC-MS system and the conditions of extraction. 14 kauran diterpenoids from *A. squamosa* fruit were isolated by Wu *et al*. (1996). These kauran diterpenoids are *ent*-kaur-16-en-19-oic acid, 16*â-ent*-kaurane-16,17, 19-triol, 17-hydroxy-16*â-ent*-kauran-19-oic acid, 17-hydroxy-16R-*ent*-kauran-19-oic acid, 16R, 17-dihydroxy-*ent*-kauran-19-oic acid, 17-acetoxy-16*â-ent*-kauran-19-oic acid, 19-formyl-*ent*-kauran-17-oic acid, 17-hydroxy-16*â-ent*-kauran-19-al, annosquamosin A, 4R-hydroxy-19-nor-*ent*-kauran-17-oic acid and annosquamosin B. These compounds were shown by the authors to have anti-HIV activity. According to another report, the fruit of this plant also contains 16α-hydroxy-(-) kauaran -19-oic acid. This compound was shown to exert antibacterial activity against *S. aureus* and *S. Pneumonia* (Hassan *et al.*, 2003).

**B. Methanolic extracts**

Methanol is the most polar solvent for the extraction of plant materials (Tiwari *et al.*, 2011). Methanolic extract from the fruit of *A. squamosa* revealed the presence of three compounds which were identified by GC-MS as 2-isoxazolidine, 4,5-octanediol and 2,3-hexanediol. Nandhakumar and Indumathi (2013) reported that phytochemical screening of methanolic extracted from the fruit of *A. squamosa* uncovered alkaloids, flavonoids, saponins, carbohydrates, phenols and glycosides. Shashirekha *et al.* (2008) detected 13 monoterpenoids, ester-like sec-butylbutanoate, benzyl alcohol and methyllinolenate in the fruit of *A. squamosa* using a GC–MS.

The major compounds found in the methanolic extract of *A. squamosa* leaves were isoquinoline, 1,6-methylphenazine and 1,2,3,4-tetrahydroisoquinoline. These compounds are alkaloids. Most phytochemical compounds which were found in *A. squamosa* leaves were alkaloids, flavonoids, carbohydrates, glycosides, tannin and phenolic compounds, steroids and sterols, as well as triterpenoids extracted into polar solvents such as methanol or water (Agrawal *et al.*, 2012; Padhi *et al.*, 2011). Vanitha *et al.* (2011) reported that GC-MS analysis of methanolic extracts of *A. squamosa* leaves collected in India revealed the presence sodium benzoate, 4,4-terta-butylcalix(4)arene, 4,4-dimethylcholesterol, butyl octylpthalate, stigmasterol acetate and isoamylacetyate. These findings are in contrast to those of this current study due to disparities in the origin of the plant and the conditions of extraction.

In the current study, the methanolic extract of *M. citrifolia* fruit revealed the presence of a variety of compound. The major compounds included 1-butanecarboxylic acid, butyric acid, N-acetylisoxazolidine, propanoic acid, carbamimidic acid, 1,3-propanediol, 4,5-octanediol, 1,3-oxazine and semicarbazone. Pino *et al.* (2010) identified ninety-six compounds in noni fruit at two stages of ripening and isolated them

using headspace solid-phase microextraction. Analysis by GC-MS by the authors revealed the major compounds to be oceanic acid and hexanoic acid. With modifications to the extraction process and using the ripe fruit, the current work identified other compounds with bioactive traits.

### 5.2. Bioactive compounds extracts

#### 5.2.1. Antibacterial activity

#### A. Anthraquinones

Anthraquinones extracts using different parts of *M. citrifolia* showed antibacterial effects against all test bacterial strain including MRSA and *H. pylori*. It is noteworthy that the anthraquinones extracted from the fruit of the plant showed significant inhibition zones compared to other extracts. According to documented literature, *M. citrifolia* contains anthraquinones in the fruit, leaves and roots (Deng *et al.*, 2009; Ee *et al.*, 2009; Takashima *et al.*, 2007). These compounds have antibacterial, antifungal and other biological activities (Zahin *et al.*, 2010). Comini *et al.* (2011) reported that anthraquinones isolated from *Heterophyllaea pustulata* Hook f. (Rubiaceae) have antibacterial activity against *S. aureus* including bacteriostatic and bactericidal effects. MIC and MBC values of anthraquinones extracts of different parts of this plants showed that the extracts are very potent against the selected test bacteria with *B. cereus* being most sensitive. However, the anthraquinones extracted from the fruit have significant values of MIC and MBC compared to extracts from other parts of *M. citrifolia*. This finding of the current study is the first report demonstrating antibacterial properties of anthraquinones extracts from different parts of *M. citrifolia* activity against *S. aureus, E. coli, B. cereus, P. aeruginosa* and including important pathogenic strains namely MRSA and *H. pylori*.

**B. Alkaloids**

The alkaloids of the fruit of *M. citrifolia* exhibited antibacterial activity against all test bacterial strains except *P. aeruginosa* while the alkaloids extract of *A. squamosa* leaves and *A. angustiloba* roots have antibacterial activity against all test bacterial strains including MRSA and *H. pylori*. These plants are known to produce alkaloids as secondary metabolites and these have antibacterial properties (Koyama *et al.*, 2008; Lim, 2013; Pandey and Barve, 2011). Costa *et al.* (2010) reported that alkaloids namely O-methylmoschatoline, lysicamine and liriodenine from the bark of *Guatteria hispida* (Annoaceae) had antibacterial activity against *S. epidermidis*. Past reports have documented that alkaloids extracted from *Mitragyna speciosa* (Rubiaceae) exhibited antibacterial activity against *S. typhi, B. subtilis*, *E. coli* and, *P. aeruginosa* (Parthasarathy *et al.*, 2009). Yang *et al.* (2012) reported that the new indole alkaloids from the twigs of *Kopsia hainanensis* exhibited antibacterial properties against *S. aureus*. The above plants belong to same families as the plants used in the current study. MIC and MBC values of alkaloids extracted from *A. squamosa* leaves and from *A. angustiloba* roots in this study showed significant antibacterial effects against all test bacterial strains with *B. cereus* being most sensitive. However, MIC and MBC values of the alkaloids extract from the fruit of *M. citrifolia* showed inhibition against all strains of bacteria except *P. aeruginosa*. According to published literature, the fruit of *M. citrifolia* has a low percentage of alkaloids compared to other compounds (Pawlus and Kinghorn, 2007; Ross, 2001). The current study is the first study on alkaloids isolated from these plants and its antibacterial activity against pathogenic bacteria including clinically important antibiotic resistant bacteria such as MRSA and *H. pylori*.

**C. Phenolic compounds and diterpenes**

Phenolic compounds extract of the APM had antibacterial activity against selected Gram-positive bacteria (*S. aureus*, *B. cereus*) and Gram-negative bacteria (*E. coli*). Previous studies have documented that leaf paste of *B. citriodora* (a plant material used in the formulation of APM) shows antibacterial activity against *Clostridium perfringens, P. aeruginosa,* MRSA, molds and fungi (Saidana *et al.*, 2008). In addition, *T. ferdinandiana* of the APM contains high levels of phenolic compounds. These substances have been demonstrated to show antibacterial activity against microorganisms (Lai *et al.*, 2010; Rodríguez-Ramos *et al.*, 2007). MIC and MBC values of phenolic compounds extract of this mixture showed that the extract is very potent against the selected test bacteria with *B. cereus* being most sensitive. These results were in agreement with the work of Dupont *et al.* (2006) who found significant values of MIC when aqueous, ethanolic and hexane extracts of these Australian native herbs were used against *E. faecalis, P. aeruginosa, E. coli, S. aureus, S. typhi, S. typhimurium,* and *L. monocytogenes* while *S. aureus* was highly sensitive. The current study is the first report of i phenolic compounds isolated from APM and tested for antibacterial activity against pathogenic bacteria with clinically important bacteria like MRSA.

Diterpenes extracted from the fruit of *A. squamosa* exhibited antibacterial activity against all the test bacterial strains except *H. pylori* in well diffusion, MIC and MBC assays. These compounds were isolated from the fruit of this plant and shown to have antibacterial activity (Pandey and Barve, 2011). Wiart *et al.* (2005) reported that 16α hydroxy-(-)-kauran-19-oic acid extracted from the fruit of this plant showed antibacterial activity against ATCC strains of *S. aureus* and *Streptococcus pneumoniae*. Diterpenes extracted from *Plectranthus* were demonstrated to possess antibacterial activity against strains of *Mycobacterium tuberculosis* (Rijo *et al.*, 2010). The purpose

of the current study is to test antibacterial activity of diterpenes extracted from the fruit of *A. squamosa* against strains of clinically relevant bacteria.

### 5.2.2. Antioxidant activity

#### A. Anthraquinones

The anthraquinones extract of *M. citrifolia* fruit had strong DPPH radical scavenging activity with significant $IC_{50}$ value compared to anthraquinones extracts from other parts of this plant (leaves or roots). In addition, anthraquinones extract of *M. citrifolia* have a high inhibition of superoxide dismutase like activity. Earlier studies have shown that the fruit of this plant are rich in anthraquinone content responsible for its high antioxidant activity (Singh, 2012). Kremer *et al.* (2012) reported anthraquinones have a strong natural antioxidant that could be extracted from plants.

#### B. Alkaloids

Antioxidant activity of alkaloids extracted from *A. squamosa* leaves exhibited higher DPPH radical scavenging activity with significant $IC_{50}$ value compared to other alkaloid extracted from fruit of *M. citrifolia* and *A. angustiloba* roots. In addition, alkaloids extracted from *A. squamosa* leaves have a higher level of inhibition of SOD-like activity. Past studies show the leaves of this plant are rich in alkaloids such as anonaine i (Li *et al.*, 2013). These compounds have antioxidant activity and show inhibition of microsomal lipid peroxidation induced by $Fe2^{+}$/ ascorbate, CCl4/NADPH or $Fe3^{+}$ ADP/NADPH. Further, these alkaloids increased deoxyribose degradation by the hydroxyl radical and increased antioxidant capacity (Ubeda *et al.*, 1993).

**C. Phenolic compounds and diterpenes**

Phenolic compound extracts of APM show high DPPH radical scavenging activity with significant $IC_{50}$ value and high levels of phenolic compounds. This suggests that the phenolic compounds contribute significantly to the antioxidant activity. In addition, the phenolic compound extracts have a high level of SOD-like activity. Previous studies showed that these compounds have hydroxyl groups attached to aromatic rings that are able to reduce agents by hydrogen-donating antioxidant and singlet oxygen quenching actions (Bagchi *et al.*, 2000; Larson, 1988; Lee *et al.*, 2006).

Diterpenes extracted from the fruit of *A. squamosa* have a high DPPH radical scavenging activity with significant $IC_{50}$ values and show high level of SOD-like activity. González-Burgos *et al.* (2013) reported the kaurane diterpenes isolated from aerial parts of *Sideritis spp* showed antioxidant activity using different assays.

### 5.2.3. Thin layer chromatography (TLC) and IR spectrometry

**A. Anthraquinones**

Anthraquinones extracts of fruit, leaves and roots of *M. citrifolia* were typically visualised as a red colouration on thin-layer plates sprayed with KOH. This study was in agreement with the work of Stawadhar *et al.* (2011) which identified some bioactive components such as anthraquinones, saponine, and scopoltein in *M. citrifolia* fruit by using the TLC technique. IR spectrometry revealed anthraquinones with functional main groups O-H, C=O and C-H in fruit, leaves and roots of *M. citrifolia*. According to published studies, anthraquinones with these functional main groups have been shown to be present in fruit, leaves and roots of *M. citrifolia* (Ee *et al.*, 2009; Lv *et al.*, 2011; Siddiqui *et al.*, 2007; Takashima *et al.*, 2007).

**B. Alkaloids**

TLC of alkaloid extracts from the plants used in this study revealed the presence of these compounds by using Dragendroff's reagent to reveal characteristic orange bands of alkaloids. *M. citrifolia, A. squamosa* and *A. angustiloba* are known for their alkaloid content (Agrawal *et al.,* 2012; Padhi *et al.,* 2011; Singh, 2012). IR spectra of alkaloids extracted from the fruit of *M. citrifolia*, leaves of *A. squamosa*, and the root *A. angustiloba* exhibited a strong O-H band, N-H band, C=O bond and C-H stretching groups in these extracts. Schulz and Baranska (2007) reported that alkaloids extracted from different plants have major functional groups O-H, N-H, C=O and C–H stretching groups of these compounds confirming the presence of alkaloids in these plants.

**C. Phenolic compounds and diterpenes**

TLC of phenolic compounds from APM and diterpenes extract from the fruit of *A. squamosa* revealed the presence of the components by using anisaldehyde $H_2SO_4$. The fruit of *A. squamosa* and APM are known to contain ditepenes and phenolic compounds (Hassan *et al.*, 2003; Lai *et al.*, 2010). IR spectra of the phenolic compounds of APM revealed the presence of O-H group, C-H stretching group, C=O group and C-H groups. A previous study has documented the phenolic compounds of APM to have O-H bending, C–H stretching, C=O stretching and C-C stretching groups (Zavoi *et al.*, 2011). IR spectra of diterpenes extracted from the fruit of *A. squamosa* indicated the presence O-H stretching, C-H stretching, C=O functional group and Bond C-H groups. Schulz and Baranska (2007) have previously reported similar results for diterpenes extracted from different plants.

### 5.2.4. LC-MS analysis

### A. Anthraquinones

LC chromatograms and MS data identified the major anthraquinones extracted from the fruit of *M. citrifolia* as 1-hydroxy-2-methylanthraquinone, 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl)anthraquinones, morindolin, 1,1-*O*i-O-methylmorindol, 1,2-dihydroxyanthraquinone, 1,3,6-trihydroxy-2-methoxyanthraquinone. Past studies have identified anthraquinones from the fruit of *M. citrifolia* such as 2-methoxy-1,3,6-trihydroxyanthraquinone, anthragallol 1,3-di-*O*-methyl ether**,** 6-hydroxyanthragallol-1,3-di-*O*-methylether, austrocortinin**,** morindone-5-*O*-methylether**,** anthragallol 2-*O*-methylether, 5,15-dimethylmorindol**,** 1,6-dihydroxy-5-methoxy-2-methoxymethyl anthraquinones**,** 1,5,7-trihydroxy-6-methoxy-2-methoxymethylanthraquinones**,** 1,3-dimethoxyanthraquinone and 1,2-dihydroxyanthraquinone**,** morindacin and lucidin (Deng *et al.*, 2009; Kamiya *et al.*, 2005; Lin *et al.*, 2007; Pawlus *et al.*, 2005; Siddiqui *et al.*, 2007). In the present study some compounds were not detected because of the differences in the methods and instrumentation for isolation and identification of the anthraquinones.

LC-MS analysis of anthraquinones extracted from *M. citrifolia* leaves identified five major compounds namely 1,2-dihydroxyanthraquinone, 1-hyroxy-2,3-methoxyanthraquinone, 2,6-diroxy-1,3-methoxyanthraquinone, 2-hydroxy-1-methoxyanthraquinone and 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl)anthraquinones. These compounds identified in the present study were also identified by other authors who used others methods and instrumentation for identification of the anthraquinones (Deng *et al.*, 2009; Takashima *et al.*, 2007).

LC chromatograms and MS data identified the major compounds of anthraquinones extracted from the roots of *M. citrifolia* as damnacanthol, morenone, 2-ethoxy-1-

hydroxyanthraquinone and damnacanthal. Other authors have isolated anthraquinones from the roots of *M. citrifolia* and identified them as monndone-5-methylether, rubiadin, morindone, anthragallol 1,2-di-*O*-methylether, ibericin, damnacanthol and soranjidiol (Ee *et al.*, 2009; Jain and Srivastava, 1992; Lv *et al.*, 2011; Pawlus and Kinghorn, 2007; Sang and Ho, 2006; Thomson, 1996). In the present study some compounds were not detected because of the differences in the methods and instrumentation for isolation and identification of the anthraquinones.

**B. Alkaloids**

LC-MS analysis of alkaloids extracted from *M. citrifolia* fruit identified five major compounds namely pelletierine, sedamine, pseudopelletierine, halosine and lycopodine. Heinicke (1985) documented that *M. citrifolia* fruit contains a natural precursor named proxeronine. This compound merges with an enzyme in the human intestine called proxeroninase to result in xeronine (Heinicke, 1985; Lim, 2013). Xeronine plays the part of directing the protein in the human body to fold into an appropriate conformation to perform properly. In this way, xeronine ensures the performance of protein in overcoming a variety of health problems (Wang *et al.*, 2002). The current study is the first report documenting that these alkaloids have antibacterial and antioxidant activities.

The major compounds of alkaloids extracted from the leaves of *A. squamosa* were identified as corydine, sanjoinine, norlaureline, norcodeine, oxanalobine and aporphine. Earlier studies isolated other compounds from the leaves of this plant and identified these as liriodenine**,** oxoanalobine**,** anonaine, isocorydine, norisocorydine, liriodenine, abenzooxyquinazoline, annosquamosin A**,** anolobine and reticuline (Bhakuni *et al.*, 1972; Lebrini *et al.*, 2010). In the present study, some of these compounds were not

detected because of the differences in the methods of extraction for isolation and identification of the alkaloids.

LC chromatograms and MS data library identified the major alkaloids extracted from the roots of *A. angustiloba* as echitamine, 3-H-indole, 1-H-indole, alstilobanine B and alstilobanine E. *A. angustiloba* is known to be rich in indole alkaloids in different parts of this plant (Goh *et al.,* 1997). Past studies isolated other indole alkaloids such as angustilobine and andranginine from the leaves and bark of this plant (Koyama *et al.*, 2008; Ku *et al.,* 2011). This is the first report of the analysis of indole alkaloids from the roots of *A. angustiloba* identified by LC-MS and FTIR assays. These bioactive extracts were then demonstrated to have both antibacterial and antioxidant activities.

**C. Phenolic compounds and diterpenes**

LC chromatograms and MS data identified the major phenolic compounds extracted from APM as hydroxybenzoic acid-hexoside, luteolin, isohamnetin, apigenin-7-O-rutinoside, quercetin, HHDP-gallogluco-pyranoside, dicaffeoyquinic acid, rosmadial, caffeic acid. This is the first work to isolate and characterise the phenolic compounds from APM based on authentic standards and literature data (Hossain *et al.,* 2010).

LC-MS analysis of diterpenes extracted from *A. squamosa* fruit identified six major compounds identified as kuaran-18-al, 16,17,19-kauranetriol, kauren-18-ol, kaur-16-ene, stigmasterol and annosquamosin. Previous studies isolated diterpenes from the fruit *A. squamosa* and identified them as 16 α hydroxy-(-)-kauran-19-oic acid, kauran-16-en-18-oic acid, annonosin A, annosquamosin C, annosquamosin D, annosquamosin E, annosquamosin F, annosquamosin G, annosquamosins B (Andrade *et al*., 2001; Wiart *et al.*, 2005). In the present study some the above compounds were not detected because of the differences in the methods of isolation and identification of the diterpenes. The findings suggest are that the bioactive extracts in this study (anthraquinones, alkaloids,

diterpenes and phenolic compounds) are suitable for use as biopharmaceutical compounds. Further, they can be useful as antioxidants. In addition, these bioactive compounds can be synthesized for use in many industrial (dyes).

### 5.2.5. Assessment of the lytic effect of bioactive extracts from selected plants by scanning electron microscopy (SEM)

#### A. Anthraquinones

SEM observations show that the anthraquinones extracted from the fruit of *M. citrifolia* causes changes and damage to the morphology of the treated bacteria. There were several mechanisms of action of anthraquinones on bacterial cells. These bioactive compounds may bind to the cell surface, penetrate the target sites and damage the bacterial cell wall or membrane by affecting the phospholipid of the cell membrane. (Denyer, 1990; Kim *et al.*, 2005). The damage to the bacterial cell wall and cytoplasmic membrane indicates a loss in structural integrity and this affects the membrane's ability to act as a permeability barrier (de Billerbeck *et al.*, 2001). As the result of this mode of action, cell death occurs because of loss of cell contents (Denyer, 1990).

#### B. Alkaloids

The effects of alkaloids extracted from the leaves of *A. squamosa* showed changes in morphology of bacterial cells such as swelling of entire cell, rupture in cell wall, cell lysis–ultimately result in cell death with the spreading of cell debris. Past studies reported that the alkaloids, berberine and piperine intercalate in cell wall and or DNA (Atta-ur-Rahman and Choudhary, 1995). Obiang-Obounou *et al.* (2011) found sanguinarine extracted from the root of *Singuinarina canadensis* cause distorted septa in MRSA with rare discerned separation and cell lysis of treated cells by using TEM.

**C. Diterpenes**

Diterpenes extracted from the fruit of *A. squamosa* could affect the morphology of cell membranes including disruption and swelling of the cells, collapse and cell lysis compared to untreated bacterial cells which showed a regular, smooth surface. The mode of action of diterpenes is by their effect on membrane disruption of target cells (Cowan, 1999; Radulovic *et al.*, 2013). These compounds could alter membrane permeability leading to drainage out of the contents of treated bacterial cells (Tyagi *et al.*, 2013). These findings help to evaluate mechanisms of action of diterpenes extracts from different selected plants.

### 5.3. Peptides extracts

#### 5.3.1. Peptide extracts from lactic acid bacteria

##### 5.3.1.1. Antibacterial activity

Peptides extracted by ammonium sulphate precipitation in the current study did not show antibacterial activity. This is perhaps due to low concentrations of peptides in these extracts. Similar results were obtained by Burianek and Yousef (2001). However, peptides extracted by the chloroform extraction method from *L. paracasei* subsp. *paracasei* 8700:2 displayed inhibitions against *E. coli, B. cereus* and *P. aeruginosa* but *S. aureu*s, MRSA and *H. pylori* were not inhibited. Previous studies have documented that the chloroform extract from *L. acidophilus* had a higher amount of bacteriocin compared to the ammonium sulphate precipitation method (Burianek and Yousef, 2001). Peptides extracts from lactic acid bacteria using acidic methanolic method showed antibacterial properties against all test strains including MRSA, *H. pylori, S. aureus*, *E. coli*, *B. cereus* and *P. aeruginosa* by using the well diffusion assay. The antibacterial peptides of lactic acid bacteria have amino acid residues (cysteine, serine and tryptophan) which can play a role in antibacterial activities against bacteria (Jack *et al.*, 1995). *L. casei* strain Shitora, found in commercial fermented milk product

(Yakult), inhibited *E. coli* 0157: H7 in infant rabbits (Asahara *et al.*, 2001; Ogawa *et al.*, 2001) and inhibited *H. pylori in vitro* and *in vivo* (Sgouras *et al.*, 2004). In the current study, *L. paracasei* subsp. *paracasei* 8700:2 showed antibacterial activity using two methods of extraction namely chloroform and acidic methanolic method extractions. A previous study reported that the supernatant of *L. paracasei* subsp. *paracasei* M3 has antibacterial activity against more than 60 strains of bacteria and yeast (Atanassova *et al.*, 2003). In addition, peptide extracted from *L. paracasei* subsp. *paracasei* BMK 2005 found in infant faeces inhibited MRSA and 32 strains of human pathogenic bacteria (Bendjeddou *et al.*, 2012). In the present study, the partially purified peptide (fraction 2) of *L. paracasei* subsp. *paracasei* 8700:2 exhibited antibacterial activity against *B. cereus*. The bound peptide purified fraction from *L. paracasei* subsp. *paracasei* 8700:2 by FPLC has antibacterial activity against *B. cereus.* Pei *et al.* (2013) reported that paracin, a bacteriocin purified from *Lactobacillus paracasei* CICC 20241 had antibacterial properties against seventeen strains of *Alicyclobacillus acidoterrestris* isolated from fruit juices and fruit juice-containing drinks.

#### 5.3.1.2. Antioxidant activity of peptide extracted from lactic acid bacteria

Peptides extracts of *L. casei* and *L. paracasei* have antioxidant activities based on DPPH scavenging with significant $IC_{50}$ values and SOD-like activity but peptides extracts from acidic methanolic method have a higher level of antioxidant activity for both species. Antioxidant peptides have groups that are electron donors which may react with free radicals to terminate the radical chain reaction (Liang *et al.*, 2010). Past studies reported that the supernatant of *L. paracasei* subsp. *paracasei* TKU010 isolated from squid pen showed antioxidant activity by using the DPPH assay (Liang *et al.*, 2010). In addition, *L. casei* has been shown to have antioxidant activity by using 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid, Butylated Hydroxyl Toluene (BHT) and NADPH (Suman and Vibha, 2006). Saadatzadeh *et al.* (2013) reported

lyophilised cell free extracts from *L. casei* have antioxidant activity by using DPPH assay. The findings of the current work involving isolation of peptides from lactic acid bacteria as antioxidants may find many applications as biopharmaceuticals.

### 5.3.1.3. IR spectrometry and LC-MS analysis

IR spectral data identified the main functional groups of the peptide extracted and purified from *L. paracasei* subsp. *paracasei* 8700:2 by the acidic methanolic method. Major functional groups identified were identified as O-H stretching. C-H stretching, C=N bending with C-C bending groups. The functional groups present matched those present in other bacteriocins analysed previously. Cerein 8A isolated from *B. cereus* by using ammonium sulphate precipitation, followed by 1-butanol extraction and ion exchange chromatography showed aliphatic chains (Bizani *et al.*, 2005). Jayachitra *et al.* (2012) analysed a bacteriocin isolated from lactic acid bacteria and found many functional groups such as phenolic, alkynes, alkenes and aromatic stretching groups. The peptide extracted from *L. paracasei* subsp. *paracasei* 8700:2 in the current study have aliphatic chains related with predominance of hydrophobic amino acids such as val, leu, ile in their structure indicating the peptide of this bacteria is paracin-like peptide in structure. This finding is similar to the results obtained by Bizani *et al.* (2005). LC-MS analysis of antibacterial peptide purified fraction from *L. paracasei* subsp. *paracasei* 8700:2 by FPLC identified a novel bacteriocin $[M + H]^+$ at *m/z* 1124.6143. This is a first report that documents a bacteriocin extracted from this bacteria by acidic methanolic method, gel filtration followed by FPLC and then detected by LC-MS. Zendo *et al.* (2008) reported that a class IIa bacteriocin extracted from lactic acid bacteria exhibited ions higher than m/z 1000. The current study isolated peptides using different methods such as ammonium sulphate, chloroform and acidic methanolic methods and demonstrated antibacterial activity against pathogenic bacteria including MRSA and *H. pylori*. MRSA was resistant to gentamicin,

tobramycin, netilmicin, amikacin, streptomycin, or erythromycin while resistance to the other compounds tested was as follows: tetracycline 86%, minocycline 76%, trimethoprim 69%, clindamycin 66%, neomycin 59%, chloramphenicol 39%, rifampicin 26%, fosfomycin 22%, ciprofloxacin 17%, fusidic acid 12%, bacitracin 2%, and novobiocin 1% (Maple *et al*., 1989). *H. pylori* was resistant to 17.5% for clarithromycin, 14.1% for levofloxacin and 34.9% for metronidazole (Megraud *et al*., 2013). In addition active peptide from specific lactic acid bacteria identified functional groups in the structure of this compound.

#### 5.3.1.4. Effect of peptide extract from lactic acid bacteria by SEM and TEM

Electron microscopy examined the effects of the paracin-like biologically active peptide from *L. paracasei* subsp. *paracasei* 8700:2 showed changes in morphology of bacterial cells (*B. cereus*, MRSA and *H. pylori*). Changes were observed the roughening of cell surface, numerous blebs, lysis of bacterial cells and accumulation of cell debris. The mode of action of these class of compounds (class 2 bacteriocin) may include interaction with the anionic surface of cytoplasmic membrane of bacterial cells by electrostatic attraction (Drider *et al.*, 2006). Furthermore, the hydrophilic N-terminal of this type of peptide is connected with hydrophobic C-terminal end as a hinge region. The hydrophobic C-terminal end of these bacteriocin inserts into the membrane and can form pores in the cell membrane, and as a result lyse bacterial cells (Cotter *et al.*, 2012; Nissen *et al.*, 2009).

TEM studies on the exposure of *H. pylori* to the active peptide gave changes in cell morphology with loss of cytoplasmic content due to lysis of the bacterial cells. The peptide could interact with bacterial cell membrane and attach and insert into membrane resulting in pore formation (Lee *et al.*, 2010; Makobongo *et al.*, 2012).

This insertion in the membrane thought to be responsible for the formation of transmembrane pores via the interaction of hydrophobic regions of the peptide with lipid components of the membrane and association of hydrophilic regions of the peptide with phospholipid head groups within the membrane of bacterial cells (Makobongo *et al.*, 2012).

### 5.3.2. Peptides extracts from medicinal plants

#### 5.3.2.1. Antibacterial activity

Peptide extracts from the fruits of *M. citrifolia* and *A. squamosa* have antibacterial properties against *B. cereus*, *P. aeruginosa* and MRSA but not against *E. coli* and *H. pylori.* Antibacterial peptides generally have cysteine or glycine residues which can play a role in their activities against bacteria (Boman, 2003; Hammami *et al.*, 2009; Wang and Wang, 2004). Peptides of *M. citrifolia* leaves showed inhibition zones against all test bacteria except *P. aeruginosa* and *H. pylori* by using well diffusion. MIC and MBC values are significant at 0.65 mg/ml for *B. cereus* and 1.30 mg/ml for S. *aureus*, *E. coli* and MRSA. The leaves of this plant are known to be rich in amino acids such as tryptophan, threonine, tyrosine, serine and alanine. These amino acids in peptides can play a role in antibacterial activities against bacteria while some bacteria show resistance to peptides (Chan-Blanco *et al.*, 2006). Peptides of *A. squamosa* leaves showed antibacterial activity against *S. aureus*, and MRSA and *B. cereus* by the well diffusion MIC and MBC assays. Employing phytochemical screening of different morphological parts of the plant, Agrawal *et al.* (2012) showed that the leaves of *A. squamosa* have protein in methanolic extracts. This protein could contain amino acids which can play a part in antibacterial activity. The peptides of *M. citrifolia* seeds showed inhibition zones against all the test bacteria except *H. pylori* while peptides of *A. squamosa* seeds showed antibacterial activity against *P. aeruginosa*, *S. aureus*, and MRSA and *B. cereus* by using well diffusion. MIC and MBC values of *A. squamosa*

seeds are significant at 0.85 mg/ml for *B. cereus* and 1.70 mg/ml for S. *aureus* and MRSA. The seeds of these plants are rich in cysteine and these compounds can play a role in antibacterial activities. However, some bacteria such as *H. pylori* show resistance against peptides from plants (Lim, 2013; Morita *et al.*, 2006; Yang *et al.*, 2007). Peptides of APM showed inhibition zones against all test bacteria except *P. aeruginosa* and *H. pylori*. APM contains organic Australian wheatgrass sprouts that are rich with a wide range of amino acids such as aspartic acid, threonine, asparagines, glutamine, proline, glycine, arginine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, tryptophan and serine. These amino acids might play a role in antibacterial activity against bacteria because these charged amino acids are related to activity against microorganisms. Antibacterial peptides from plants have 17% of amino acids are positively or negatively charged while 70% of these charged amino acid is arginines and/or lysine while 30% of amino acids are negatively charge aspartic acid and glutamic acid. These charged residues play a role against microorganisms (Wang and Wang, 2004). Fraction 1 of APM purified by gel filtration chromatography showed antibacterial activity against all test bacteria except *P. aeruginosa* and *H. pylori.* Of the bacteria which showed inhibition the most sensitive was *B. cereus*. The finding of is the first report of documenting isolation of antibacterial peptides from *M. citrifolia, A. squamosa* and APM and their activity against pathogenic bacteria.

#### 5.3.2.2. Antioxidant activity of peptides extracted from *M. citrifolia* and *A. squamosa* and APM

Peptides extracts of *M. citrifolia* and *A. squamosa* have antioxidant activities based on the DPPH and SOD assays with significant values of $IC_{50}$ comparable with ascorbic acid. Peptide extracts from the seeds and fruits of *M. citrifolia* have comparatively higher levels of antioxidant. This plant has been shown to be rich in certain amino acids

which may contribute to the antioxidant activity (Chan-Blanco *et al.*, 2006)). Results of the current study show peptide extracts of APM have a high DPPH radical scavenging activity with significant $IC_{50}$ values. In addition, the peptide extracts have a high level of SOD-like activity. The components of APM are known to be rich in antioxidant amino acids (Singh *et al.*, 2012).

There are 2-20 amino acids residues in antioxidant peptides related to their molecular weight, constituents and sequences of amino acids (Grimble, 1994). These peptides possess some metal chelation or hydrogen electron donating property. This reaction could allow them to interact with free radicals and terminate the radical chain initiation (Liu *et al.*, 2010; Ren *et al.*, 2008).

**5.3.2.3. LC- MS/MS analysis of peptide extracted from APM**

LC-MS/MS results showed active fraction purified by HPLC matched to pathogenesis-related-protein-2 OS of *Phaseolus vulgaris* with the amino acid sequence IVEGNGGPGTIK. This peptide had antibacterial activity against pathogenic bacteria such as *B. cereus*. The peptides have amino acid residues, which may play a role in antibacterial activities against bacteria (Boman, 2003; Hammami *et al.*, 2009; Wang and Wang, 2004). Wong *et al.* (2006) reported this peptide (defensin) to have antibacterial activity against *Mycobacterium phlei, Bacillus megaterium, Bacillus subtilis* and *Proteus vulgaris* because this peptide have amino acid residues in its structure. Furthermore, it was shown to have antifungal activity against *Botrytis cinerea, Fusarium oxysporum* and *Mycosphaerella arachidicola*. Also Defensin has antifungal properties against *M. arachidicola, Setospaeria turcica* and *Bipolaris maydis* (Lin *et al.*, 2009).

### 5.3.2.4. Effect of fraction 1 of AMP on *B. cereus* and MRSA cells by SEM analysis

The SEM observations show that the peptide from APM causes changes which include roughening in bacterial cell surface, numerous blebs, pore formation and lysis of bacterial cells and accumulation of cell debris characteristic of the barrel-stave mechanism of action. Based on the barrel-stave model, the peptides interact with the hydrophobic portion of membrane and bind with acyl chains of phospholipid residues while the hydrophilic portion constitutes the interior of the pore (Dong *et al.*, 2012; Rahnamaeian, 2011). Thus peptides open the pore into the membrane and destroy bacterial cells by altering permeability. Pore formation depend on the concentration of peptides (Okorochenkov *et al.*, 2011). Other studies reported that the mode of action of antibacterial peptides appear to follow a membrane disruption model because peptide-mediated membrane disruption depended on the peptide integrity of the cell membrane or peptide translocation into the cytoplasm (Friedrich *et al.*, 2000; Larson, 1988).

# 6. CONCLUSION

Hot or cold aqueous extracts of *M. citrifolia* fruit and leaves, *A. angustiloba* roots and Australian plant mixture showed no antibacterial activity against all strains of test bacteria (*S. aureus* (RF 122), *E. coli* (UT181), *B. cereus* (ATCC 14579), *P. aeruginosa* (PA7), methicillin- resistant *S. aureus* (ATCC BA-43) and *H. pylori* (ATCC 43504). However, the cold or hot aqueous extracts of *A. squamosa* fruit showed significant antibacterial activity against Gram-positive and Gram-negative bacteria along with antioxidant activity. Methanolic extracts of *M. citrifolia* leaves, *A. angustiloba* roots and APM showed no inhibition against all strains of test bacteria. However, methanolic extracts of *M. citrifolia* fruit showed inhibition zones against the test bacteria including MRSA and *H. pylori*. The methanolic extracts from the fruit and leaves of *A. squamosa* showed antibacterial activity against the test bacteria except *H. pylori*. It was noted in this current study that the methanol extraction was better than aqueous extraction for antibacterial and antioxidant activities. GC-MS analysis showed that aqueous extract from *A. squamosa* fruit has active diterpenes, such as ent-spathulenol, kauran-18-al, kaur-16-ene, kauren-18-ol and trihydroxycholan-24-oic acid while the methanolic extract of the fruit of *A. squamosa* revealed the presence of three active compounds named 2-isoxazolidine, 4,5-octanediol and 2,3-hexanediol. The methanolic extracts of *A. squamosa* leaves revealed the presence of alkaloids such as isoquinoline, 1,6-methylphenazine and 1,2,3,4-tetrahydroisoquinoline. However, the methanolic extract of *M. citrifolia* fruit revealed the presence of many compounds such as 1-butanecarboxylic acid, butyric acid, N-acetylisoxazolidine, propanoic acid, carbamimidic acid, 1,3-propanediol, 4,5-octanediol, 1,3-oxazine and semicarbazone. These compounds were identified by GC-MS analysis from different morphological parts of these plants.

Anthraquinones extract of the fruit, leaves and roots of *M. citrifolia* have antibacterial effects against all pathogenic bacteria including important resistant bacteria such as MRSA and *H. pylori* while anthraquinones extract of the fruit gave significant antibacterial properties compared to other anthraquinones extracts. Also anthraquinones extract from the fruit of this plant exhibited a high level of antioxidant activity with significant value of $IC_{50}$ 5.48 mg/ml compare to leaves $IC_{50}$ 7.76 mg/ml and roots at 19.85 mg/ml. IR spectra of anthraquinones extracted from the morphological parts of *M. citrifolia* revealed the major functional groups were O-H stretching, C-H stretching and C=O groups. LC-MS analysis of anthraquinones extracted from the fruit of *M. citrifolia* identified that the major compounds were identified 1-hydroxy-2-methylanthraquinone, 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl)anthraquinones, morindolin, 1,1-*O*i-O-methylmorindol, 1,2-dihydroxyanthraquinone, 1,3,6-trihydroxy-2-methoxyanthraquinone. LC-MS analysis of anthraquinones extracted from *M. citrifolia* leaves identified the five major compounds as 1,2-dihydroxyanthraquinone, 1-hyroxy-2,3-methoxyanthraquinone, 2,6-diroxy-1,3-methoxyanthraquinone, 2-hydroxy-1-methoxyanthraquinone and 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl)anthraquinones. LC-MS analysis of anthraquinones extracted from the roots of *M. citrifolia* identified the major compounds as damnacanthol, morenone, 2-ethoxy-1-hydroxyanthraquinone and damnacanthal. SEM analysis of anthraquinones extracted from *M. citrifolia* indicated a bactericidal effect of these compounds related to breakage in cell wall and membrane with roughening in cell surface and leakage of cytoplasmic contents.

Alkaloids extracts of *M. citrifolia* fruit, *A. squamosa* leaves and *A. angustiloba* roots had antibacterial activity against all strains of test bacteria including MRSA and *H. pylori*. However, *P. aeruginosa* has resistance against alkaloid extracts of *M. citrifolia* fruit. Alkaloids extract of *A. squamosa* leaves have a high level of antioxidant activity

with significant value of $IC_{50}$ (5.51 mg/ml) compared to alkaloids extracts of *A. angustiloba* roots ($IC_{50}$ 10.04 mg/ml) and alkaloid extract from *M. citrifolia* fruit (18.05 mg/ml). IR spectra of alkaloids extracted from the fruit of *M. citrifolia*, leaves of *A. squamosa*, and the root *A. angustiloba* exhibited showed the presence of O-H band, N-H band, C=O band and C-H stretching groups. LC-MS analysis of alkaloids extracted from *M. citrifolia* fruit identified five major compounds: pelletierine, sedamine, pseudopelletierine, halosine and lycopodine. LC chromatograms and MS data identified the major compounds of alkaloids extracted from the leaves of *A. squamosa*, as corydine, sanjoinine, norlaureline, norcodeine, oxanalobine and aporphine. The major compounds in the alkaloids extracted from the roots of *A. angustiloba* were identified as echitamine, 3-H-indole, 1-H-indole, alstilobanine B and alstilobanine E. SEM observation of the mode of action of alkaloids extracted from the leaves of *A. squamosa* on bacterial cells showed changes in cell morphology such as swelling of cells, rupture in cell wall and cell lysis.

The phenolic compounds extracted from APM have antibacterial activity against most test bacterial strains but *P. aeruginosa* and *H. pylori* were resistant. Diterpenes extracted from *A. squamosa* fruit showed significant antibacterial activity against pathogenic bacteria and MRSA. However, *H. pylori* showed resistance against these extracts. Diterpenes of *A. squamosa* fruit and phenolic compounds extracted from APM exhibited a high level of DPPH radical scavenging activity and SOD-like activity with significant $IC_{50}$ values of 5.40 mg/ml and 5.74 mg/ml respectively. APM showed the highest amounts of phenolic compounds. The IR spectra of the phenolic compounds from APM showed O-H group, C-H stretching and C=O groups. IR spectra of diterpenes extracted from *A. squamosa* fruit showed O-H stretching, C-H stretching, C=O group, C-H group and C-O functional groups.

LC chromatograms and MS data identified the major phenolic compounds extracted from APM as hydroxybenzoic acid-hexoside, luteolin, isohamnetin, apigenin-7-O-rutinoside, quercetin, HHDP-gallogluco-pyranoside, dicaffeoyquinic acid, rosmadial, caffeic acid. LC-MS analysis of diterpenes extracted from *A. squamosa* fruit identified six major compounds: kuaran-18-al, 16,17,19-kauranetriol, kauren-18-ol, kaur-16-ene, stigmasterol and annosquamosin. The mechanism of action of diterpenes extracted from *A. squamosa* fruit on bacterial cells includes alteration of membrane permeability leading to draining out of the inner contents and cell lysis.

In this study, the peptides extracted from lactic acid bacteria (*L. casei* BL 23, *L. casei* ATCC 11578, *L. paracasei* subsp. *paracasei* 25302, *L. paracasei* subsp. *paracasei* 8700:2) by the acidic methanolic method showed antibacterial activity against all pathogenic bacteria including important resistant bacteria such as MRSA and *H. pylori. L. paracasei* subsp. *paracasei* 8700:2 showed significant antibacterial activity using two methods namely chloroform and acidic methanolic extractions. The peptides extracts of *L. casei* and *L. paracasei* have antioxidant activities based on the DPPH and SOD assays but peptides extracts from the cell acidification method have a higher level of antioxidant activity. IR spectrum data identified the main functional groups of peptide extract such as O-H stretching. C-H stretching, C=N bending with C-C bending groups. LC-MS analysis of antibacterial peptide purified fraction from *L. paracasei* subsp. *paracasei* 8700:2 by FPLC was identified a novel bacteriocin. Electron microscopy studies on the effects of active peptide from *L. paracasei* subsp. *paracasei* 8700:2 on *B. cereus* and MRSA showed several changes in cell surface morphology, numerous blebs and lysis. TEM of the exposure of *H. pylori* to active peptide gave changes in cell morphology and cell membrane with loss of cytoplasmic contents due to cell lysis of bacterial cells.

Peptides extracted from the medicinal plants showed antibacterial activity while the peptides of *M. citrifolia* seeds showed antibacterial activity against the test bacteria with the exception of *H. pylori*. Fraction 1 of APM purified by gel filtration chromatography showed antibacterial activity against the test bacteria except *P. aeruginosa* but was most effective on *B. cereus*. Peptides extracted from medicinal plants had antioxidant activities. LC-MS analysis of active fraction purified by HPLC showed the peptide to be part of pathogenesis-related protein 2 of *Phaseolus vulgaris* has the sequences IVEGNGGPGTIK. This peptide showed antibacterial activity against *B. cereus*. The SEM results indicated that the bacteriolytic properties of this active peptide were related to membrane disruption with bubble-like formations followed by cell lysis.

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# LIST OF PUBLICATIONS AND PAPERS PRESENTED

## PUBLICATIONS

1. Shami, A. M., Philip, K. & Muniandy, S. (2013). Synergy of antibacterial and antioxidant activities from crude extracts and peptides of selected plant mixture. *BMC Complementary and Alternative Medicine, 13,* 360-375. doi:10.1186/1472-6882-13-360

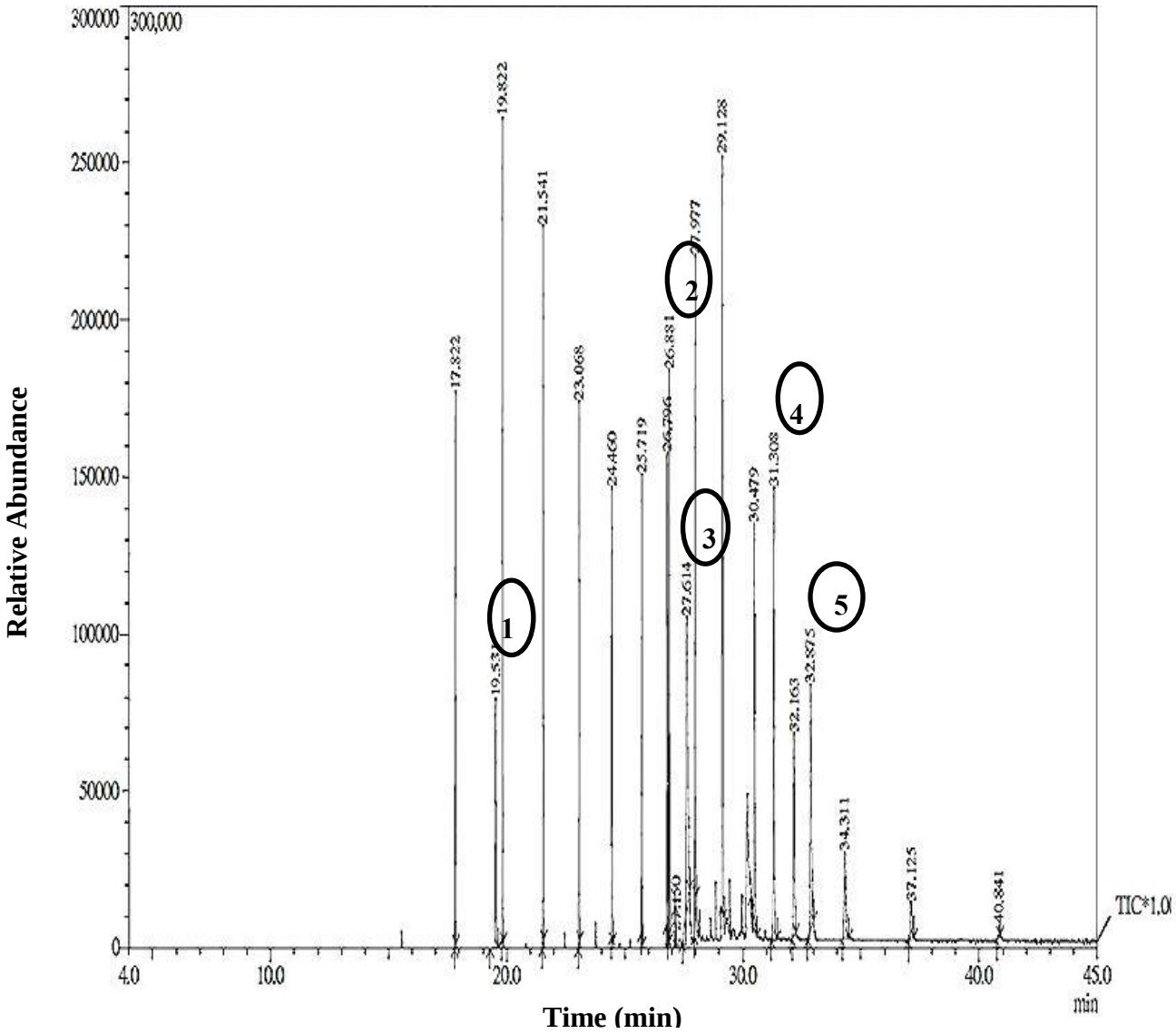
## MANUSCRIPTS SUBMITTED FOR REVIEW

1. Antibacterial and antioxidant properties and mode of action of peptides extracted from *Lactobacillus spp.* (submitted to LWT-Food science and Technology).
2. Antibacterial and antioxidant effects of anthraquinones extracted from *Morinda citrifolia* L. (Rubiaceae). (Submitted to Evidence-Based Complementary and Alternative Medicine).
3. Antibacterial, antioxidant activities and LC-MS analysis of diterpenes from the fruit of *Annona squamosa* (Annonaceae). (Submitted to Molecules).
4. Antibacterial and antioxidant effects of alkaloids extracted from local selected plants in Malaysia. ). (Submitted to Ethnopharmacology).
5. *In Vitro* antibacterial, antioxidant and GC-MS analysis of *Morinda citrifolia* and *Annona squamosa* extracts. (Submitted to Evidence-Based Complementary and Alternative Medicine).

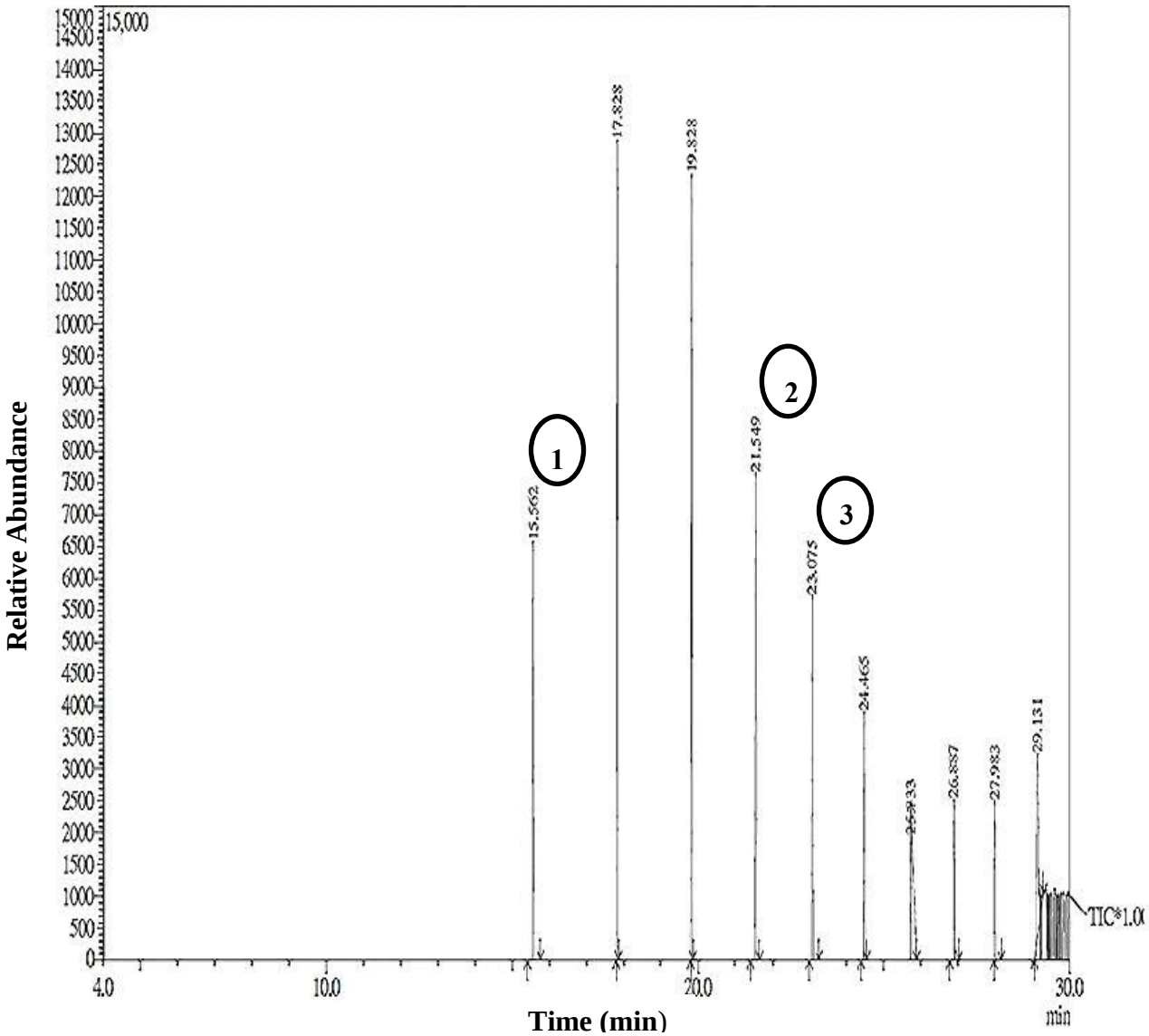
**PAPERS PRESENTED**

1- Shami, A. M., Philip, K. & Muniandy, S. (2011).Antibacterial effect, antioxidant potential and content of phenolic compounds in the aqueous and ethanolic extracts of Australian plant mixture against selected microorganisms. QR22 M4ICMSM International Congress of the Malaysian Society for Microbiology 2011 (8-11 Dec 2011: Batu Ferringhi, Penang). Proceedings, editors: Raha Abdul Rahim ... [*et al*.] (Serdang : Malaysian Society for Microbiology, 2011), Vol.1 (348-35)

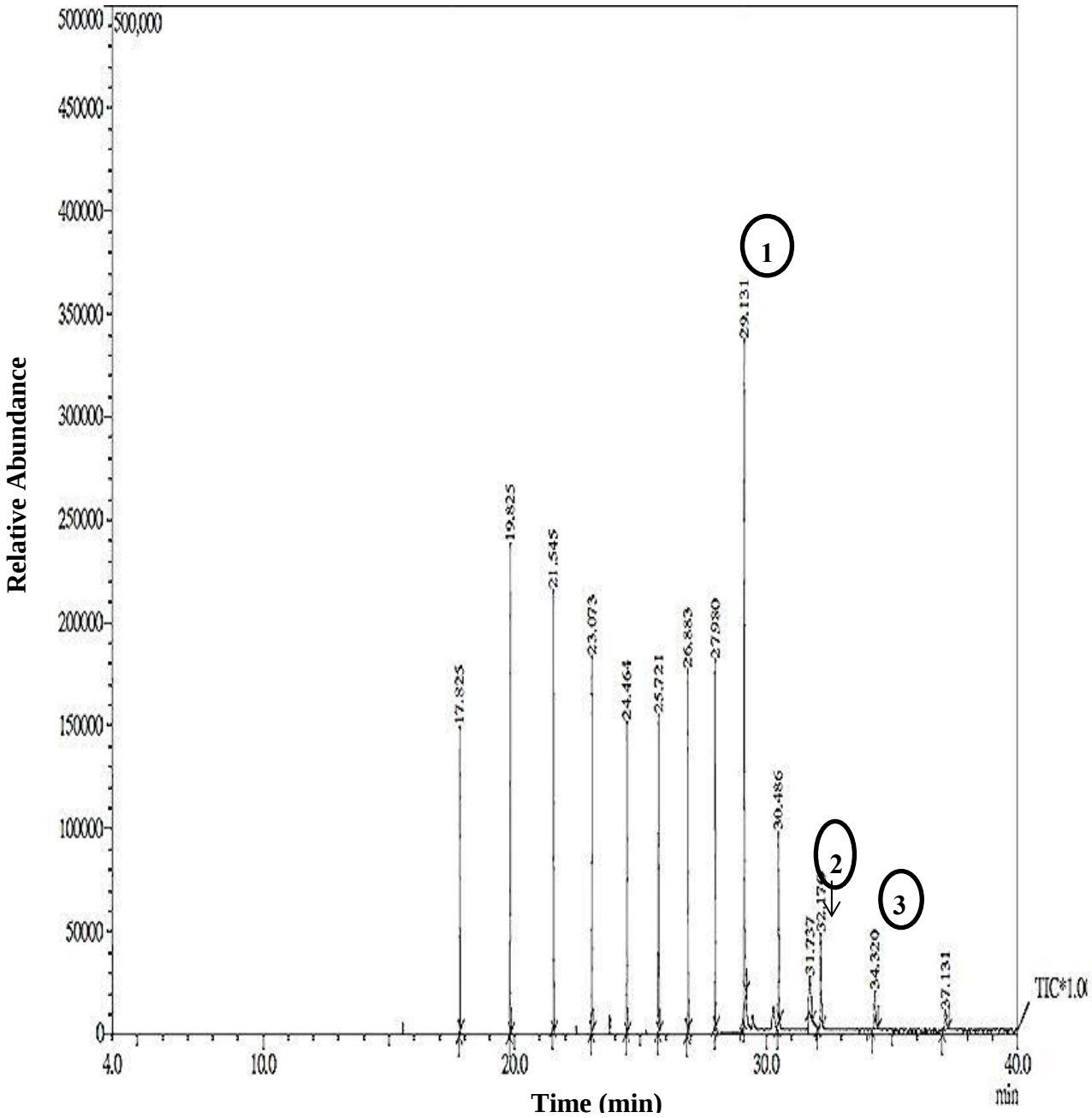
# APPENDICES



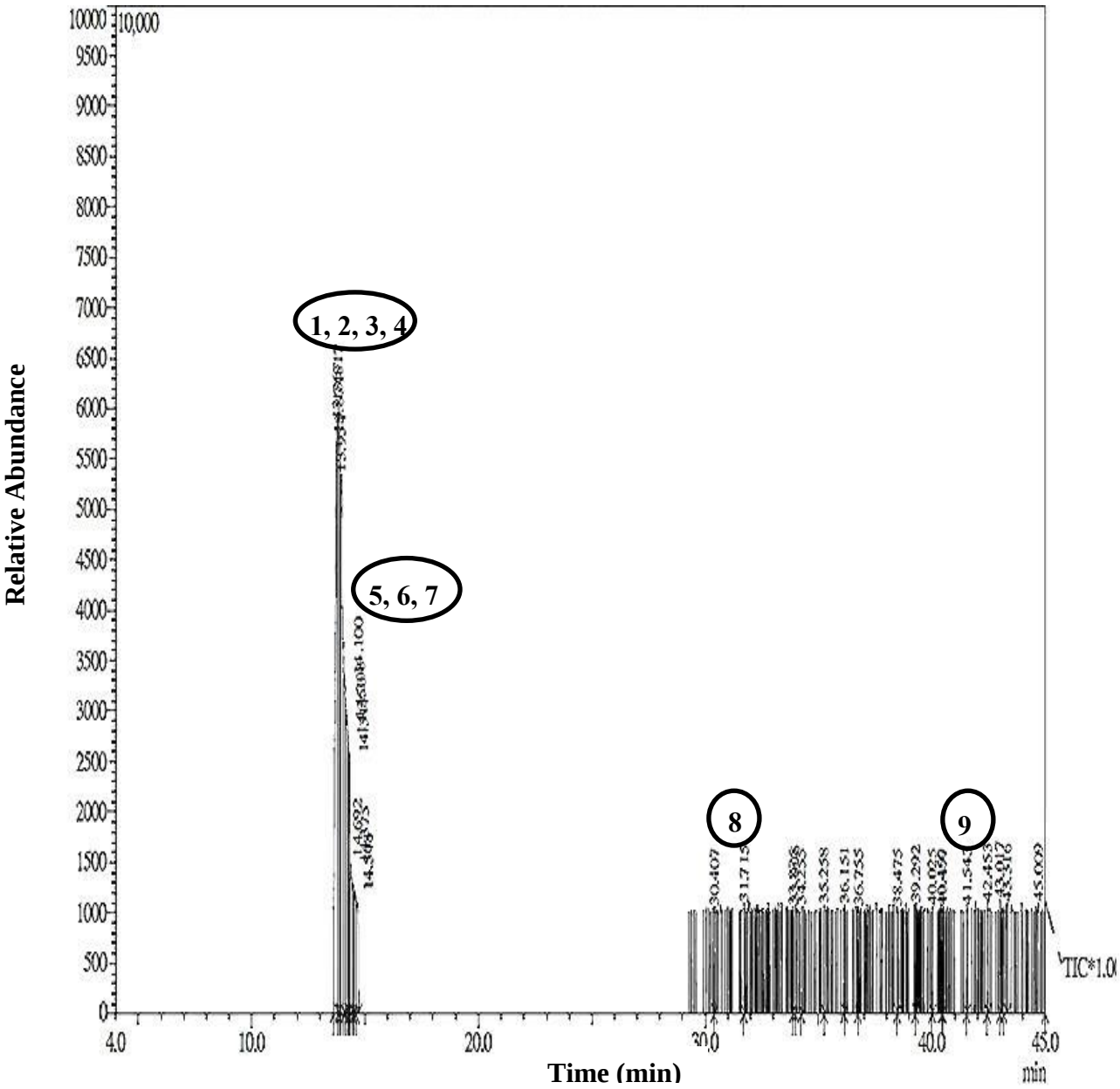

**Appendix 1.** GC-MS chromatogram of aqueous extract from *A. squamosa* fruit. (**1**) Ent-spathulenol at 19.53 min; (**2**) Kauran-18-al at 26.79 min; (**3**) Kaur-16-ene at 27.61min; (**4**) Kauren-18-ol at 31.30 min and **(5**) Trihydroxychola-n-24-oic acid at 32.87 min.



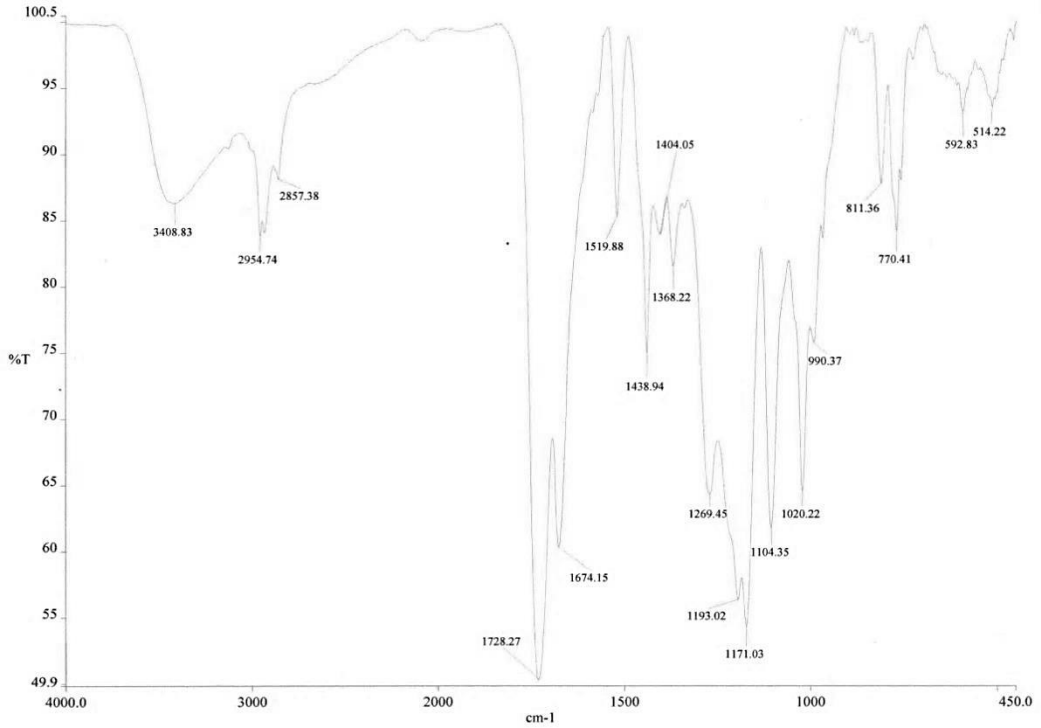

**Appendix 2.** GC-MS chromatogram of the methanolic extract of *A. squamosa* fruit. (**1)** 2-Isoxazolidine at 15.56 min; (**2**) 4,5-Octanediol at 21.54 min and (**3**) 2,3-Hexanediol at 23.07 min.



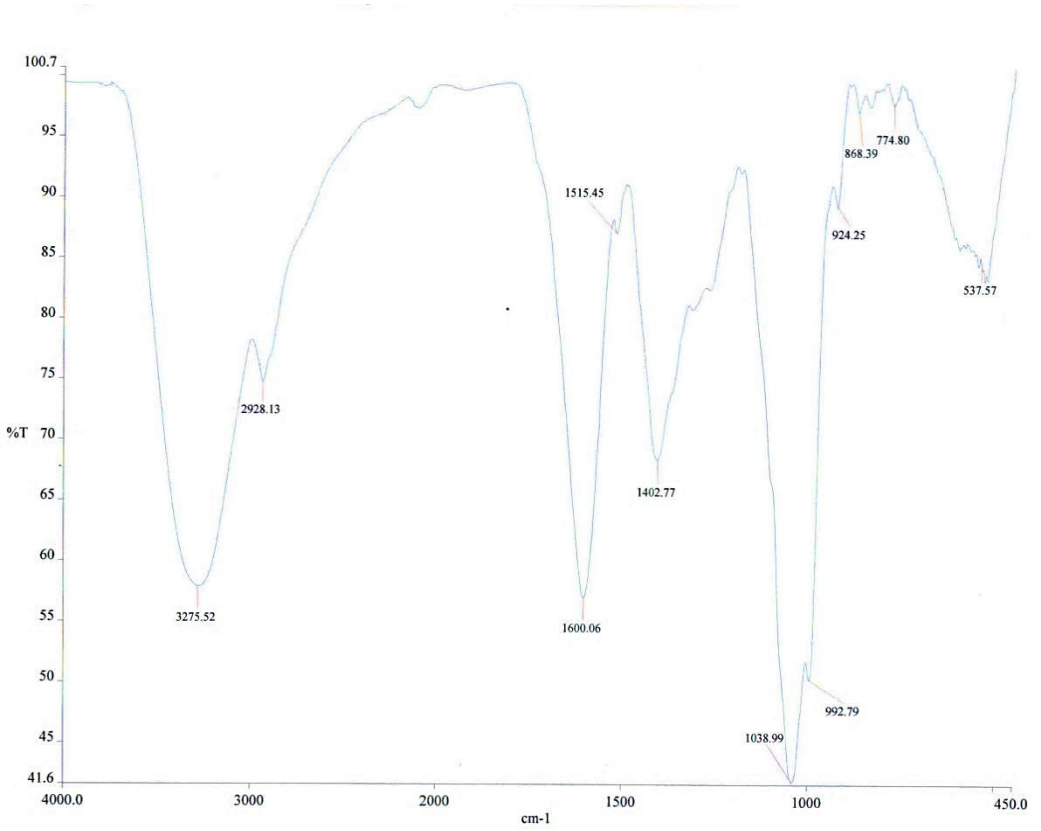

**Appendix 3.** GC-MS chromatogram of the methanolic extracts of *A. squamosa* leaves. (**1)** Isoquinoline at 29.13 min; (**2**) 1,6-Dimethylphenazine at 31.73 min and (**3**) 1,2,3,4-Tetrahydroisoquinoline at 34.32 min.



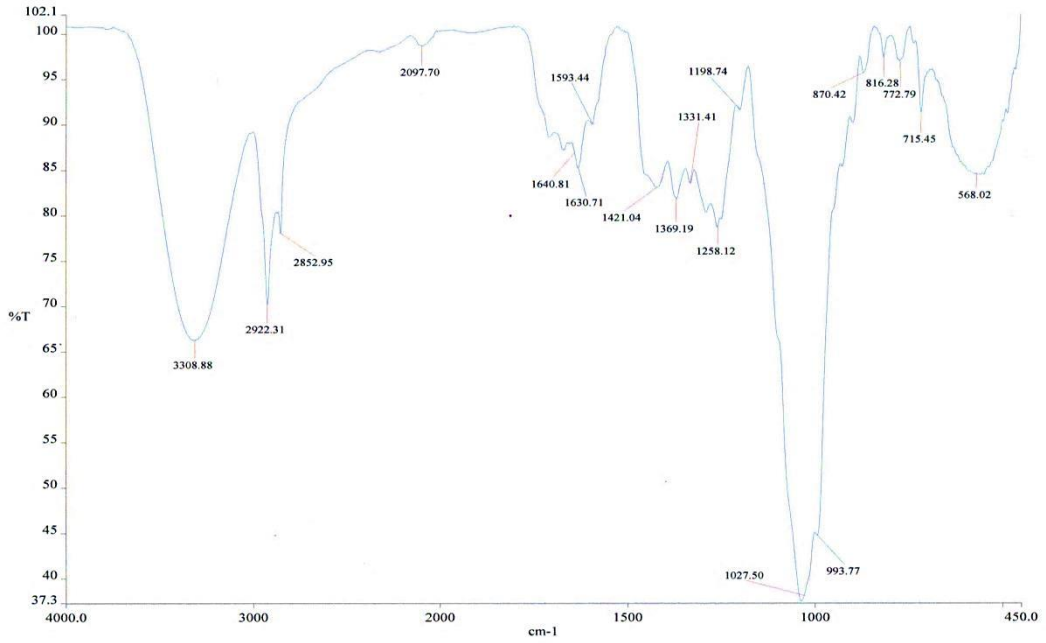

**Appendix 4.** GC-MS chromatogram of the methanolic extracts of *M. citrifolia* fruit. **(1)** 1-Butanecarboxylic acid at 13.76 min; (**2**) Butyric acid at 13.81 min; (**3**) N-Acetylisoxazol-dine at 13.87 min; (**4)** Propanoic acid at 13.95 min; (**5**) Carbamimidic acid at 14.10 min; (**6**) 1,3-Propanediol at 14.20 min; (**7)** 4,5-Octanediol at 14.25 min; (**8**) 1,3-Oxazine at 30.40 min and (**9**) Semicarbazone at 40.02 min.



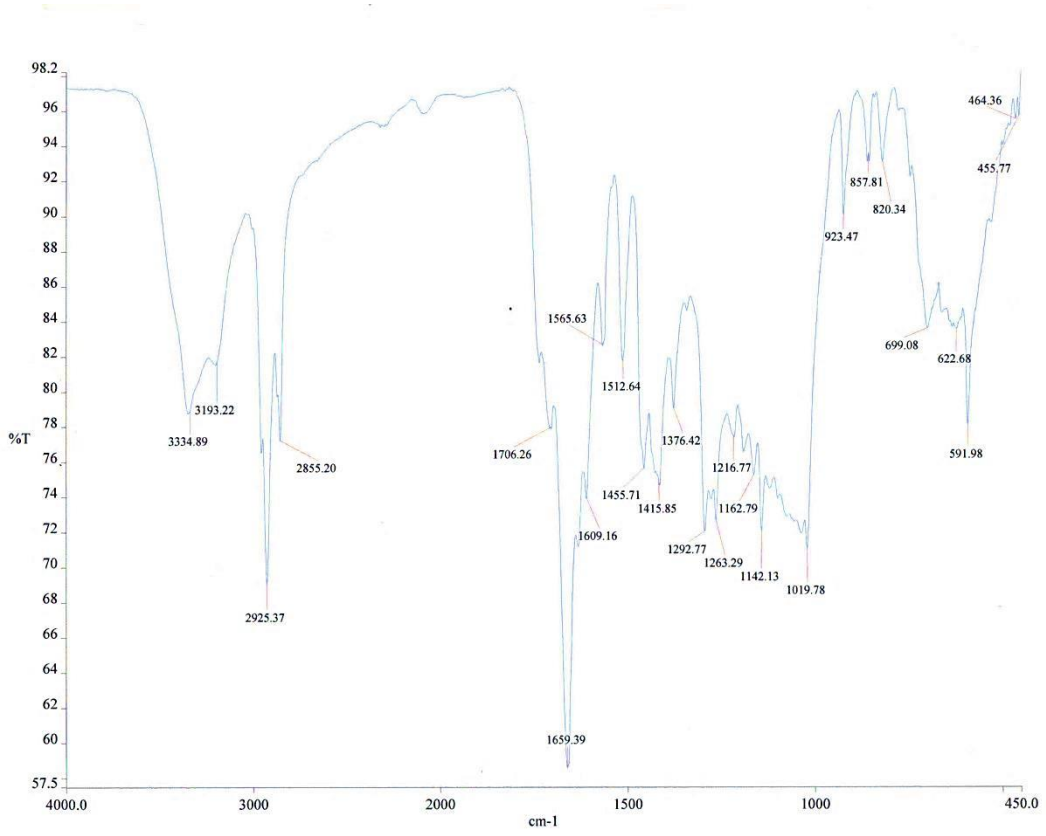

**Appendix 5.** IR spectra of anthraquinones extracted from the fruit of *M. citrifolia*.



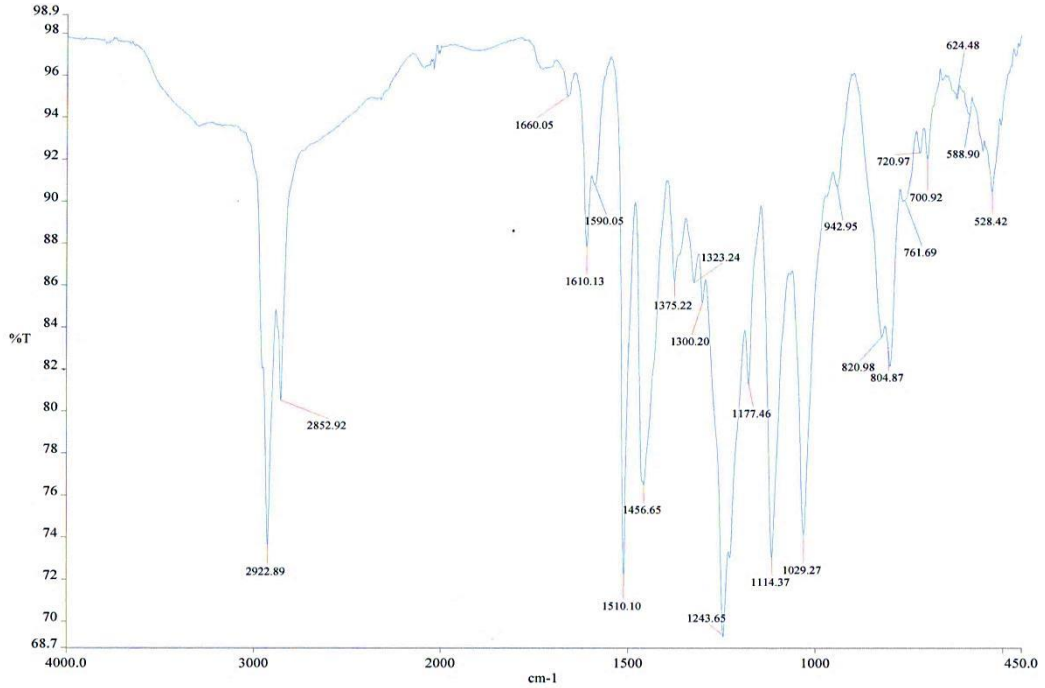

**Appendix 6.** IR spectra of anthraquinones extracted from the leaves of *M. citrifolia*.



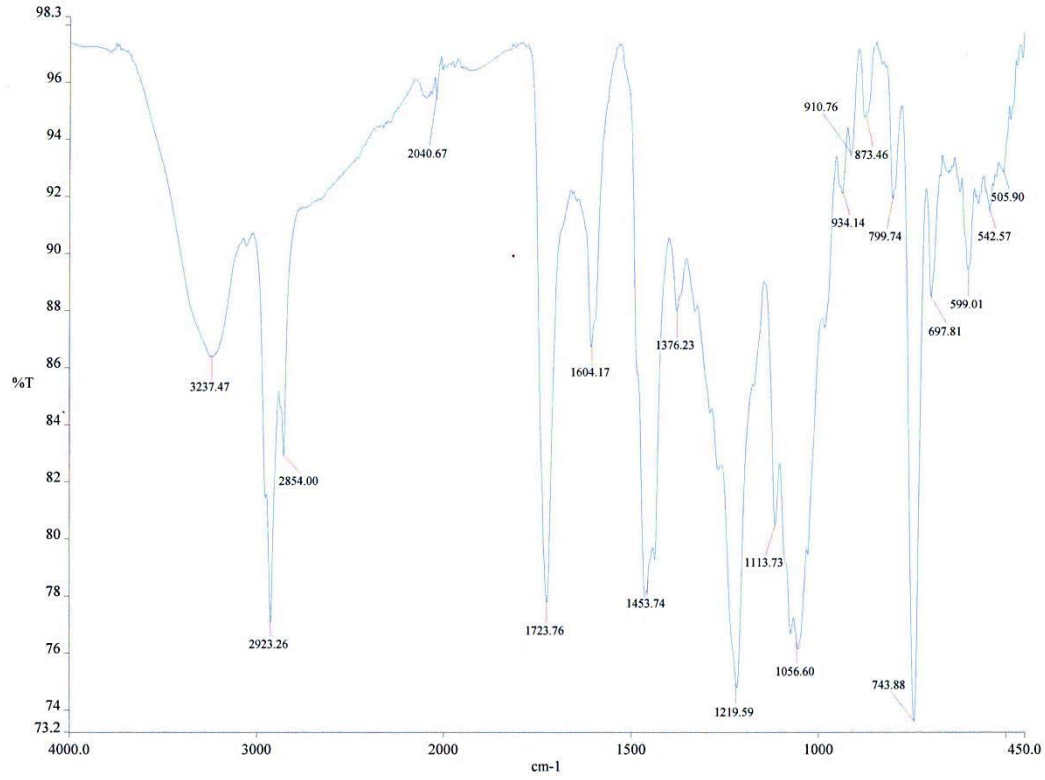

**Appendix 7.** IR spectra of anthraquinones extracted from the roots of *M. citrifolia*.



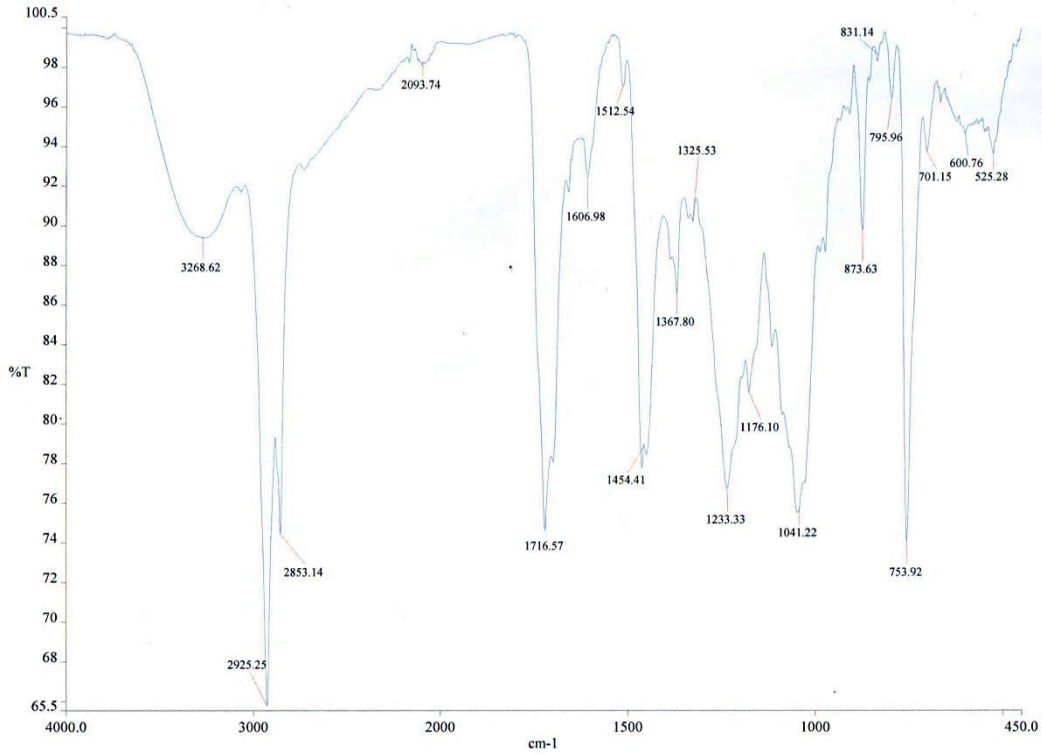

**Appendix 8.** IR spectra of alkaloids extracted from the fruit of *M. citrifolia.*



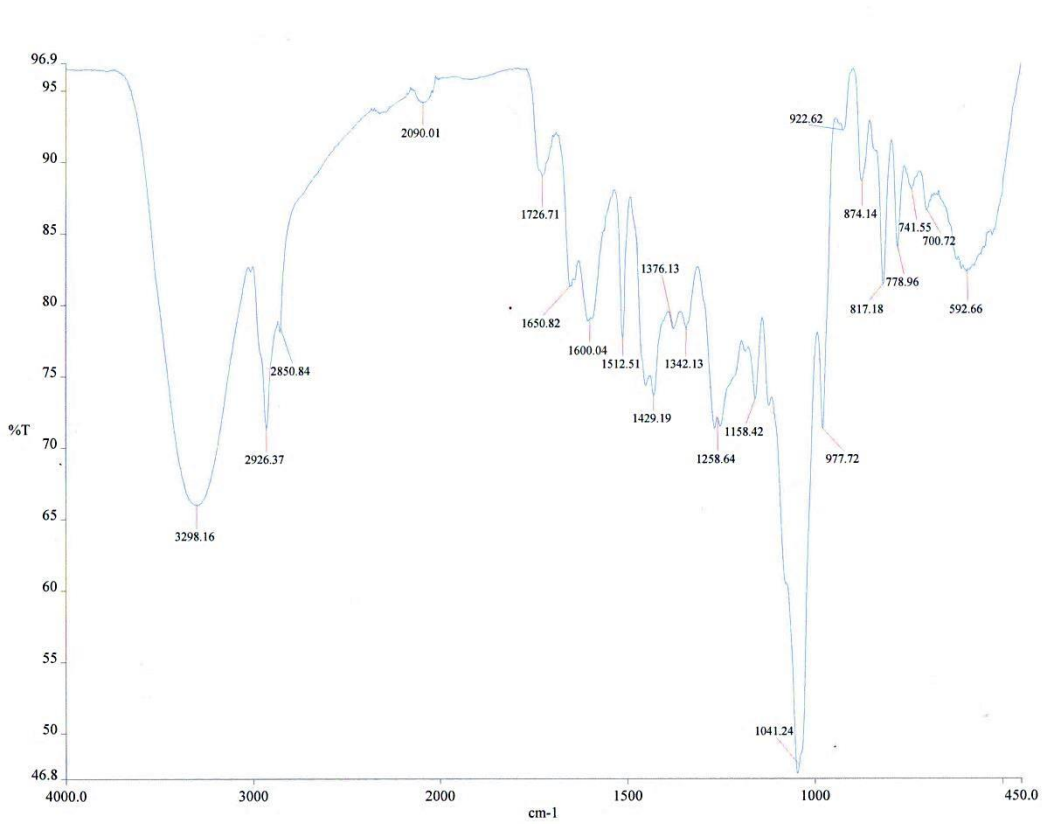

**Appendix 9.** IR spectra of alkaloids extracted from the leaves of *A. squamosa.*



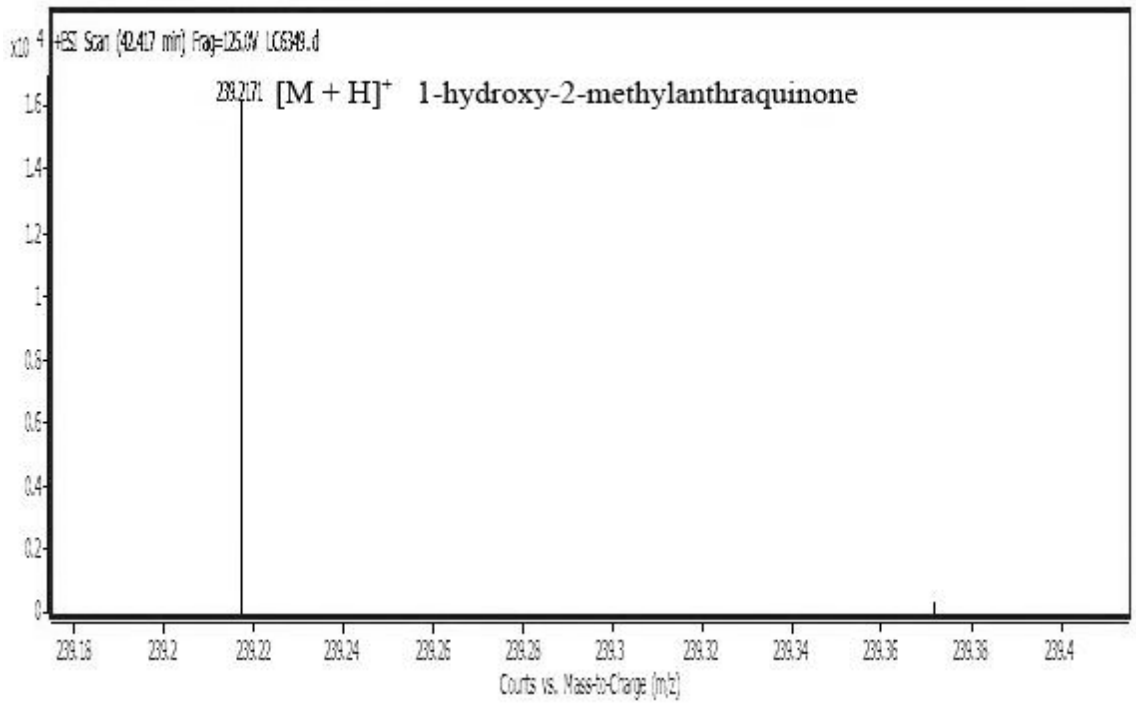

**Appendix 10.** IR spectra of alkaloids extracted from the roots of *A. angustiloba.*



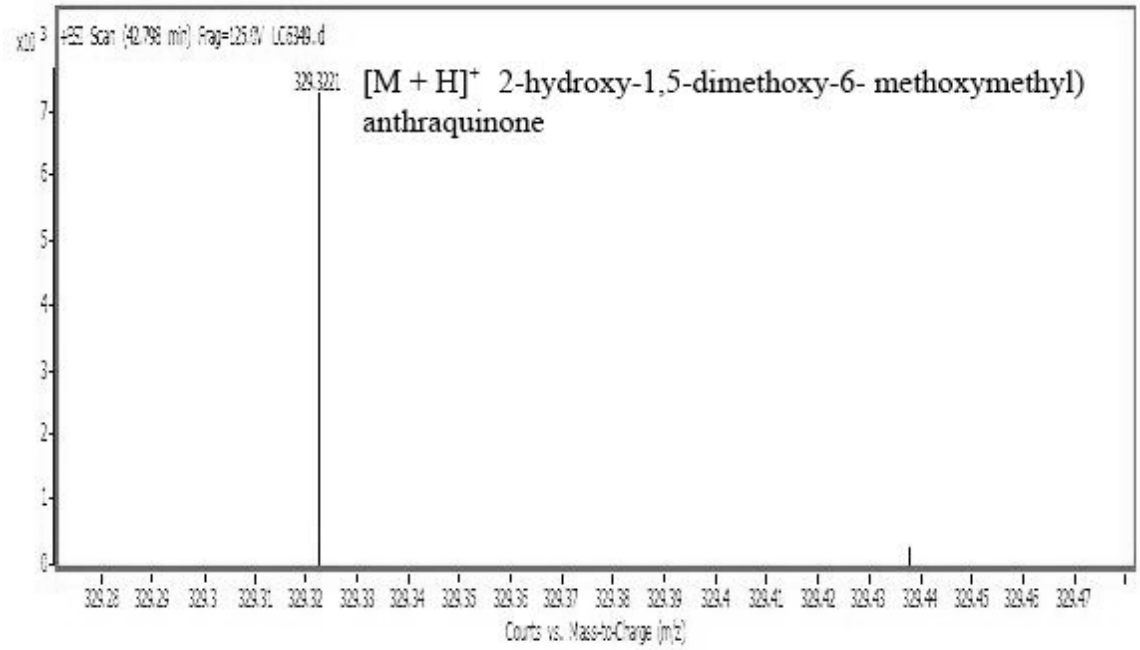

**Appendix 11.** IR spectra of diterpens extract from the fruit of *A. squamosa.*



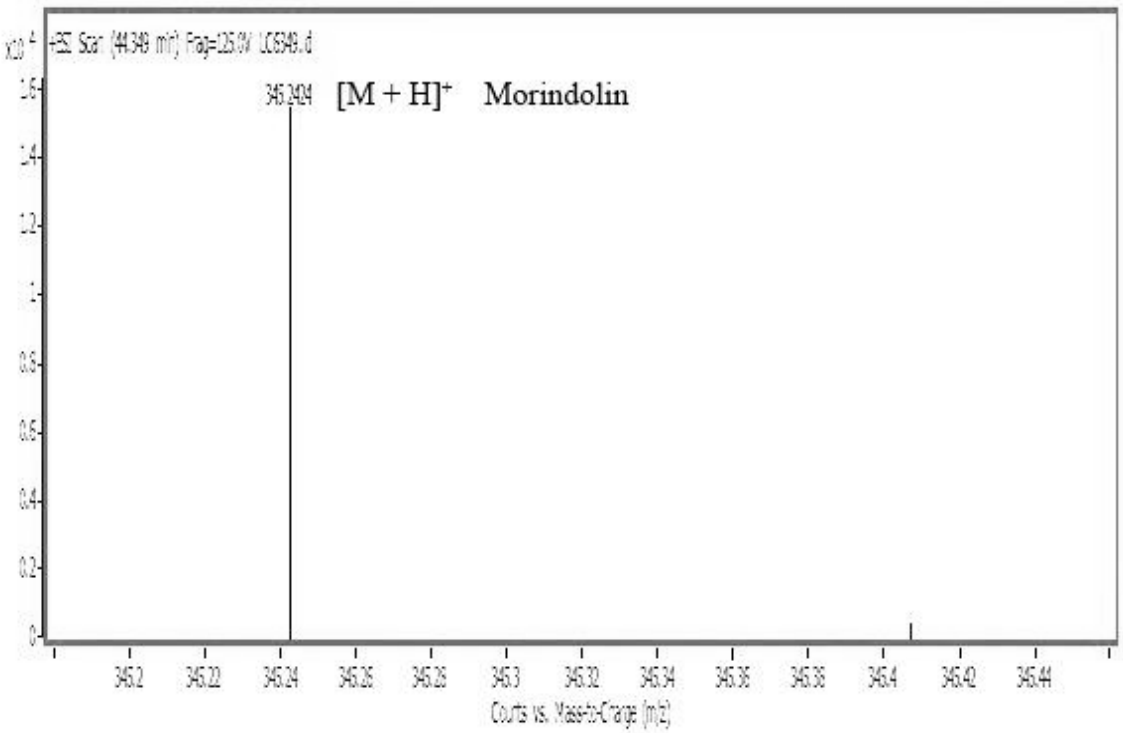

**Appendix 12.** IR spectra of the phenolic compounds from Australian plant mixture.



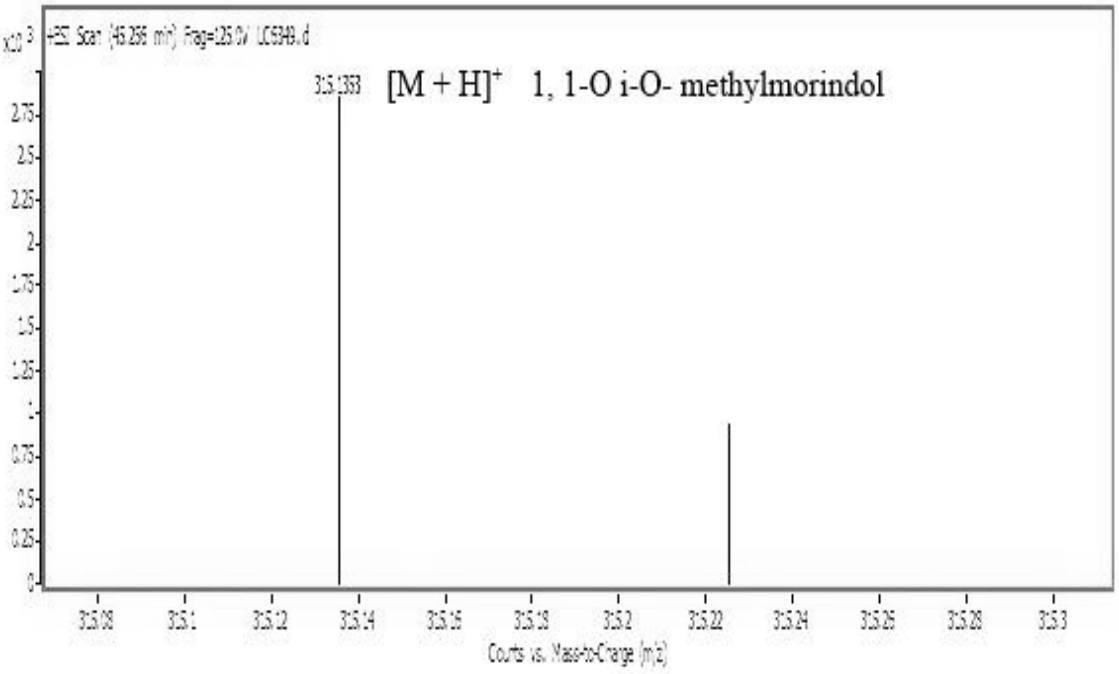

**Appendix 13.** MS/MS spectrum of 1-hydroxy-2-methylanthraquinone extracted from the fruit of *M. citrifolia* at *m/z* 239.2117.



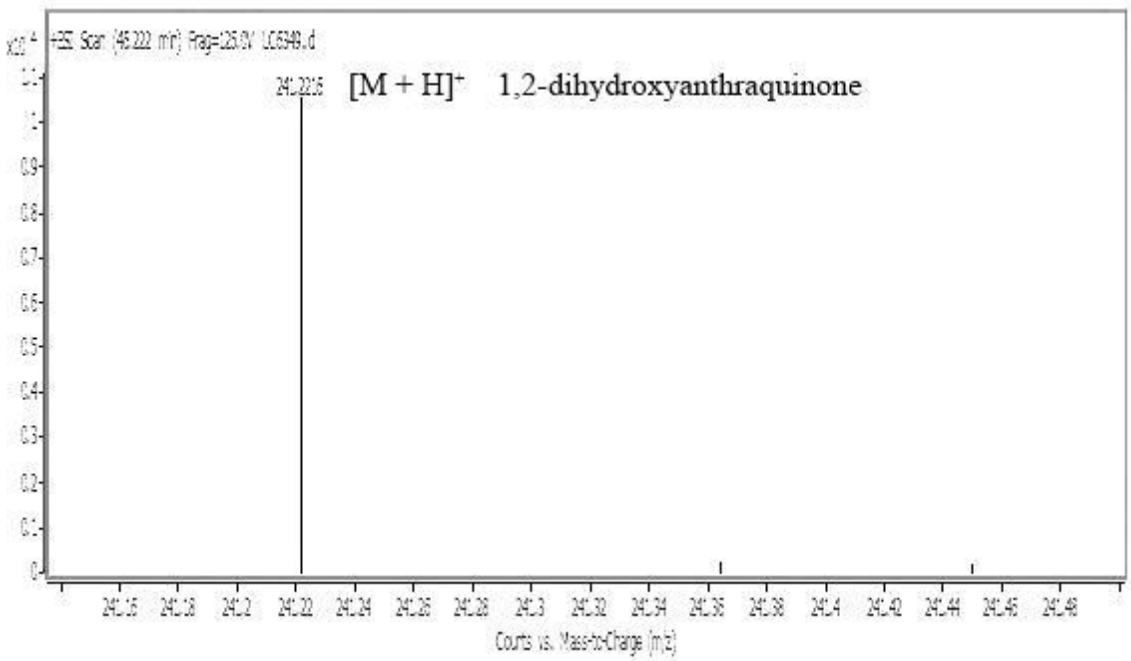

**Appendix 14.** MS/MS spectrum of hydroxy-1,5-dimethoxy-6-methoxymethyl) anthraquinones extracted from the fruit of *M. citrifolia* at *m/z* 329.3221.



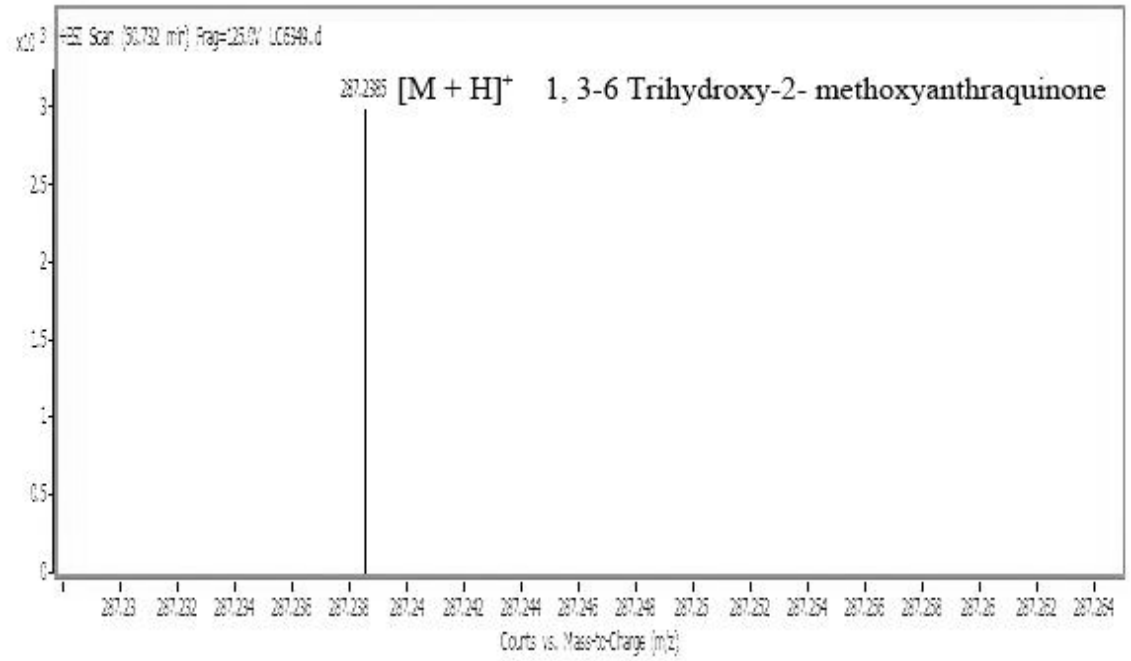

**Appendix 15.** MS/MS spectrum of morindolin extracted from the fruit of *M. citrifolia* at *m/z* 345.2424.



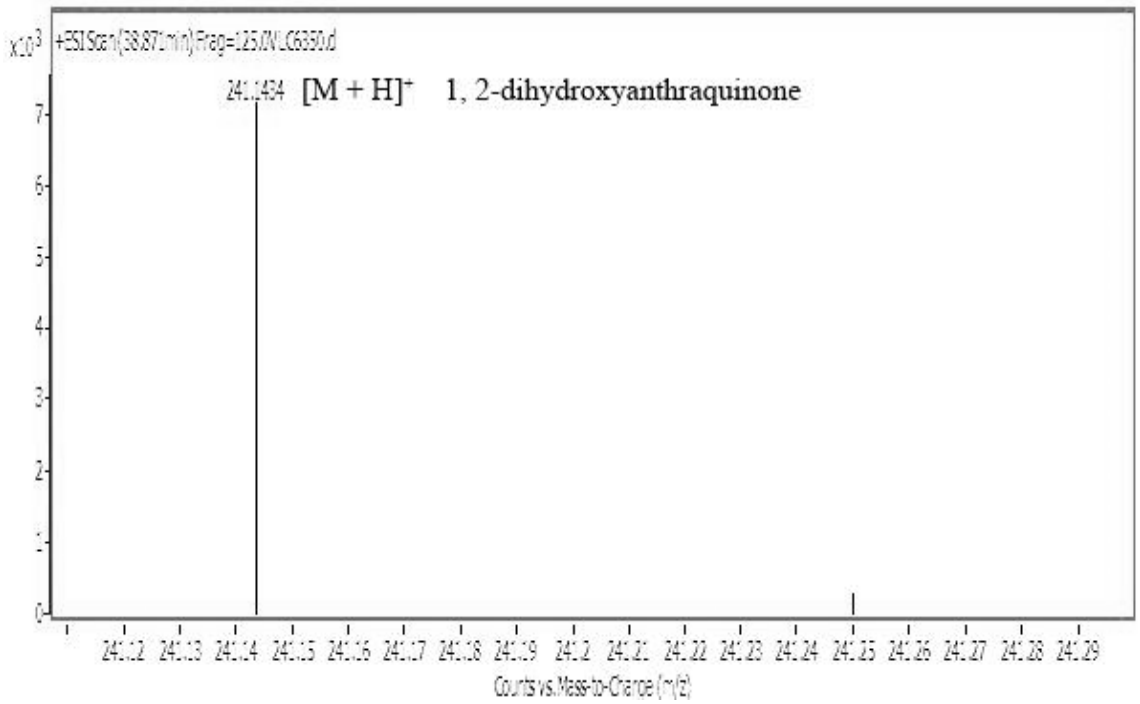

**Appendix 16.** MS/MS spectrum of 1,1-*Oi*-O-methyl morindol extracted from the fruit of *M. citrifolia* at *m/z* 315.1353.



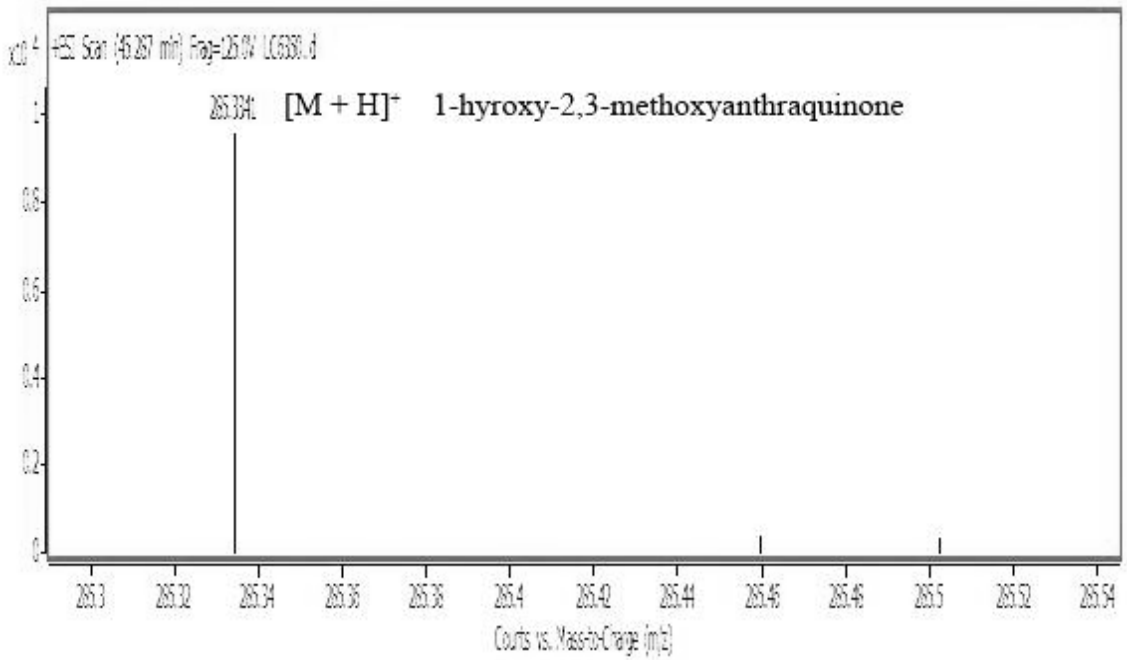

**Appendix 17.** MS/MS spectrum of 1,2-dihydroxyanthraquinone extracted from the fruit of *M. citrifolia* at *m/z* 241.221.



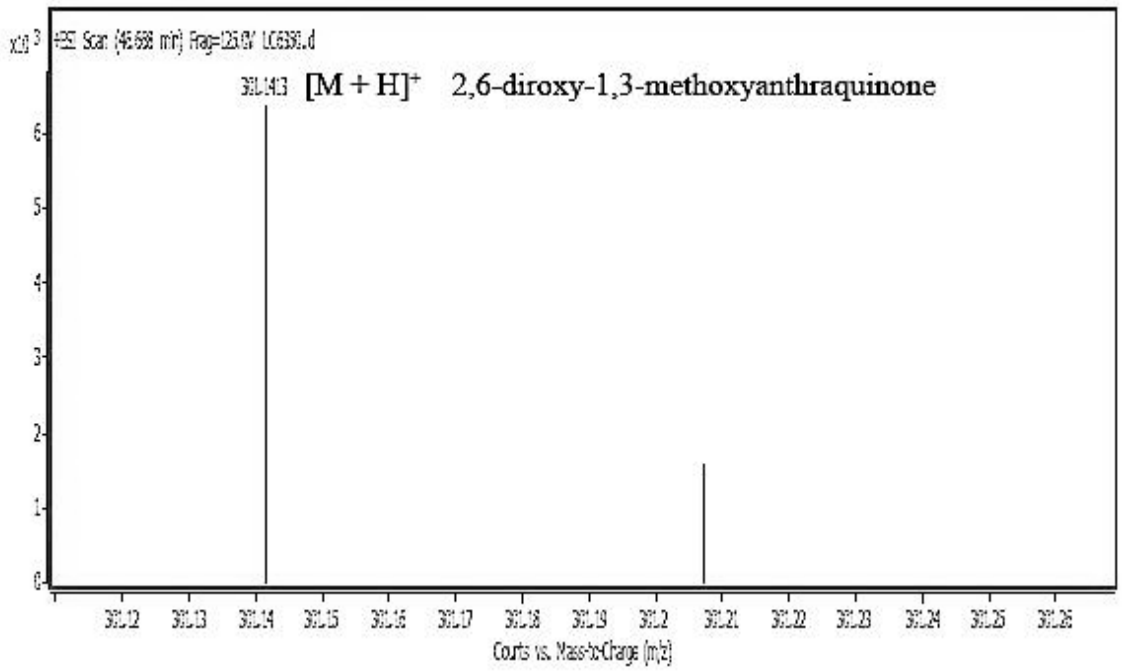

**Appendix 18.** MS/MS spectrum of 1,3-6 Trihydroxy-2-methoxyanthraquinone extracted from the fruit of *M. citrifolia* at *m/z* 287.2385.



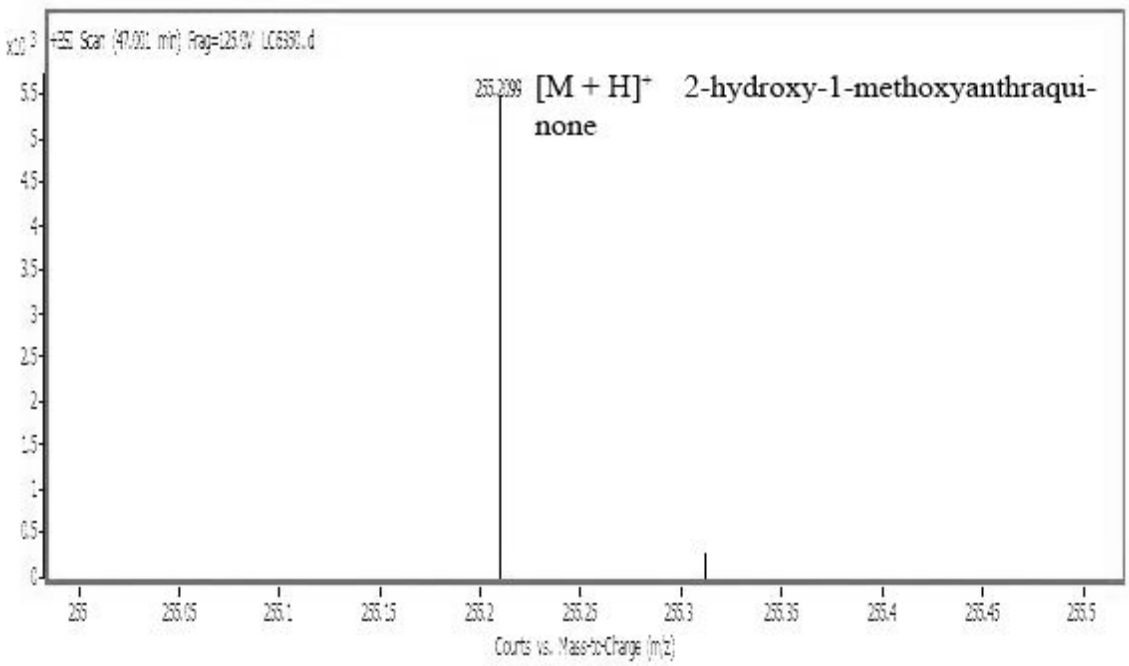

**Appendix 19.** MS/MS spectrum of 1,2-dihydroxyanthraquinone extracted from the leaves of *M. citrifolia* at *m/z* 241.1434.



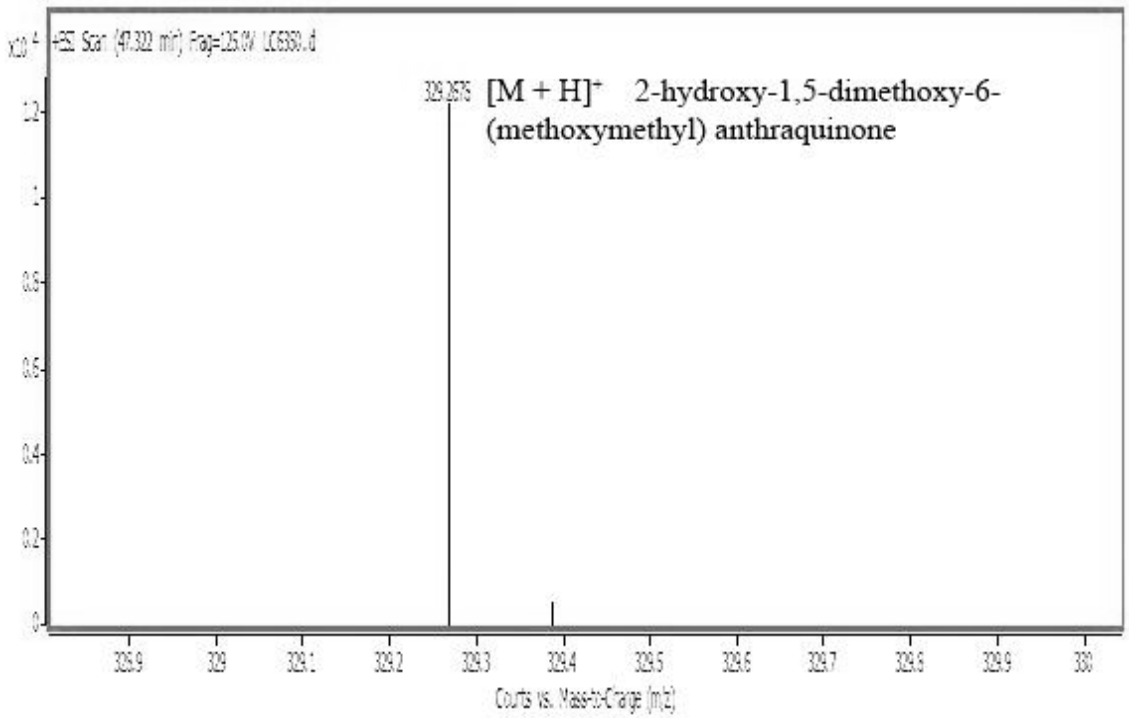

**Appendix 20.** MS/MS spectrum of 1-hyroxy-2,3-methoxyanthraquinone extracted from the leaves of *M. citrifolia* at *m/z* 285.3341.



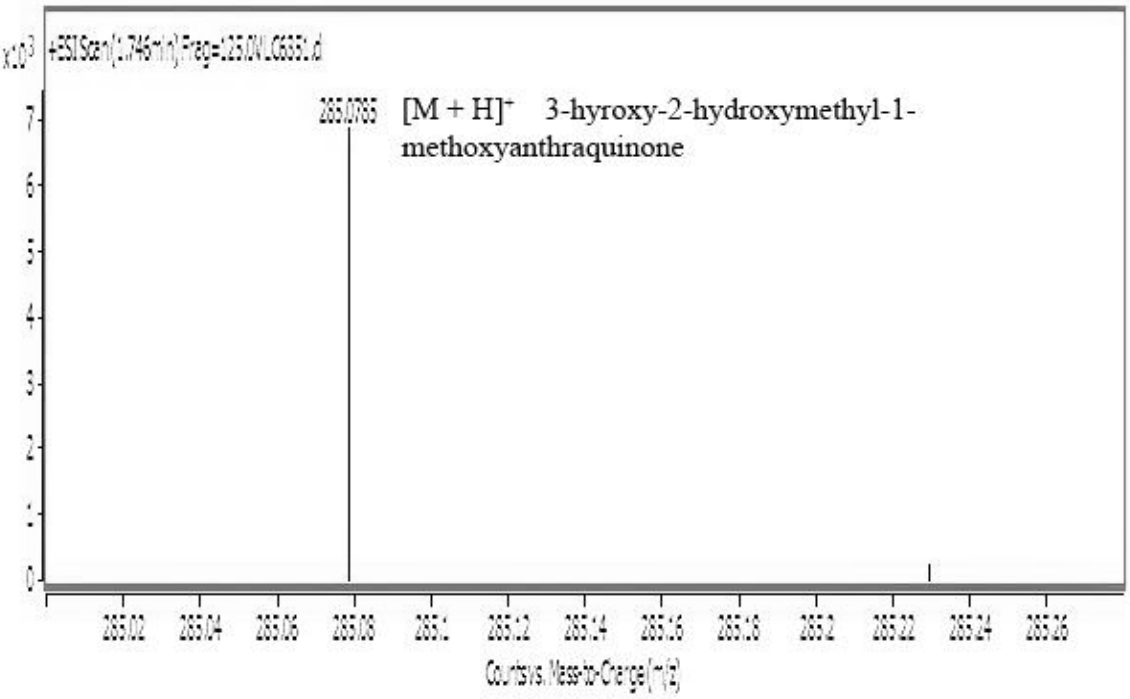

**Appendix 21.** MS/MS spectrum of 2,6-diroxy-1,3-methoxyanthraquinone extracted from the leaves of *M. citrifolia* at *m/z* 301.1413.



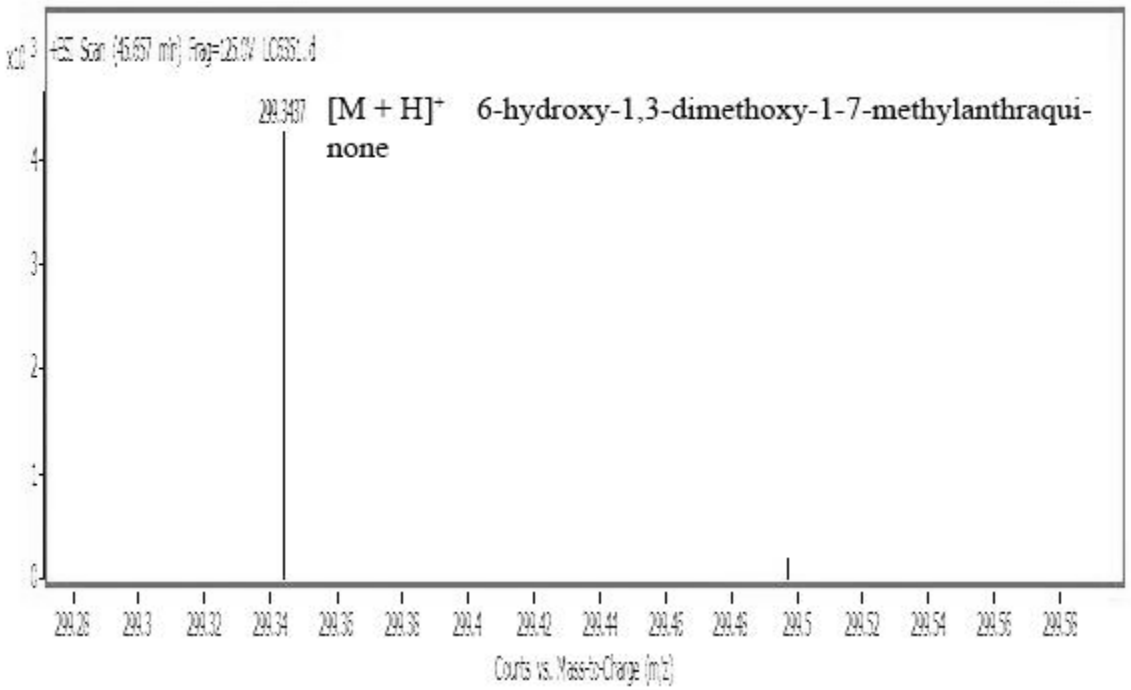

**Appendix 22.** MS/MS spectrum of 2-hydroxy-1-methoxyanthraquinone extracted from the leaves of *M. citrifolia* at *m/z* 255.2099.



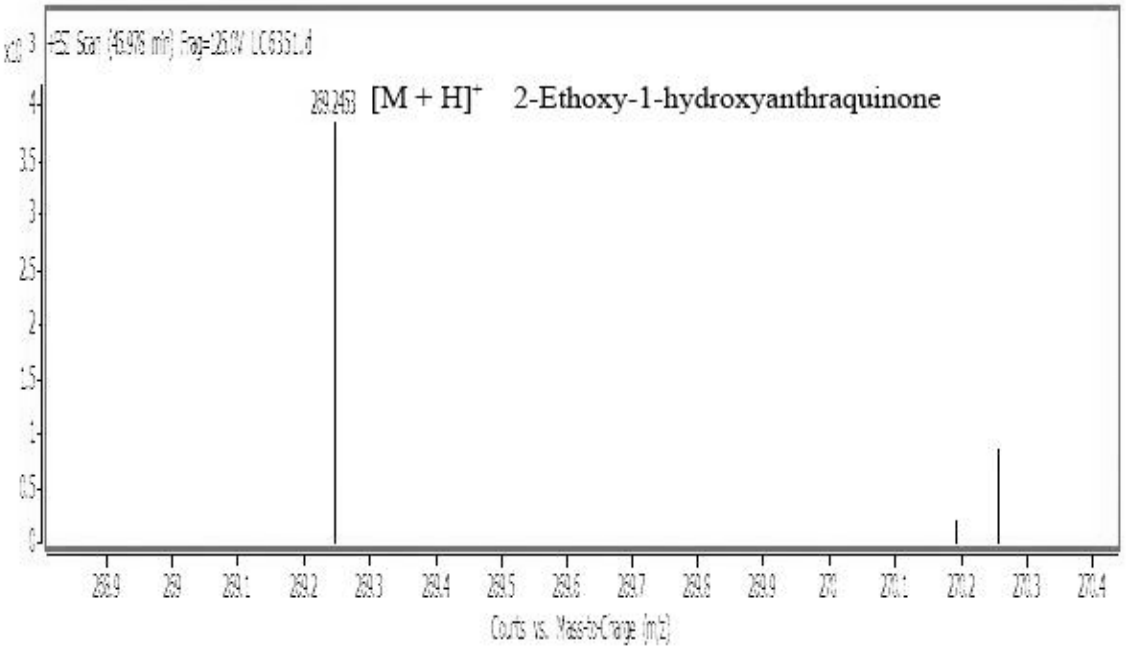

**Appendix 23.** MS/MS spectrum of 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl) anthraquinone extracted from the leaves of *M. citrifolia* at *m/z* 329.2678.



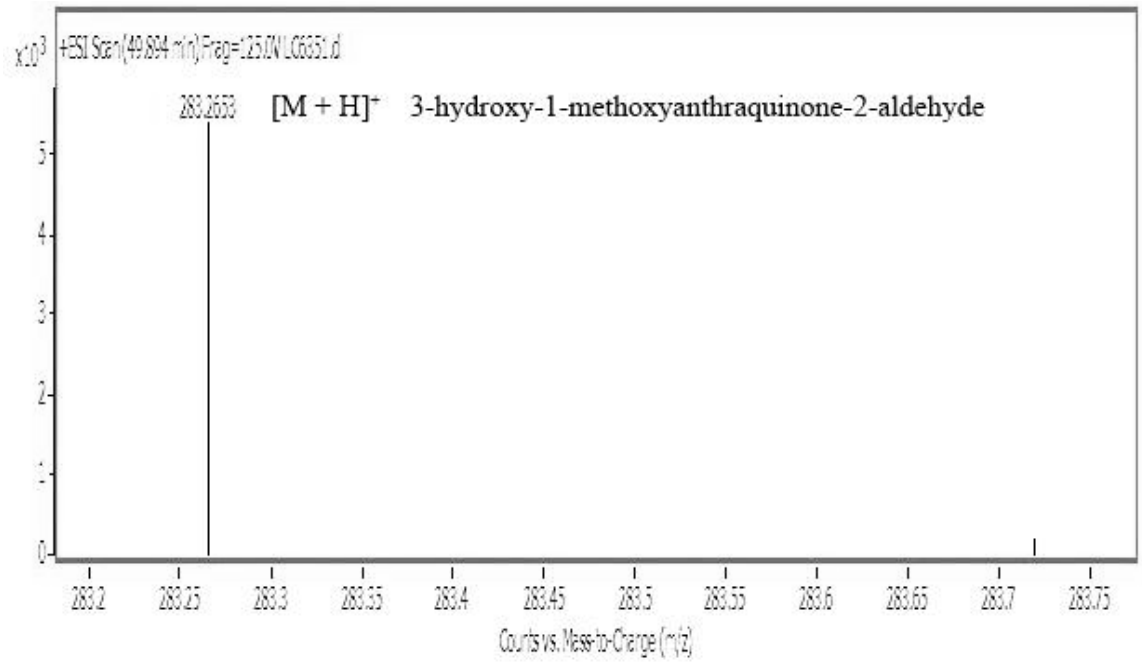

**Appendix 24.** MS/MS spectrum of 3-hyroxy-2-hydroxymethyl-1-methoxyanthraquinone extracted from the roots of *M. citrifolia* at *m/z* 285.0785.



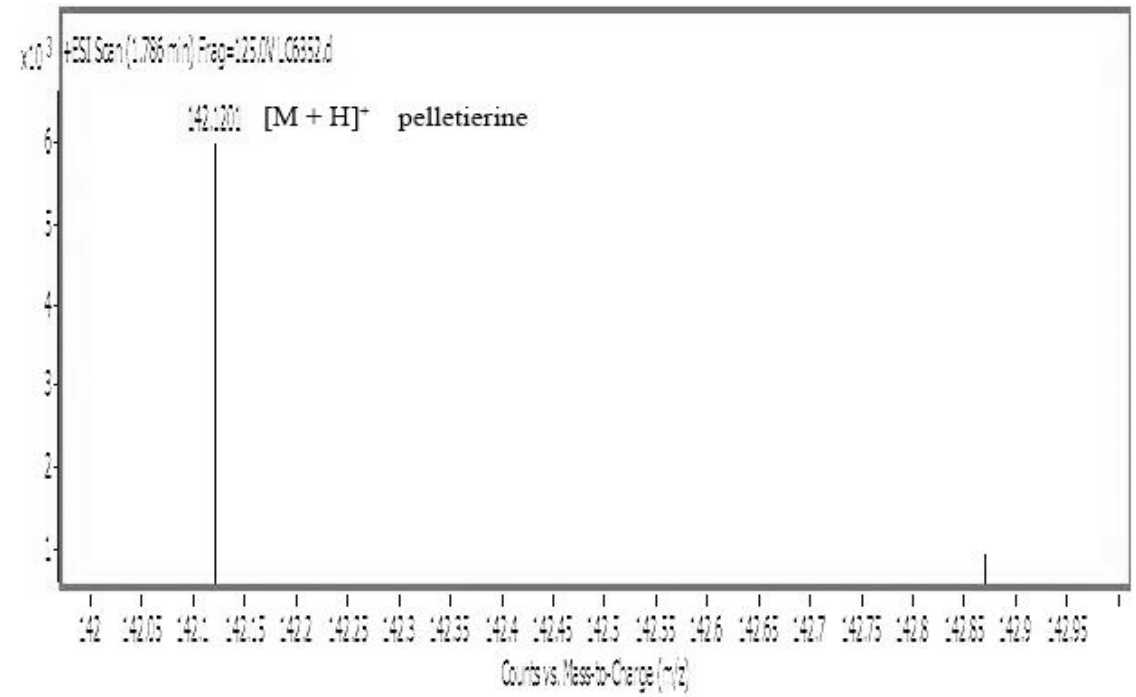

**Appendix 25.** MS/MS spectrum of 6-hydroxy-1,3-dimethoxy-1-7-methylanthraquinone extracted from the roots of *M. citrifolia* at *m/z* 299.3437.



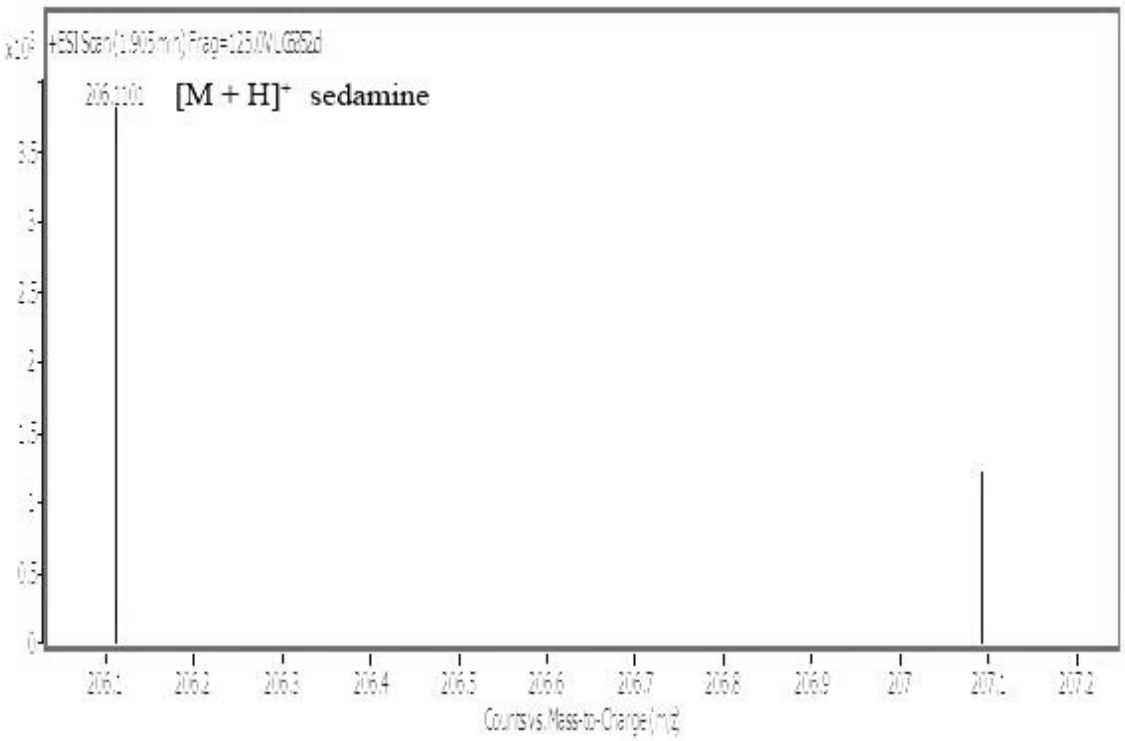

**Appendix 26.** MS/MS spectrum of 2-Ethoxy-1-hydroxyanthraquinone extracted from the roots of *M. citrifolia* at *m/z* 269.2453.



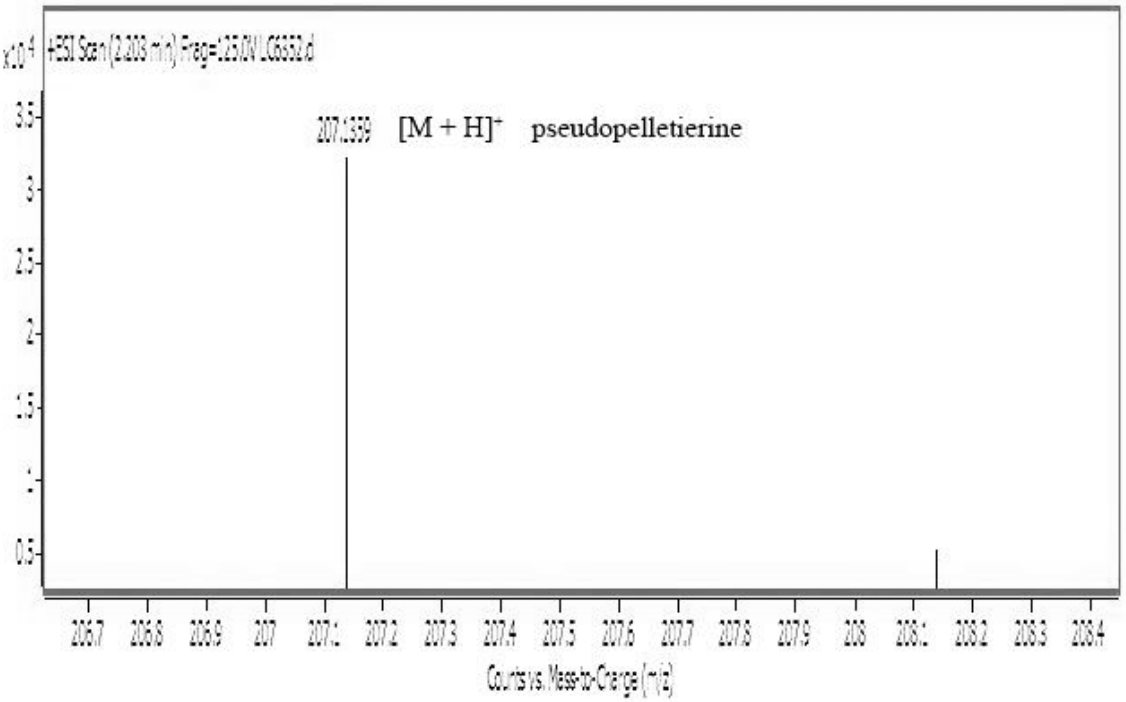

**Appendix 27.** MS/MS spectrum of 3-hydroxy-1-methoxyanthraquinone-2-aldehyde extracted from the roots of *M. citrifolia* at *m/z* 283.2653.



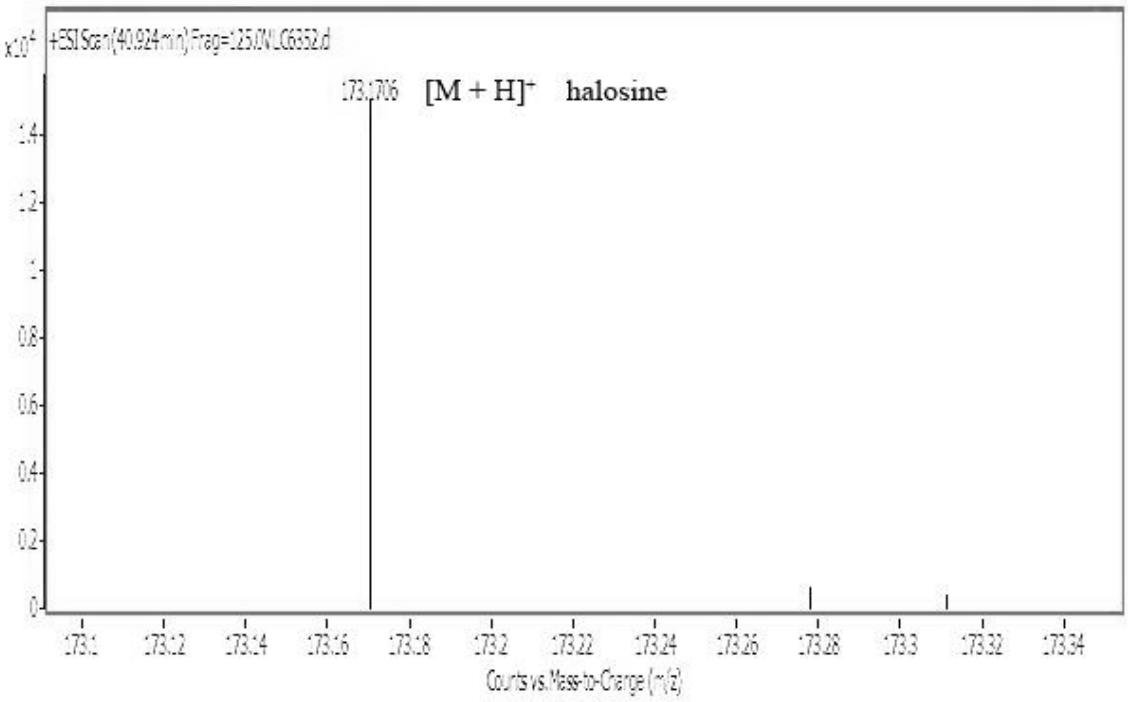

**Appendix 28.** MS/MS spectrum of pelletierine extracted from the fruit of *M. citrifolia* at *m/z* 142.1201.



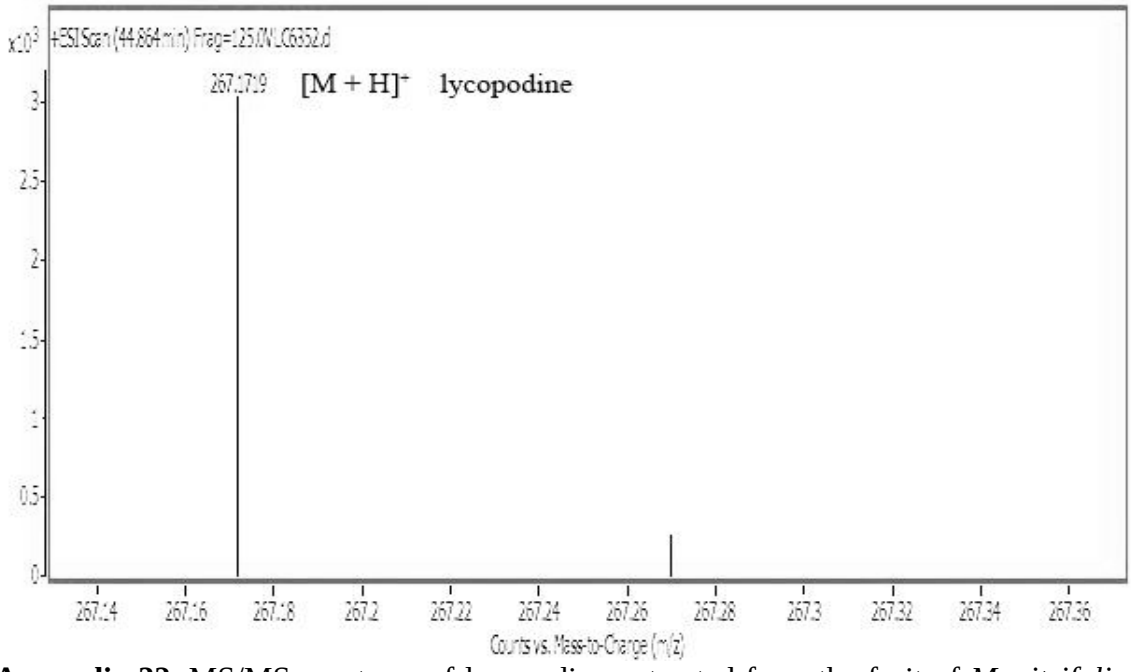

**Appendix 29.** MS/MS spectrum of sedamine extracted from the fruit of *M. citrifolia* at *m/z* 206.1101.



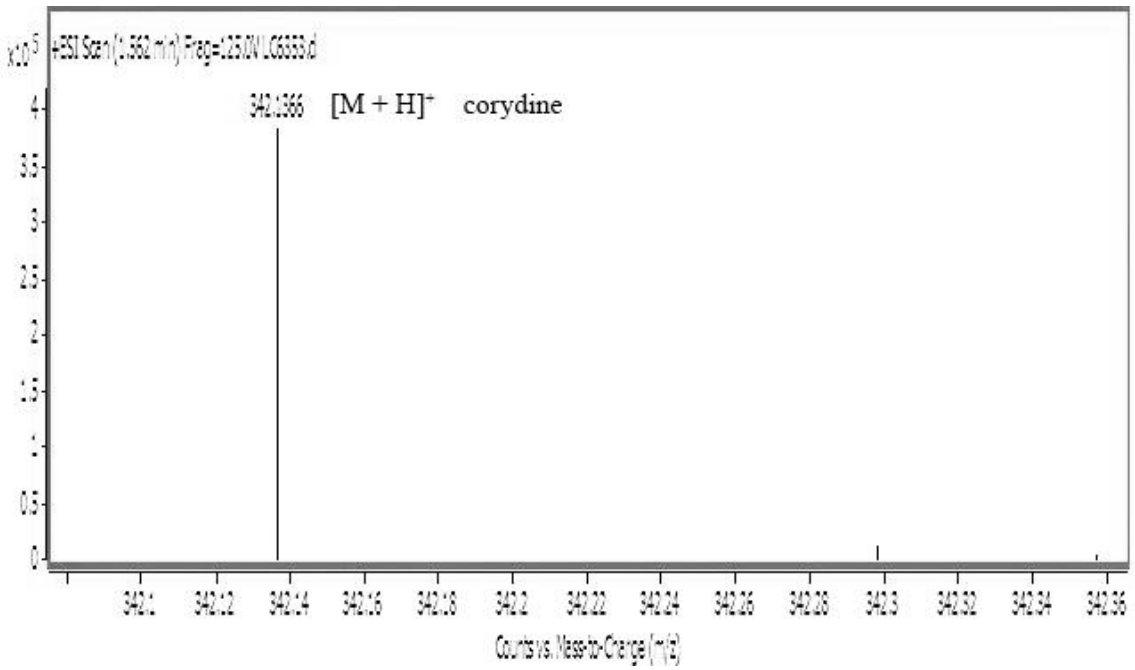

**Appendix 30.** MS/MS spectrum of pseudopelletierine extracted from the fruit of *M. citrifolia* at *m/z* 207.1359.



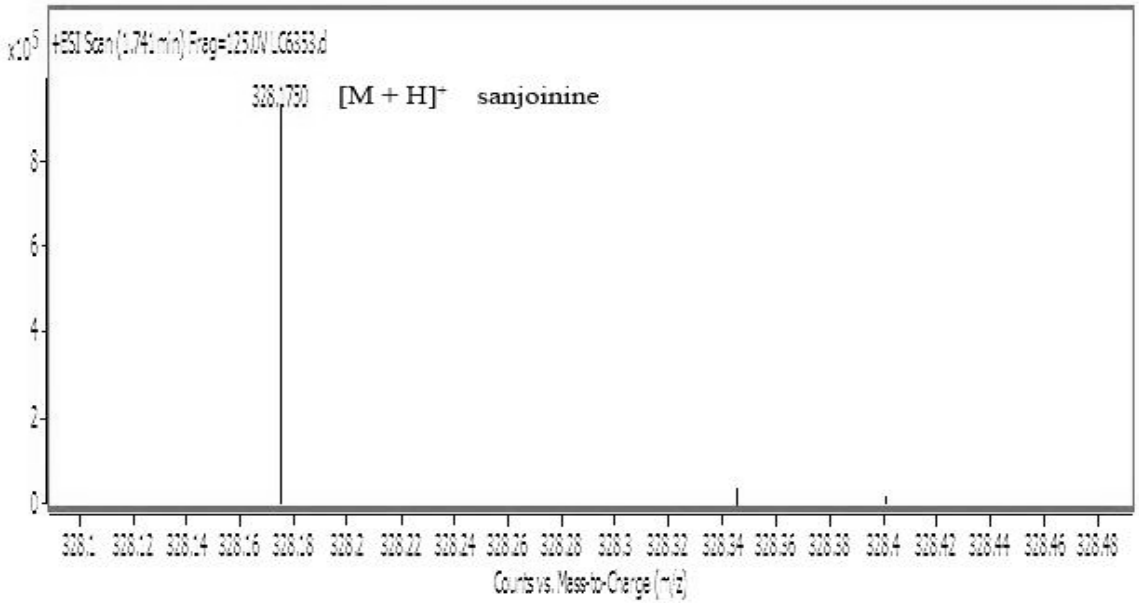

**Appendix 31.** MS/MS spectrum of halosine extracted from the fruit of *M. citrifolia* at *m/z* 173.1413.



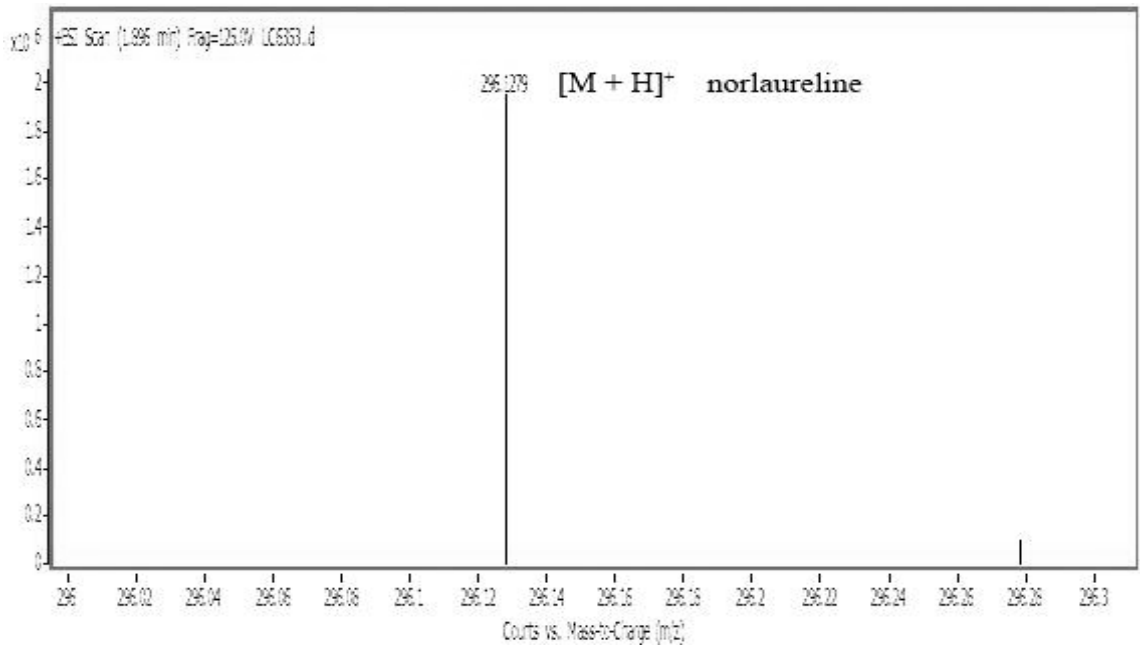

**Appendix 32.** MS/MS spectrum of lycopodine extracted from the fruit of *M. citrifolia* at *m/z* 267.1764.



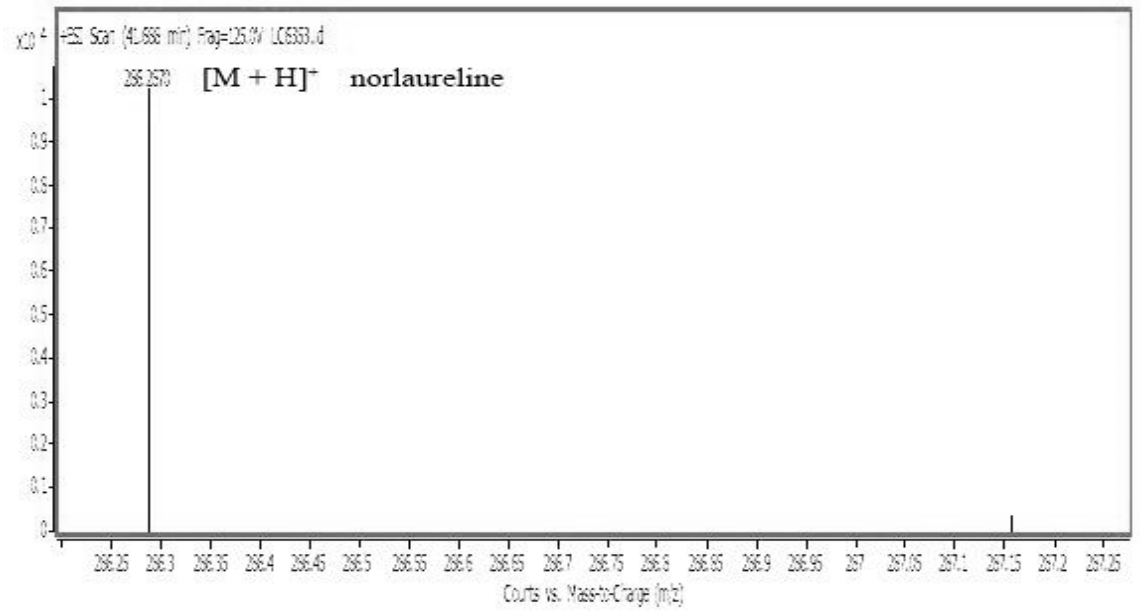

**Appendix 33.** MS/MS spectrum of corydine extracted from the leaves of *A. squamosa* at *m/z* 342.1366.



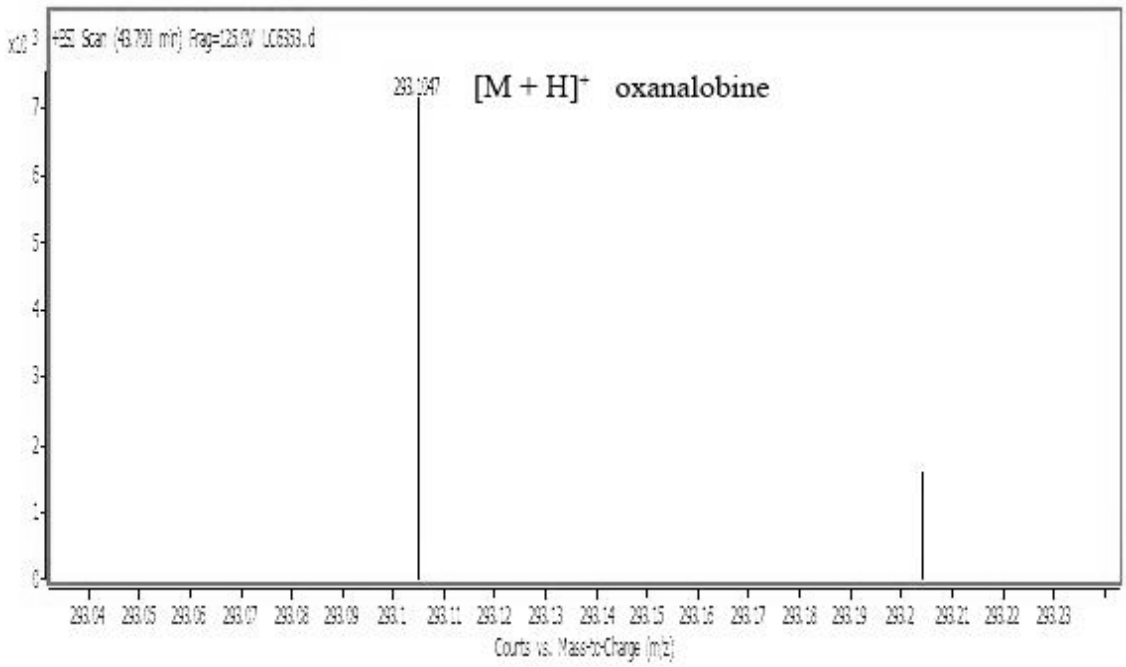

**Appendix 34.** MS/MS spectrum of sanjoinine extracted from the leaves of *A. squamosa* at *m/z* 328.1750.



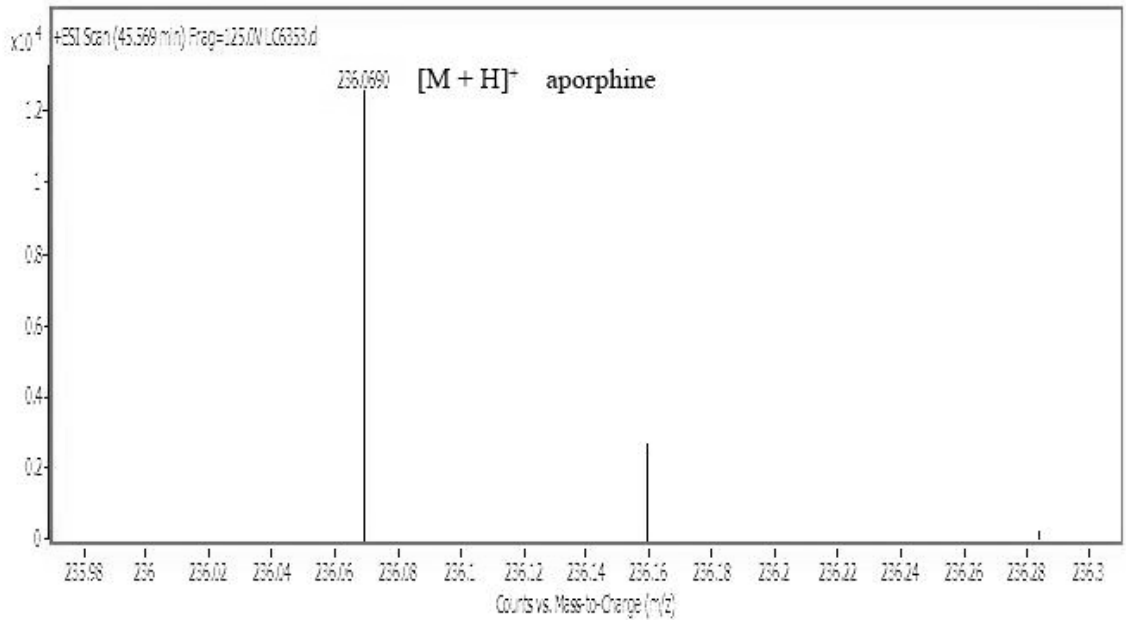

**Appendix 35.** MS/MS spectrum of norlaureline extracted from the leaves of *A. squamosa* at *m/z* 296.1270.



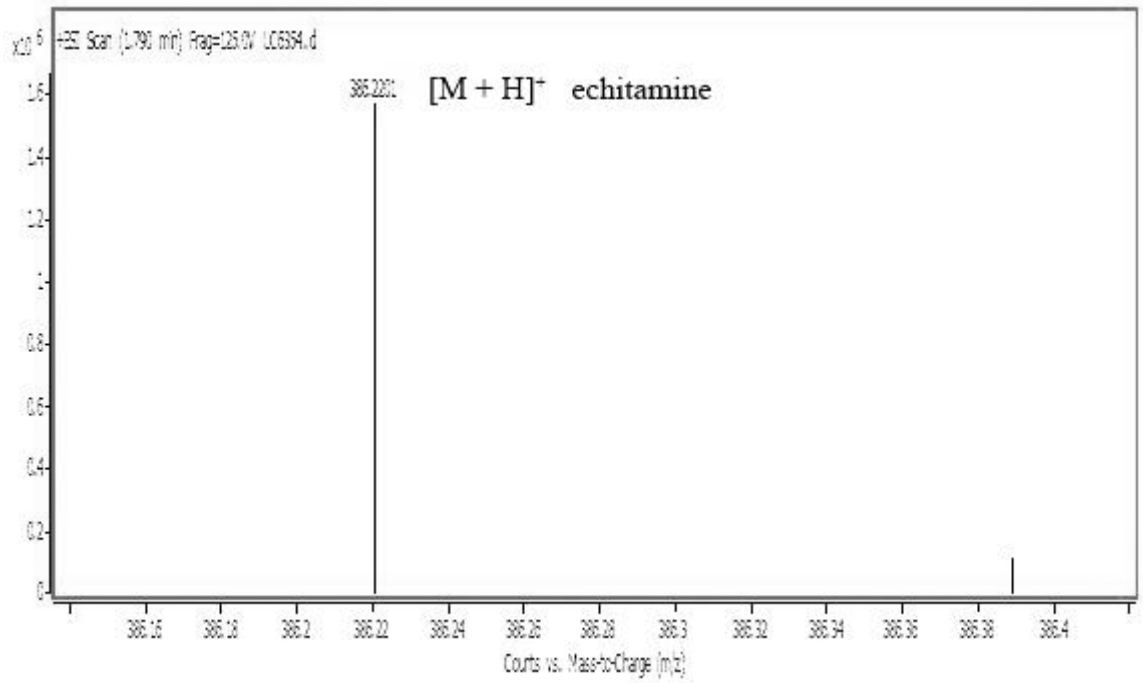

**Appendix 36.** MS/MS spectrum of norcodeine extracted from the leaves of *A. squamosa* at *m/z* 286.2870.



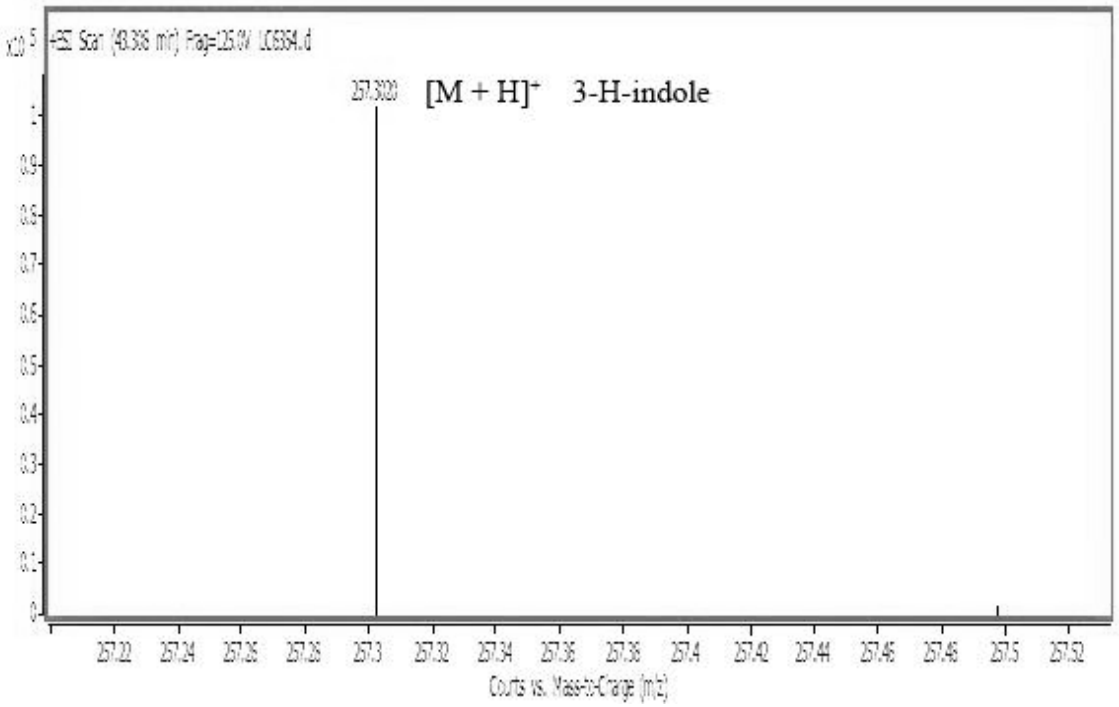

**Appendix 37.** MS/MS spectrum of oxanalobine extracted from the leaves of *A. squamosa* at *m/z* 293.1047.



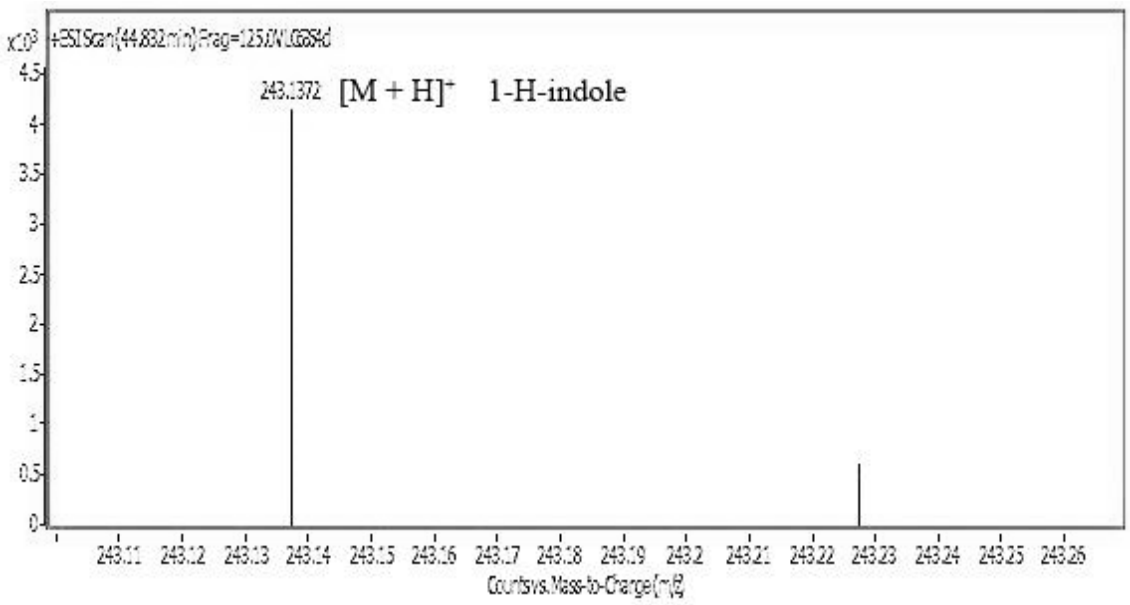

**Appendix 38.** MS/MS spectrum of aporphine extracted from the leaves of *A. squamosa* at *m/z* 236.0960.



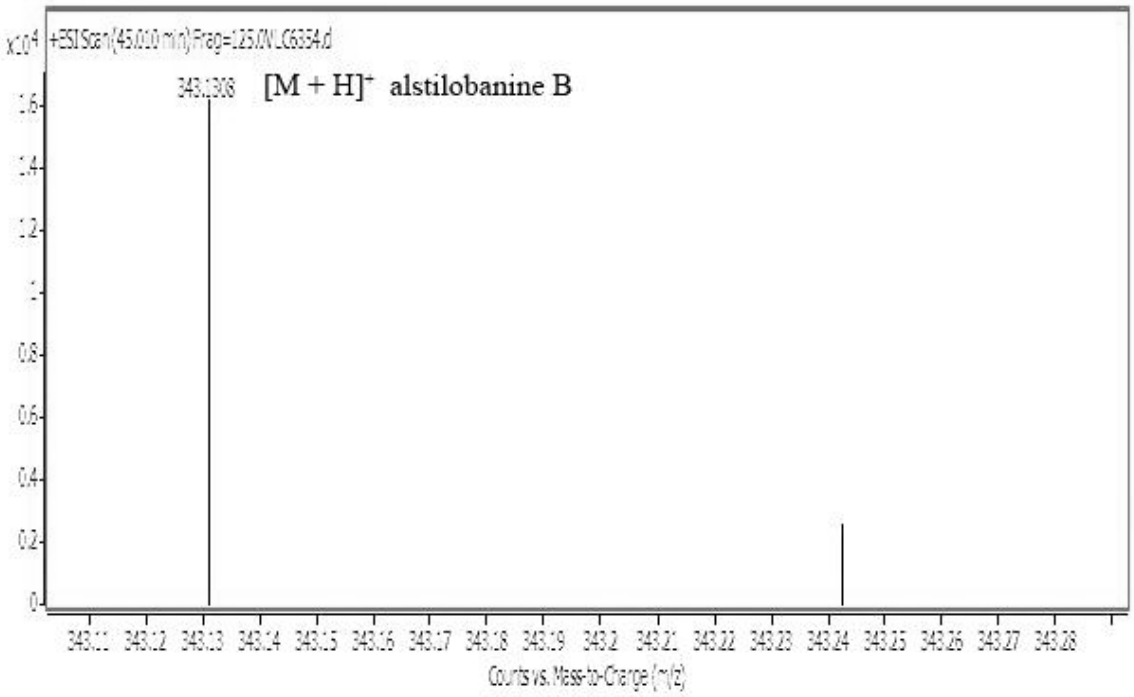

**Appendix 39.** MS/MS spectrum of echitamine extracted from the roots of *A. angustiloba* at *m/z* 386.2201.



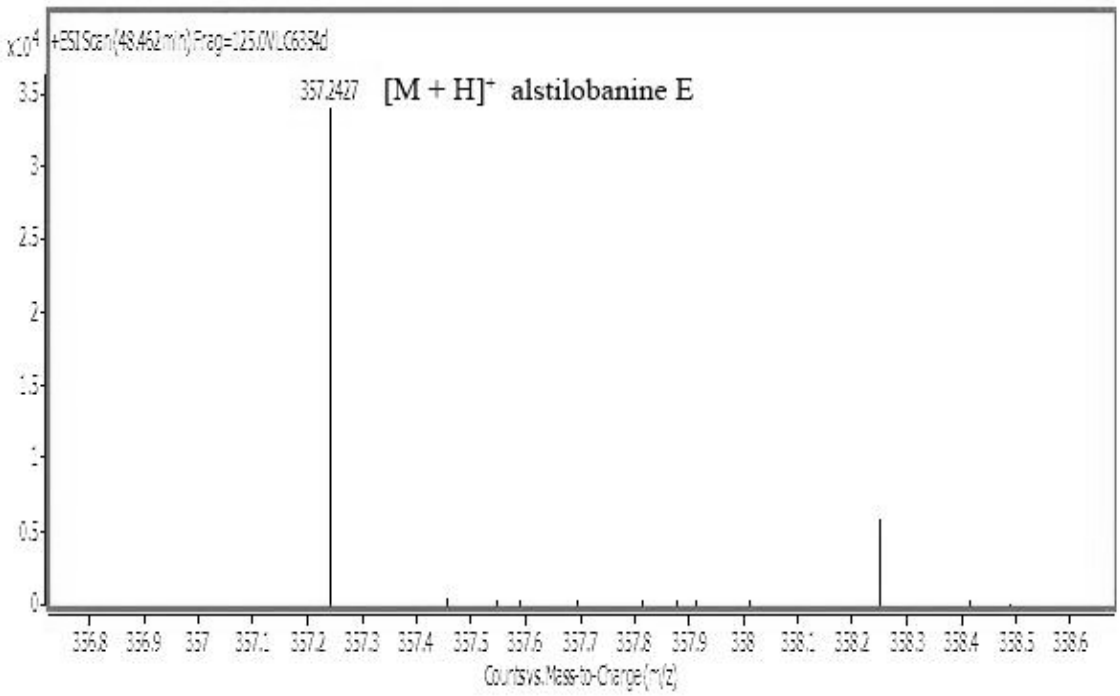

**Appendix 40.** MS/MS spectrum of 3-H-indole extracted from the roots of *A. angustiloba* at *m/z* 257.3020.



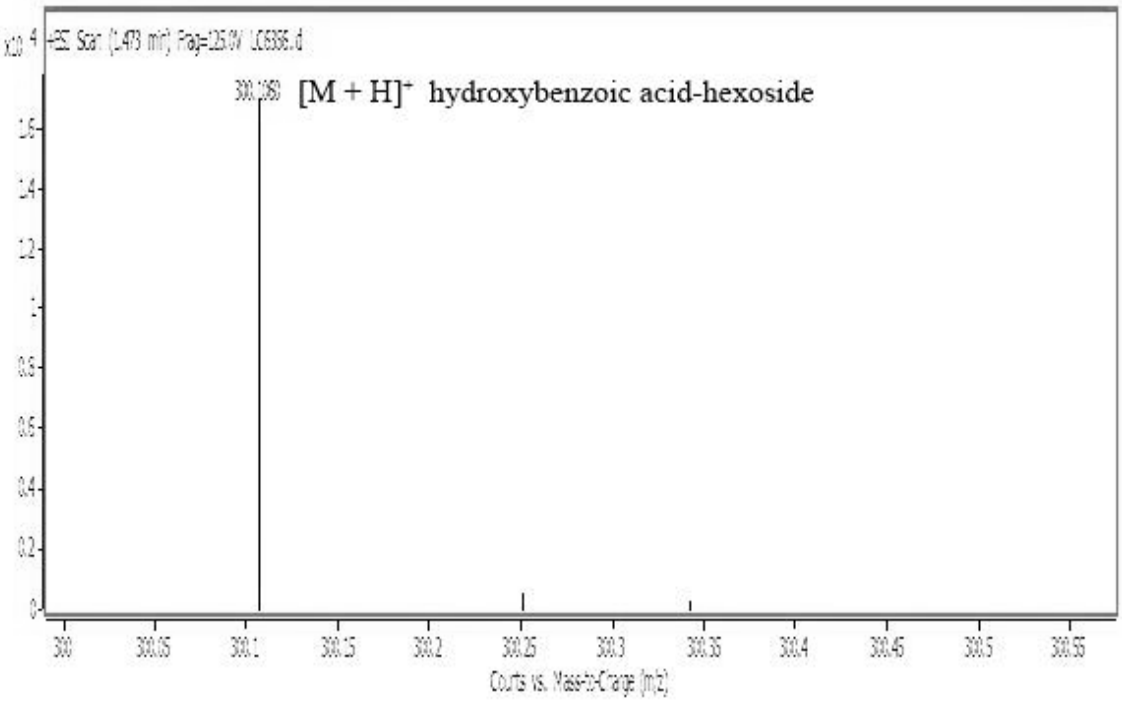

**Appendix 41.** MS/MS spectrum of 1-H-indole extracted from the roots of *A. angustiloba* at *m/z* 243.1372.



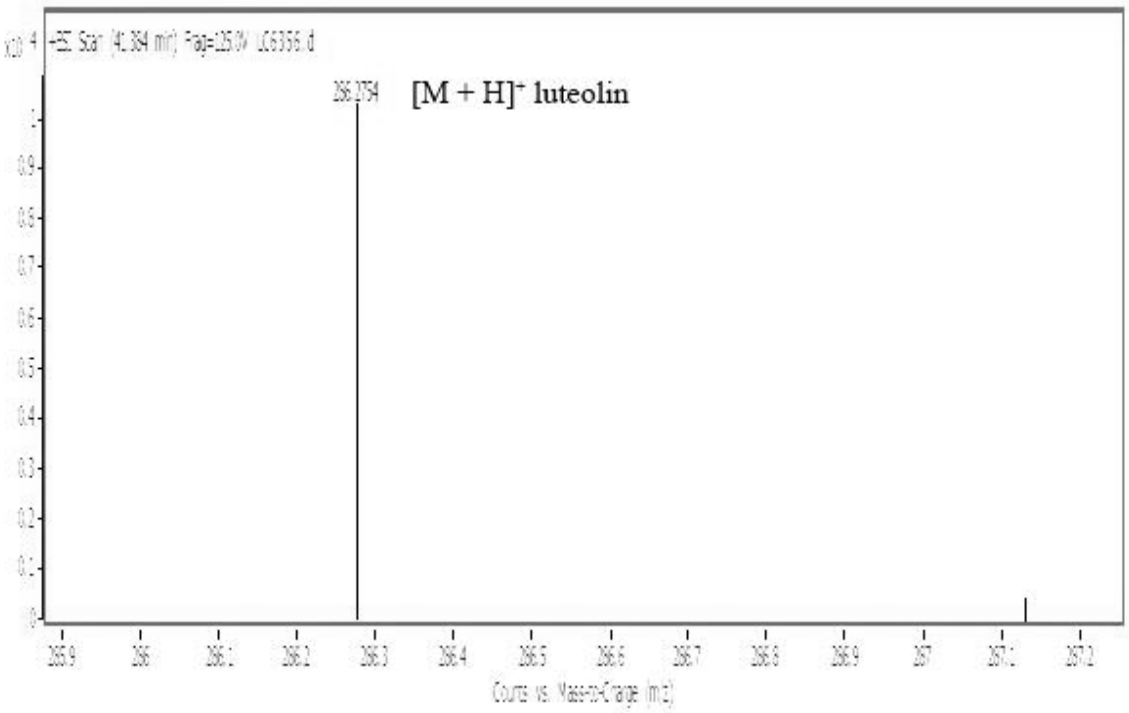

**Appendix 42.** MS/MS spectrum of alstilobanine B extracted from the roots of *A. angustiloba* at *m/z* 343.1308.



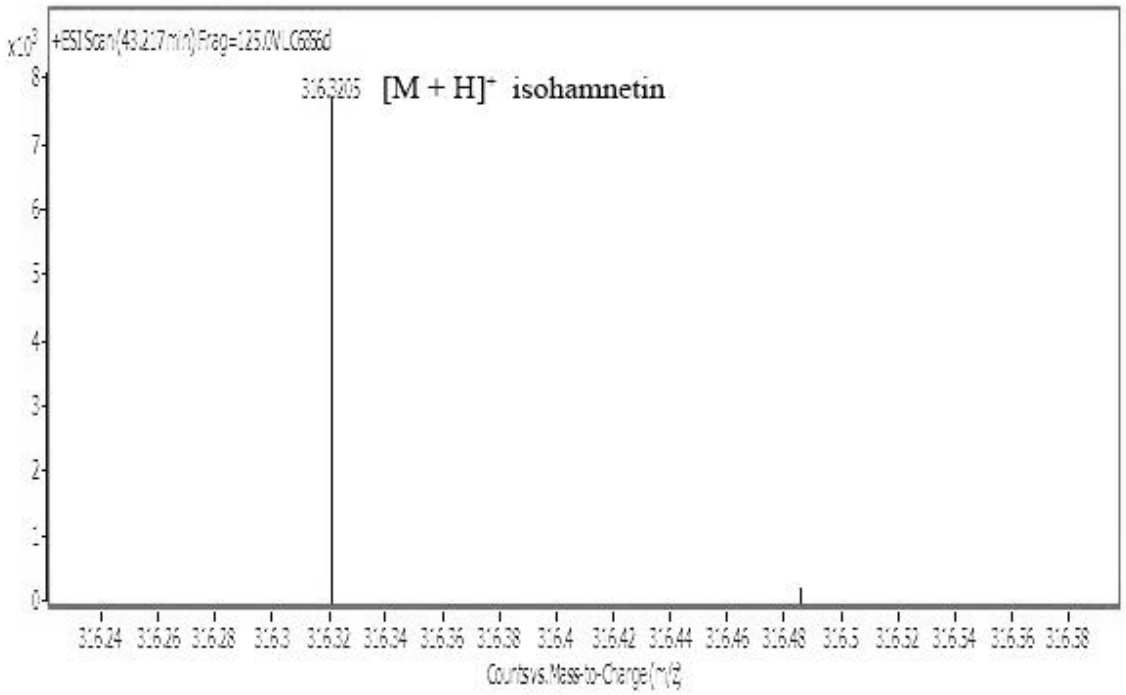

**Appendix 43.** MS/MS spectrum alstilobanine E extracted from the roots of *A. angustiloba* at *m/z* 357.2427.



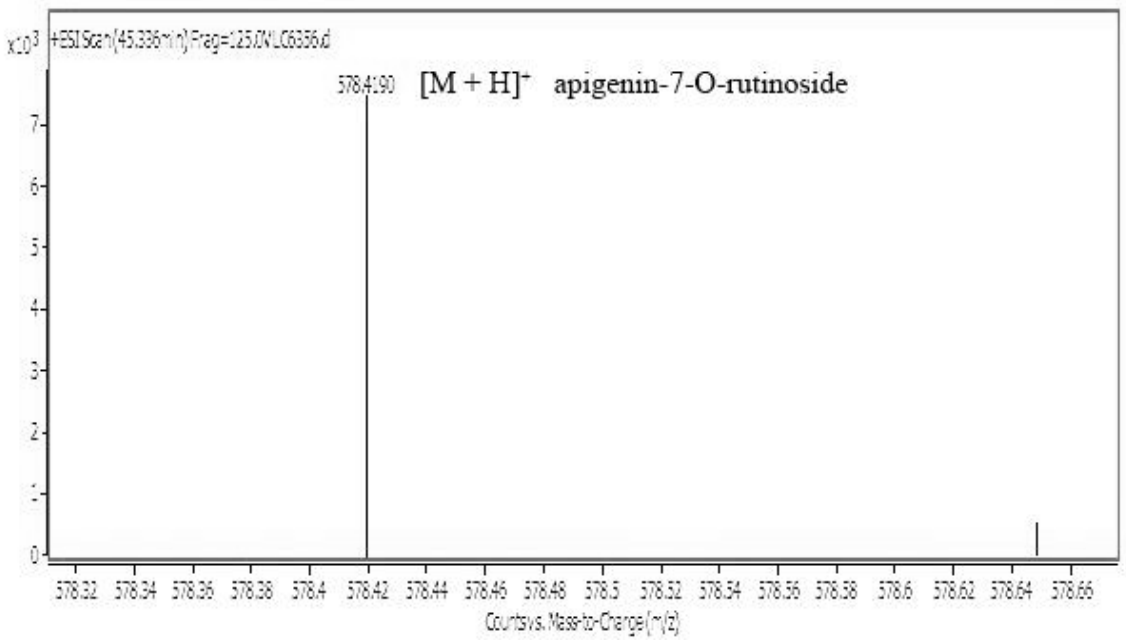

**Appendix 44.** MS/MS spectrum of hydroxybenzoic acid-hexoside extracted from the Australian plant mixture at *m/z* 300.1060.



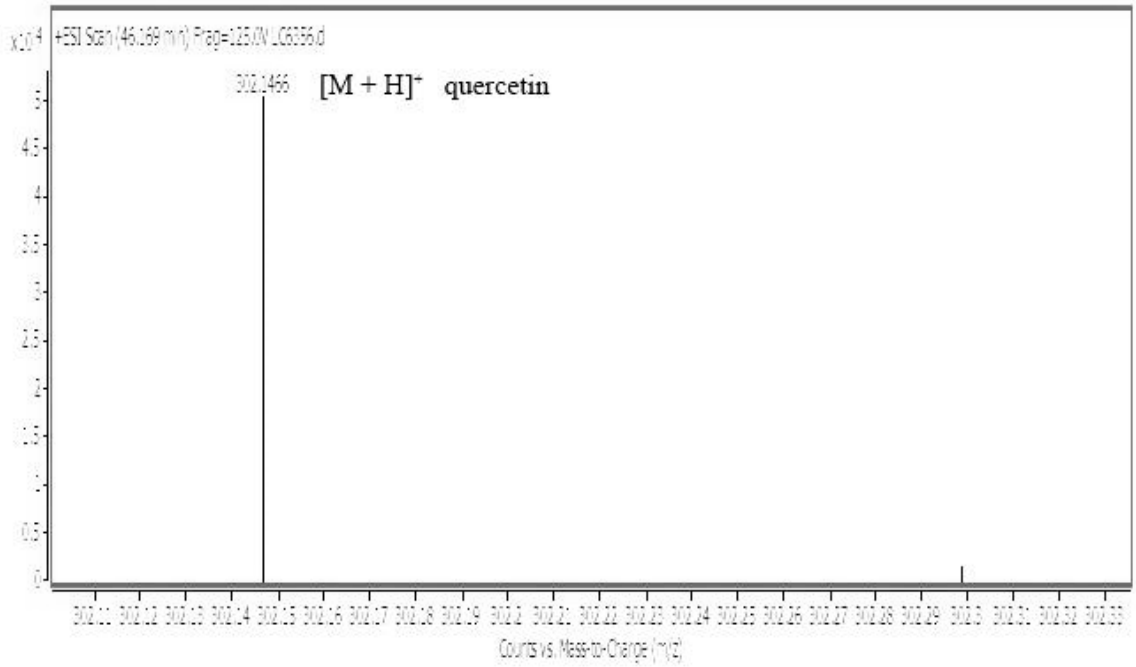

**Appendix 45.** MS/MS spectrum of luteolin extracted from the Australian plant mixture at *m/z* 286.2754.



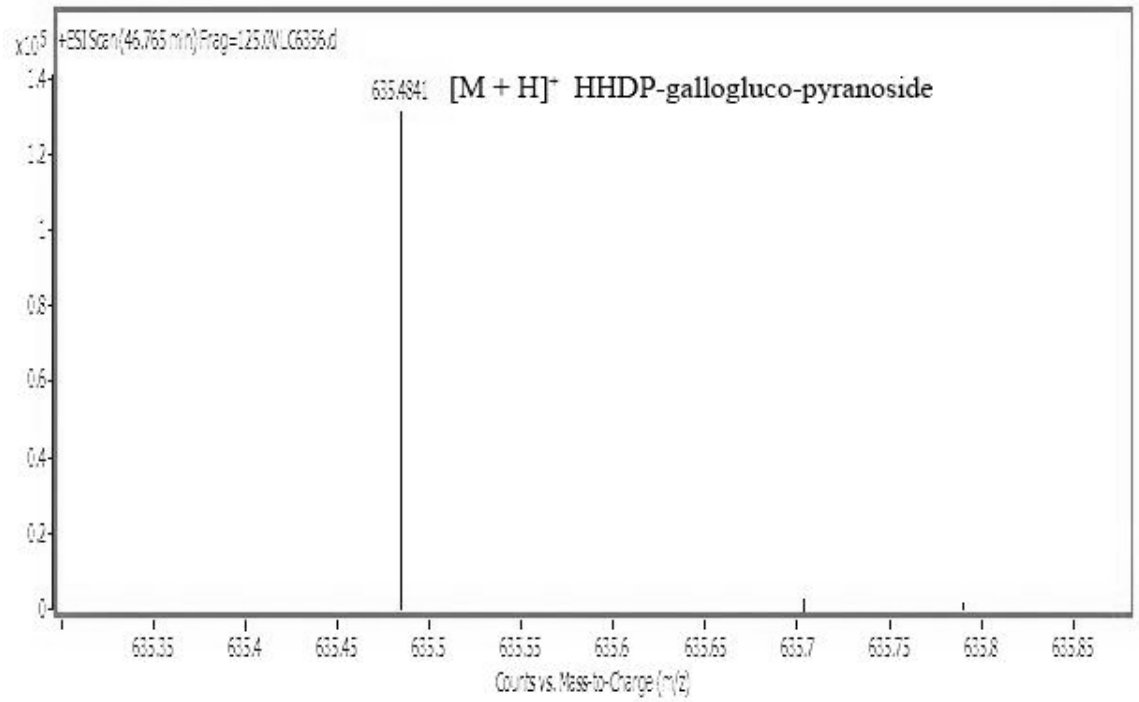

**Appendix 46.** MS/MS spectrum of isohamnetin extracted from the Australian plant mixture at *m/z* 316.3205.



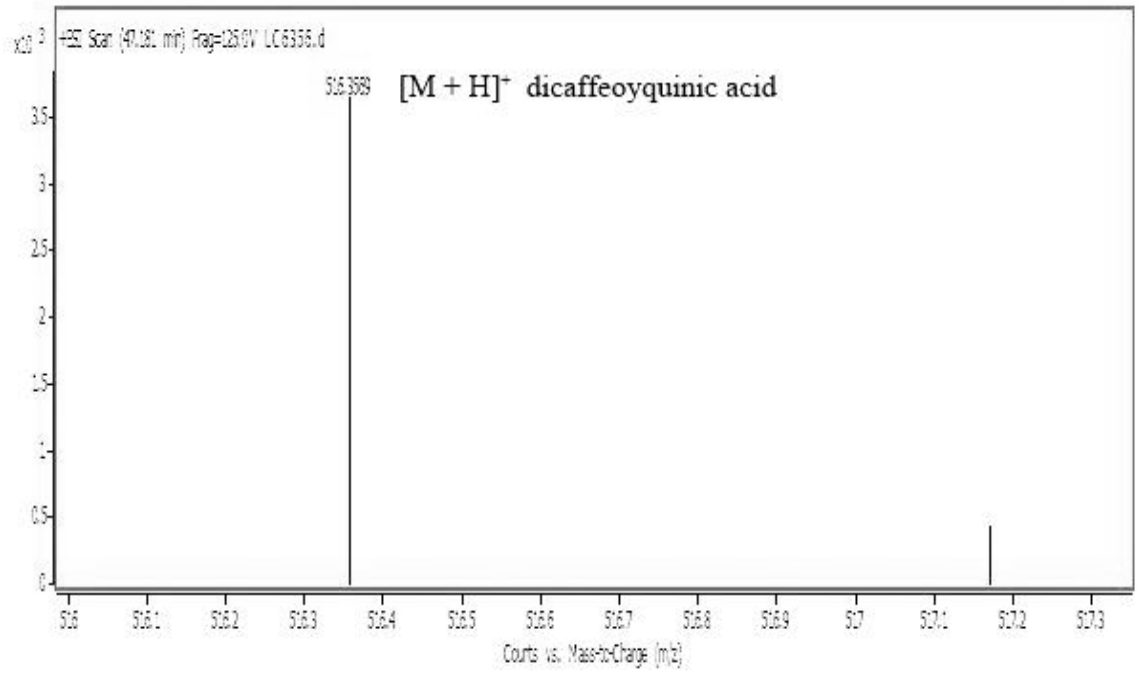

**Appendix 47.** MS/MS spectrum of apigenin-7-O-rutinoside extracted from the Australian plant mixture at *m/z* 578.4190.



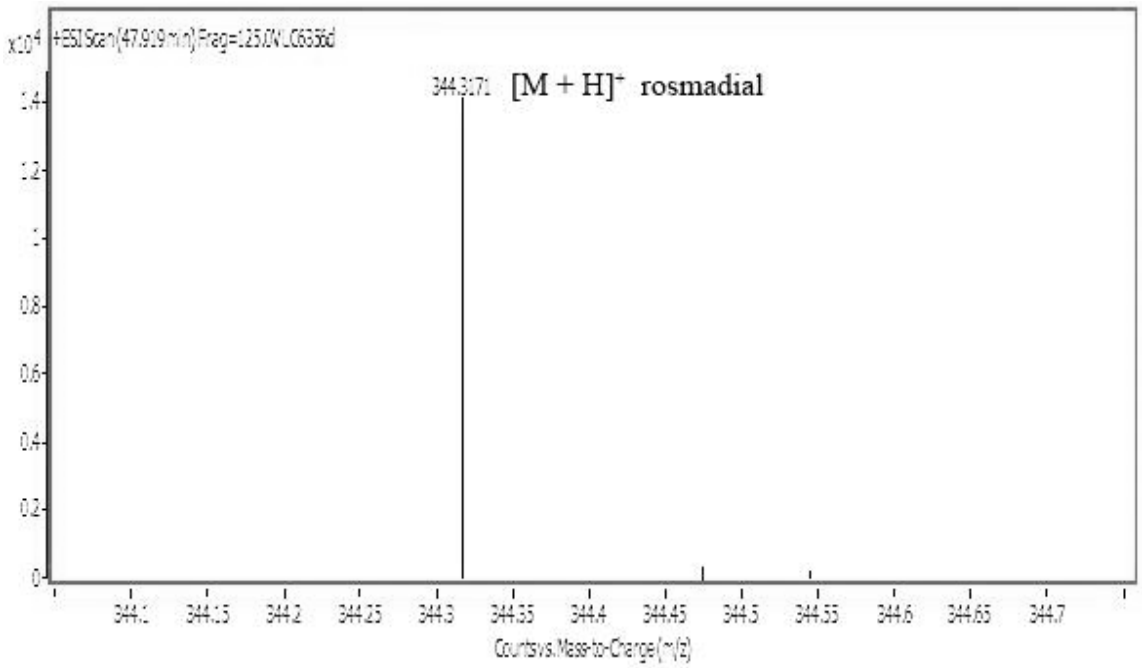

**Appendix 48.** MS/MS spectrum of quercetin extracted from the Australian plant mixture at *m/z* 302.1466.



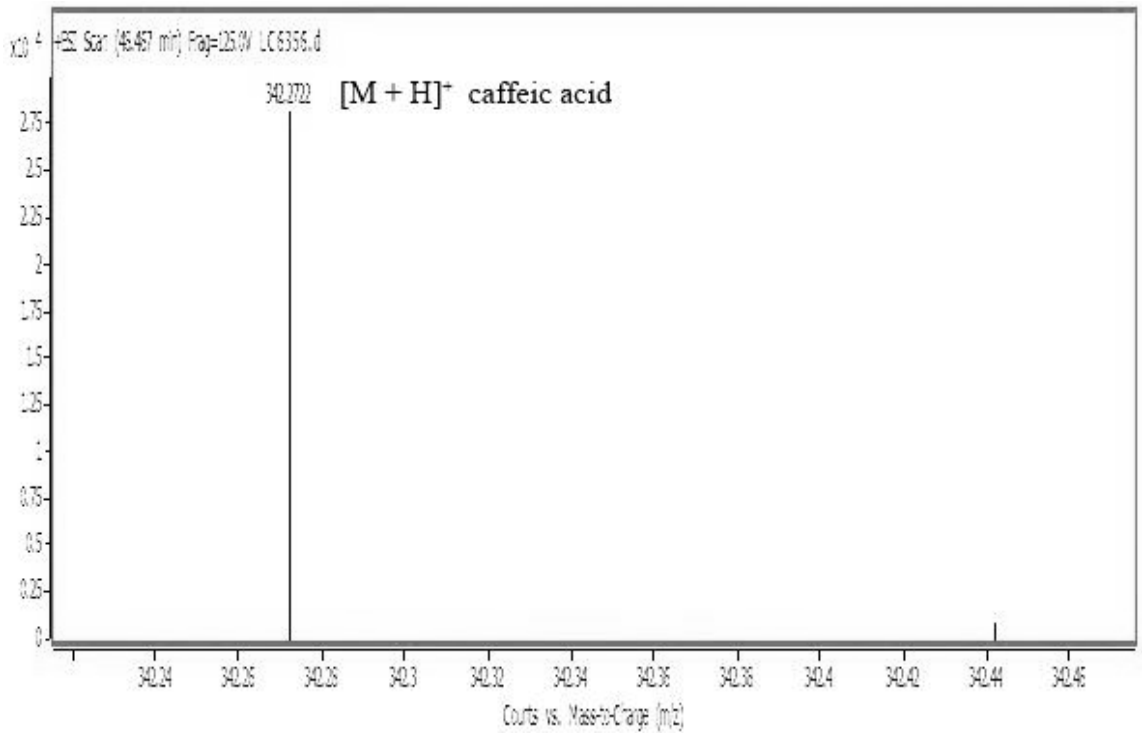

**Appendix 49.** MS/MS spectrum of HHDP-gallogluco-pyranoside extracted from the Australian plant mixture at *m/z* 635.4841.



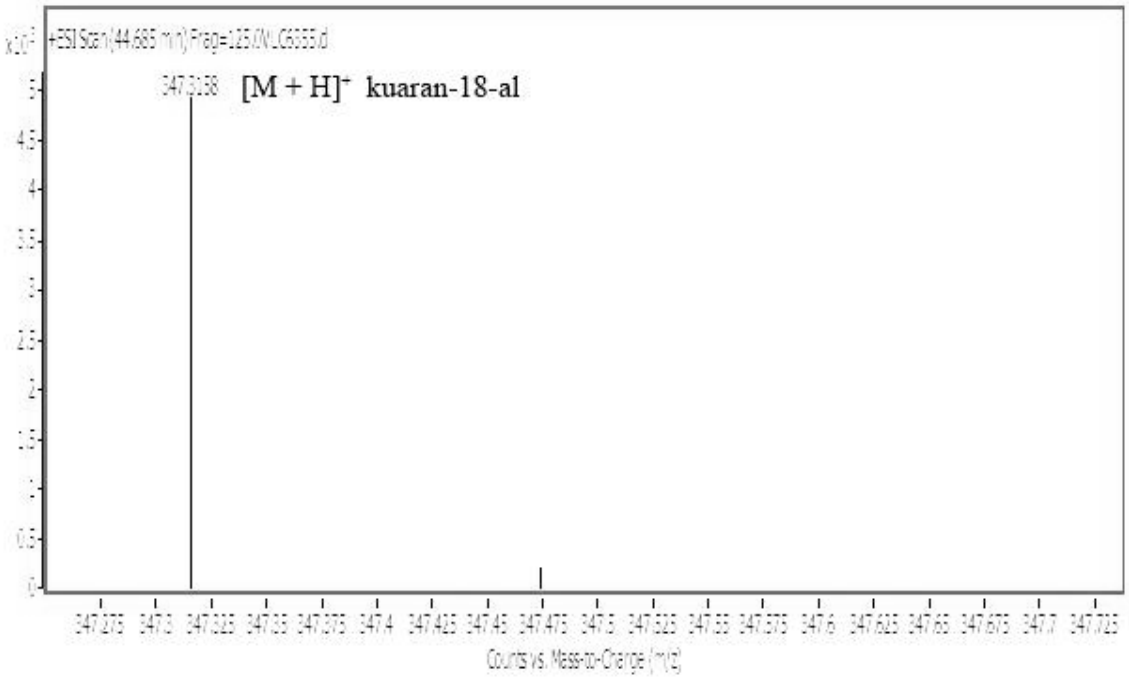

**Appendix 50.** MS/MS spectrum of dicaffeoyquinic acid extracted from the Australian plant mixture at *m/z* 516.3569.



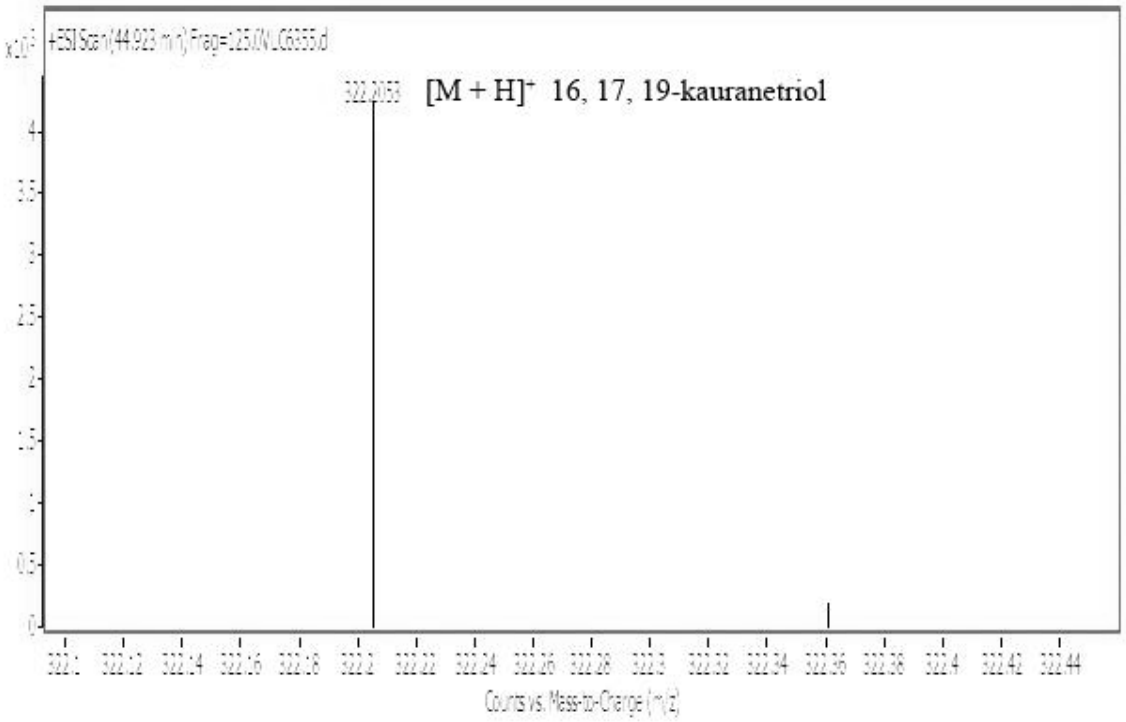

**Appendix 51.** MS/MS spectrum of rosmadial extracted from the Australian plant mixture at *m/z* 344.3171.



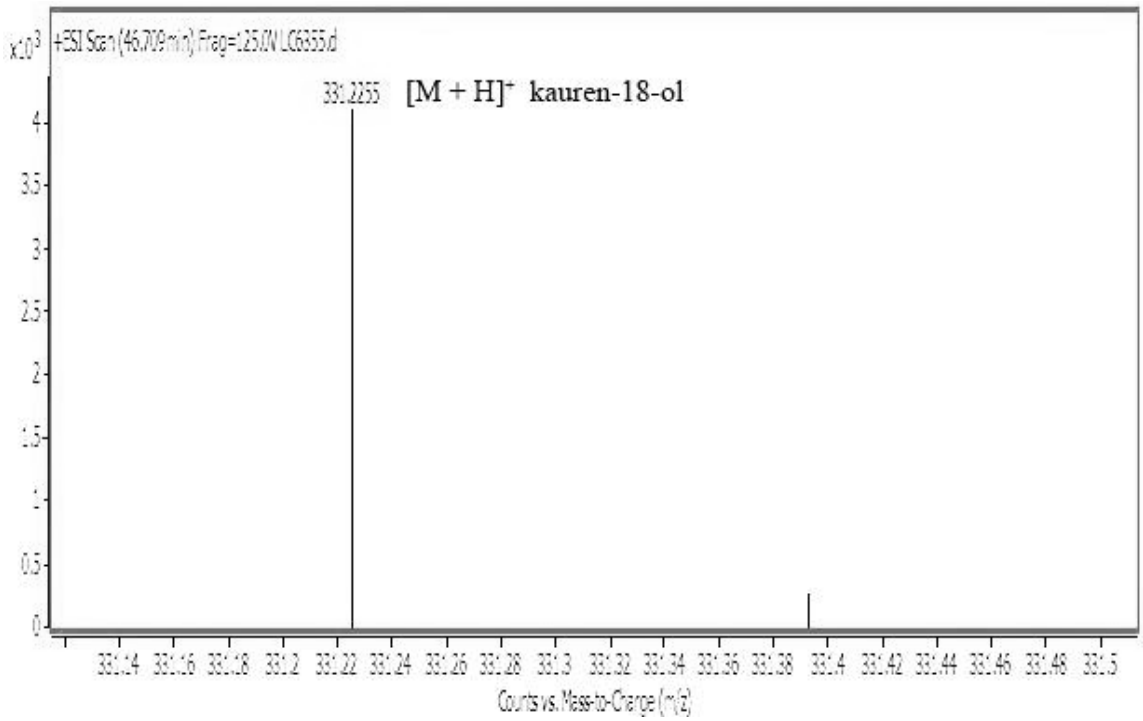

**Appendix 52.** MS/MS spectrum of caffeic acid extracted from the Australian plant mixture at *m/z* 342.2722.



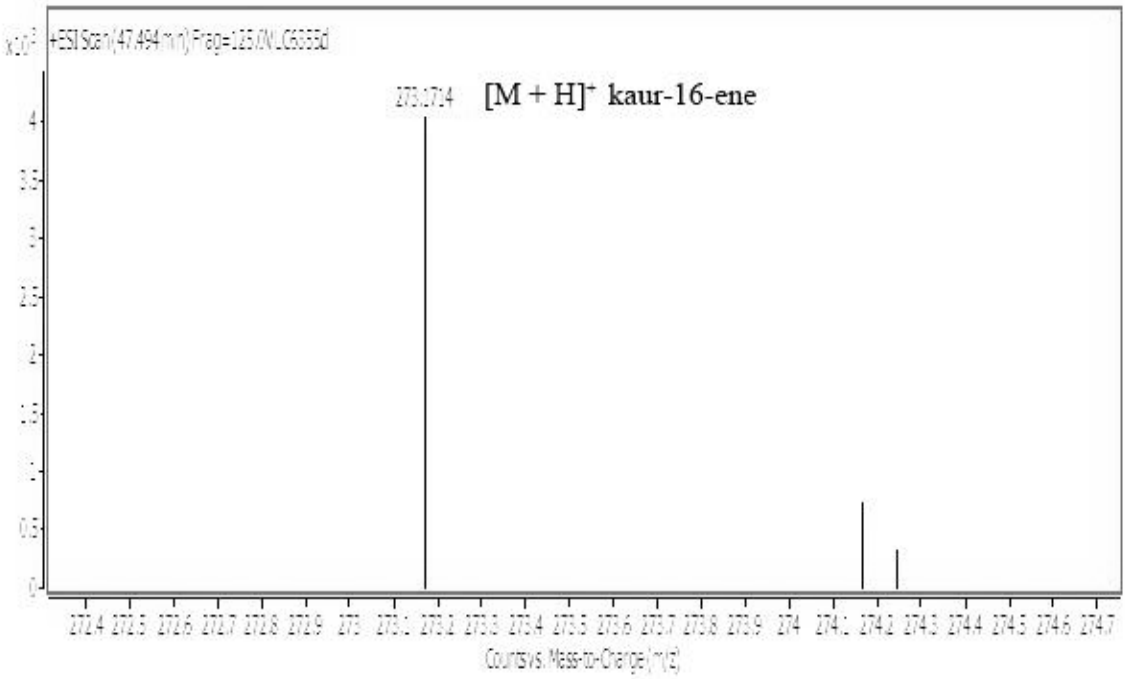

**Appendix 53.** MS/MS spectrum of kuaran-18-al extracted from the fruit of *A. squamosa* at *m/z* 347.3158.



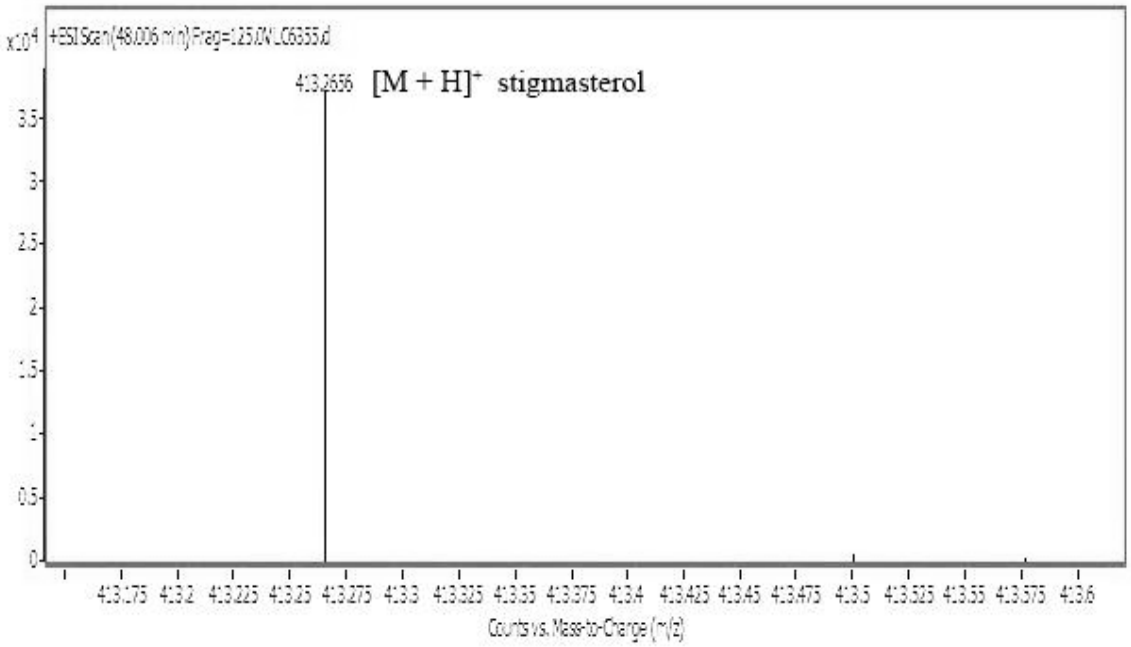

**Appendix 54.** MS/MS spectrum of extracted from 16,17,19-kauranetriol the fruit of *A. squamosa* at *m/z* 322.2053.



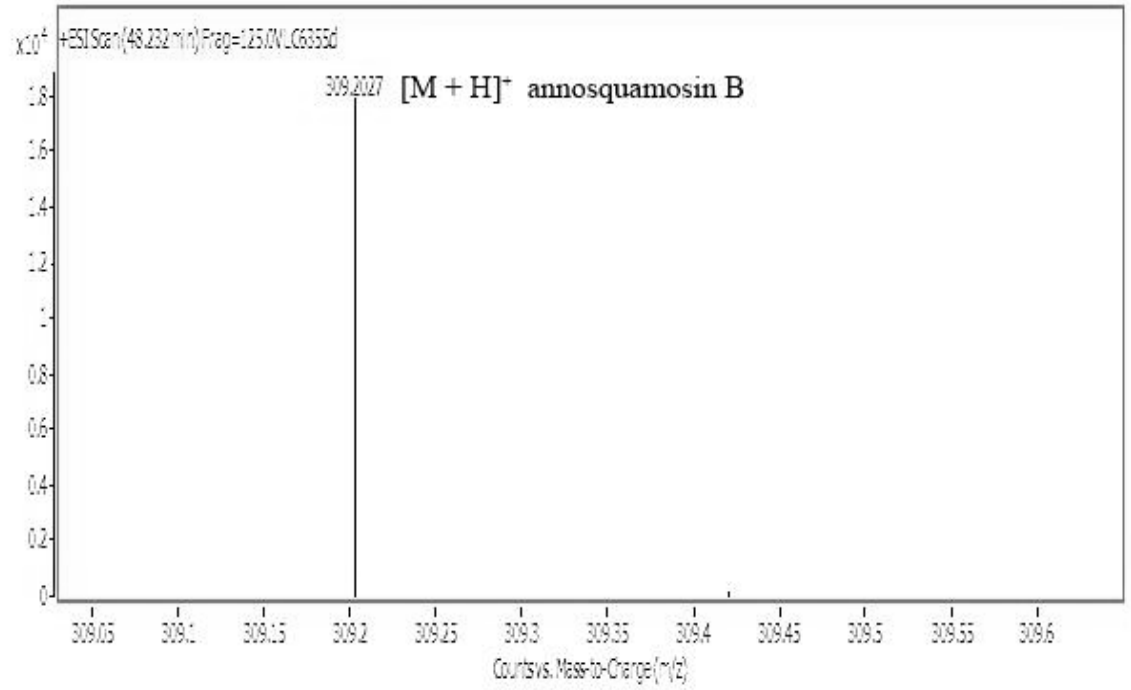

**Appendix 55.** MS/MS spectrum of kauren-18-ol extracted from the fruit of *A. squamosa* at *m/z* 331.2253.




**Appendix 56.** MS/MS spectrum of kaur-16-ene extracted from the fruit of *A. squamosa* at *m/z* 273.1714.




**Appendix 57.** MS/MS spectrum of stigmasterol extracted from the fruit of *A. squamosa* at *m/z* 413.2656.




**Appendix 58.** MS/MS spectrum of annosquamosin B extracted from the fruit of *A. squamosa* at *m/z* 309.2027.

ATTTGTCACCTTAGACGGCTCGCTCCCTAAAAGGGTTACGCCACCGGCTTCG
GGTGTTACAAACTCTCATGGTGTGACGG
GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCG
ATTACTAGCGATTCCGACTTCGTGTAGGC
GAGTTGCAGCCTACAGTCCGAACTGAGAATGGCTTTAAGAGATTAGCTTGA
CCTCGCGGTCTCGCAACTCGTTGTACCAT
CCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACG
TCATCCCCACCTTCCTCCGGTTTGTCACC
GGCAGTCTTACTAGAGTGCCCAACTAAATGCTGGCAACTAGTCATAAGGGT
TGCGCTCGTTGCGGGACTTAACCCAACAT
CTCACGACACGAGCTGACGACAACCATGCACCACCTGTCATTTTGCCCCCG
AAGGGGAAACCTGATCTCTCAGGTGATCA
AAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACA
TGCTCCACCGCTTGTGCGGGCCCCCGTC
AATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAATGCTTAA
TGCGTTAGCTGCGGCACTGAAGGGCGGA
AACCCTCCAACACCTAGCATTCATCGTTTACGGCATGGACTACCAGGGTATC
TAATCCTGTTCGCTACCCATGCTTTCGA
GCCTCAGCGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTC
CATATATCTACGCATTTCACCGCTACAC
ATGGAGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGATGCGCT
TCCTCGGTTAAGCCGAGGGCTTTCACA
TCAGACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGATA
ACGCTTGCCACCTACGTATTACCGCGGCT
GCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTGGATACCGTCACGCCGACAC
AGTTACTCTGCCGACCATTCTCTCAACA
ACAGAGTTTTACGACCCGAAAGCTTCTTCACTCACGCGGCGTGCTCATCAGA
ACTTGCGTCCATGTGAGATCCCTACTGC
TGCCTCCCGTAGGATTTGGGCAGTTCTCAGTCCCATGTGTCGAATCACCTCT
CAGTCGCTACGTATCATCGCCTGGGTGA
CCGATACTCACACTAGCCTTAATGCGCCCGGGTTCCATCTCCAAAGGCGATA
GAGT

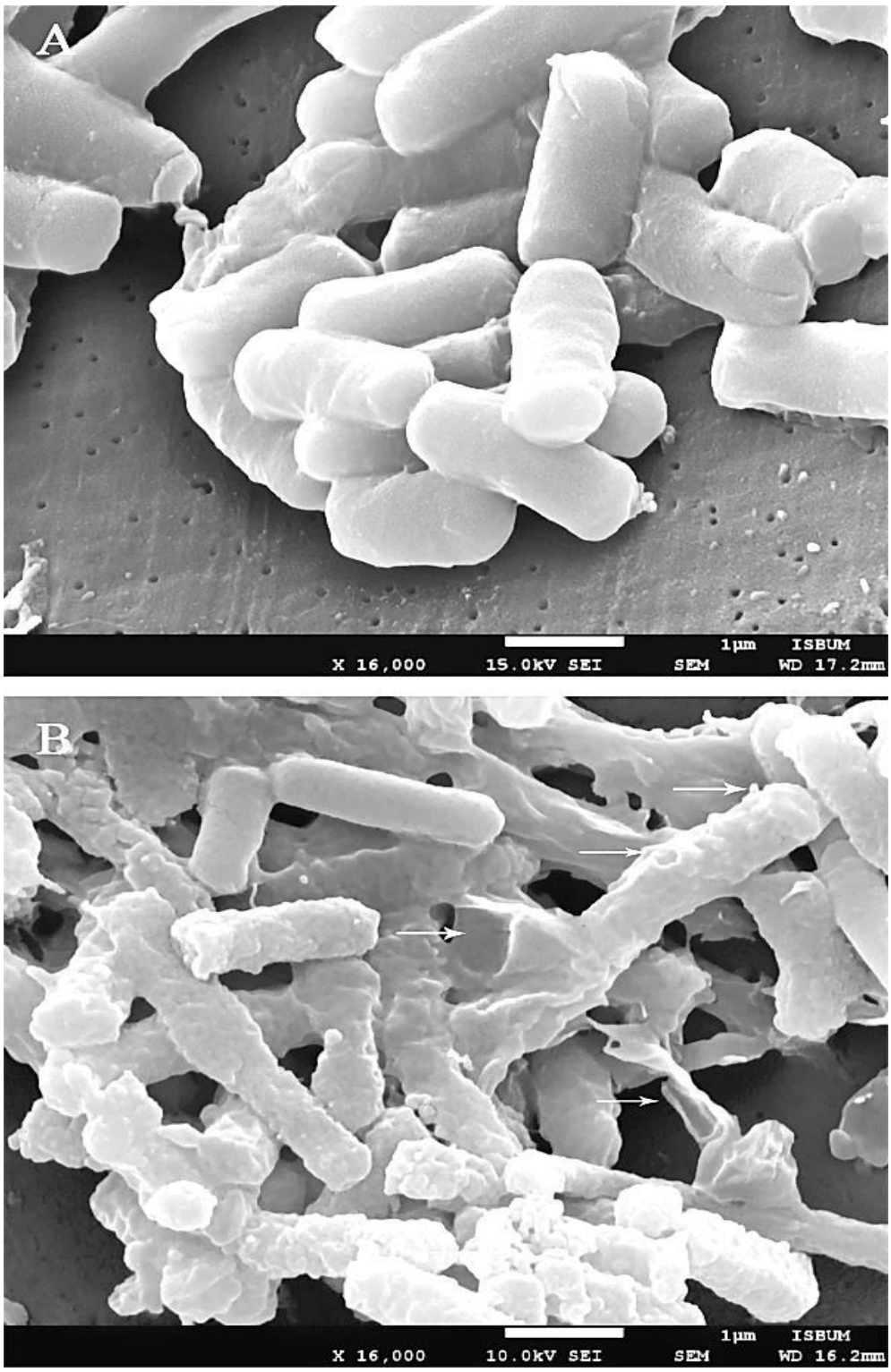
**Appendix 59.** 16S rDNA sequences of *Lactobacillus casei* BL 23 isolated from fermented soy milk.

ATTTGGTTCACCCTTAGACGGCTCGCTCCCTAAAAGGGTTACGCCACCGGCT
TCGGGTGTTACAAACTCTCATGGTGTGA
CGGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCC
GCGATTACTAGCGATTCCGACTTCGTGTA
GGCGAGTTGCAGCCTACAGTCCGAACTGAGAATGGCTTTAAGAGATTAGCT
TGACCTCGCGGTCTCGCAACTCGTTGTAC
CATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTC
ACCGGCAGTCTTACTAGAGTGCCCAACTAAATGCTGGCAACTAGTCATAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAA
CATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCATTTTGCCCC
CGAAGGGGAAACCTGATCTCTCAGGTGA
TCAAAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACC
ACATGCTCCACCGCTTGTGCGGGCCCCC
GTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAATGCT
TAATGCGTTAGCTGCGGCACTGAAGAGC
GGAAACCCTCCAACACCTAGCATTCATCGTTTACGGCATGGACTACCAGGG
TATCTAATCCTGTTCGCTACCCATGCTTT
CGAGTCTCAGCGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTC
TTCCATATATCTACGCATTTCACCGCTA
CACATGGAGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGATGC
GCTTCCTCGGTTAAGCCGAGGGCTTTC
ACATCAGACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGG
ATAACGCTTGCCACCTACGTATTACCGCG
GCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTGGATACCGTCACGCCGA
CACAGTTACTCTGCCGACCATTCTTCTC
CACACAGAGTTTTACGACCCGAAAGCCTTCTTCACTCACGCGGCGTGCTCCA
TCAGACTGCGTCCATGTGAAGATCCCTA
CTGCTGCCTCCCGTAGGAGTTTGGCGTGTCTCAGTCCAATGTGGCCGGATCA
CCTCTCAGTCGCTACGTATCATCGCTTG
CCGAACCGATACTTCACCACTTAGCCTAATACGCCCGGCGGTCCATTCCAAA
GGCGAA

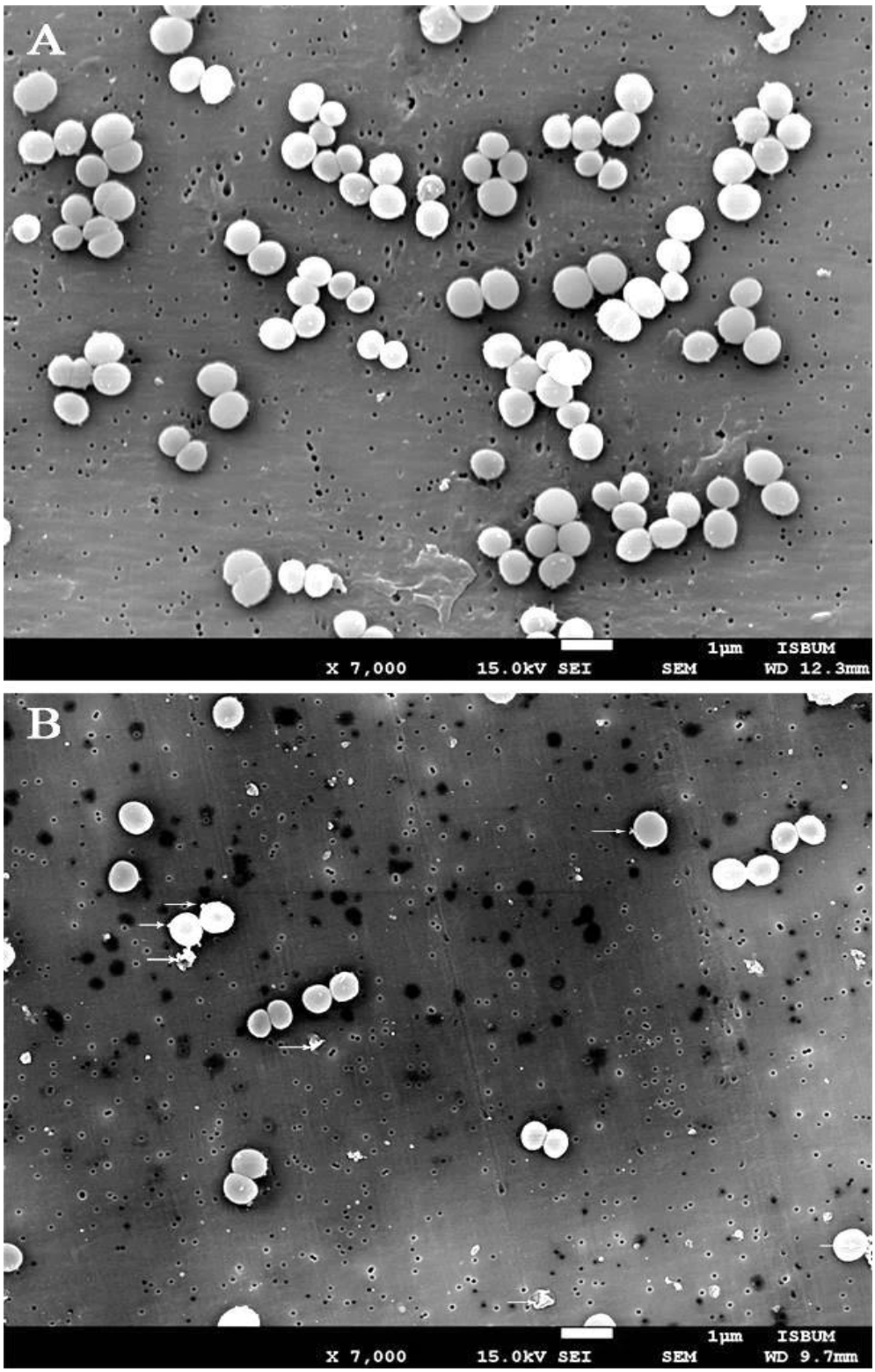
**Appendix 60.** 16S rDNA sequences of *Lactobacillus paracasei* subsp. *paracasei* 25302 isolated from cow milk.

TTTGTCACTTAGACGGCTCGCTCCCTAAAAGGGTTACGCCACCGGCTTCGG
GTGTTACAAACTCTCATGGTGTGACGGGC
GGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGA
TTACTAGCGATTCCGACTTCGTGTAGGCGA
GTTGCAGCCTACAGTCCGAACTGAGAATGGCTTTAAGAGATTAGCTTGACC
TCGCGGTCTCGCAACTCGTTGTACCATCC
ATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC
ATCCCCACCTTCCTCCGGTTTGTCACCGG
CAGTCTTACTAGAGTGCCCAACTAAATGCTGGCAACTAGTCATAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAACCATGCACCACCTGTCATTTTGCCCCCGA
AGGGGAAACCTGATCTCTCAGGTGATCAAA
AGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACAT
GCTCCACCGCTTGTGCGGGCCCCCGTCAA
TTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAATGCTTAAT
GCGTTAGCTGCGGCACTGAAGGGCGGAAA
CCCTCCAACACCTAGCATTCATCGTTTACGGCATGGACTACCAGGGTATCT
AATCCTGTTCGCTACCCATGCTTTCGAGC
CTCAGCGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTCC
ATATATCTACGCATTTCACCGCTACACAT
GGAGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGATGCGCTT
CCTCGGTTAAGCCGAGGGCTTTCACATC
AGACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGATAA
CGCTTGCCACCTACGTATTACCGCGGCTGC
TGGCACGTAGTTAGCCGTGCTTTCTGGTTGGATACCGTCACGCGACAACAG
TTACTCTGCGACCATTCTCTCAACAACAG
AGTTTACGACCGAAAGCTTCTCACTCACGCGCGTGCTCATCAGACTTGCGT
CCATTGTGGAGATCCCTACTGCCTGCCTC
CGGTAGAGAGTTGGGCGGGTCTCAGTCCATGTGGCGAATCAACCCTCTCA
GTCCGCCTACGATTATTATCGCCTGTGGAC
CTTACTCACACCATAGCTAATAGCGCGGTCTATCTTCTAAAAGCCG

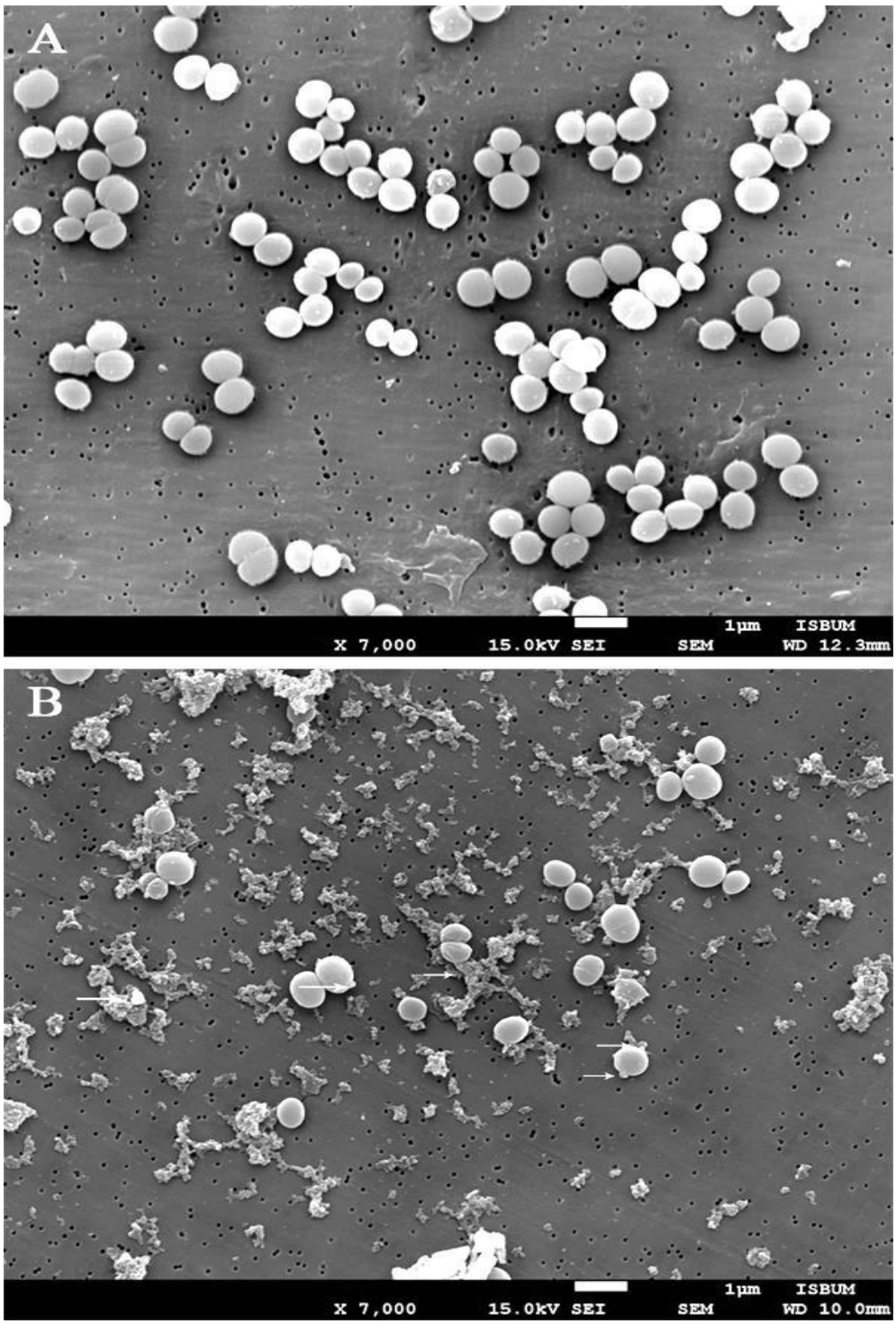
**Appendix 61.** 16S rDNA sequences of *Lactobacillus paracasei* subsp. *paracasei*. 8700:2 isolated from cow milk.



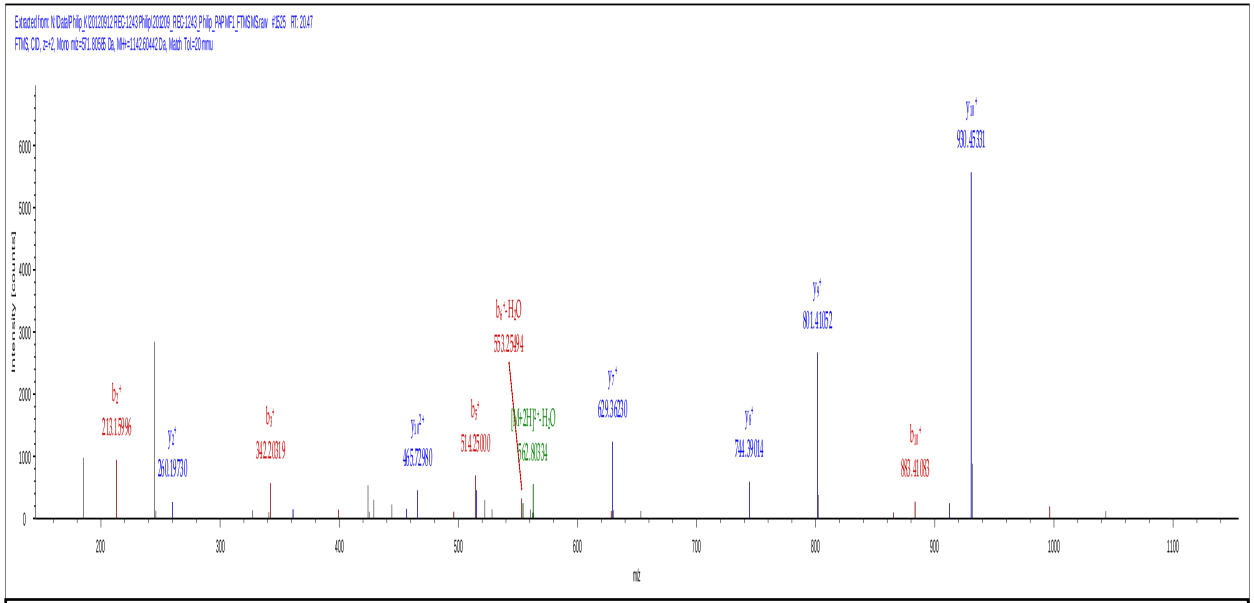

**Appendix 62**. Effect of active peptide extracted (50 µg/ml) from *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by scanning electron microscope. (A) Control: *B. cereus*. (B) *B. cereus* treated with peptide. (B) Arrows show significant alterations in cell morphology and shape include roughening of the cell surface, the formation of numerous blebs and lysis accompanied with an accumulation of cell debris.




**Appendix 63**. Effect of active peptide extracted (50 µg/ml) from *Lactobacillus paracasei* subsp. *paracasei* 8700:2 by scanning electron microscope. (A) Control: MRSA. (B) MRSA treated with peptide. (B) Arrows show significant alterations in cell morphology and shape include roughening of the cell surface, the formation of numerous blebs and lysis accompanied with an accumulation of cell debris.

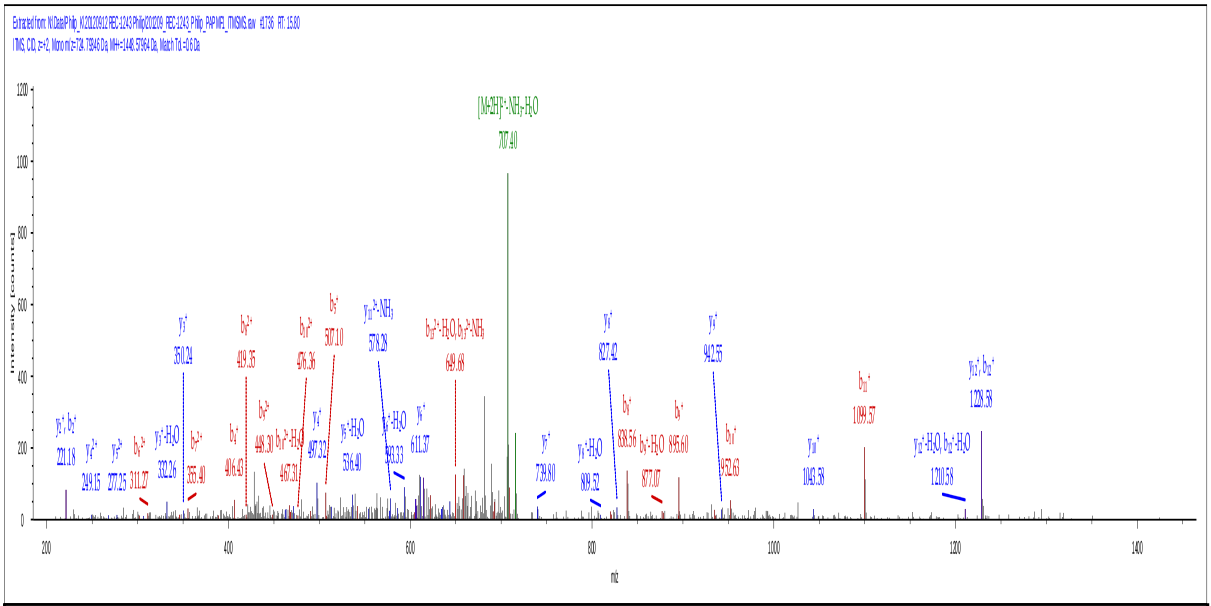



**Appendix 64**. Effect of active peptide extracted (50 µg/ml) from APM by scanning electron microscope. (A) Control: MRSA (B) MRSA treated with active peptide**.** (B) Arrows show significant alterations in cell morphology and shape include roughening of the cell surface, the formation of numerous blebs and lysis accompanied with an accumulation of cell debris.



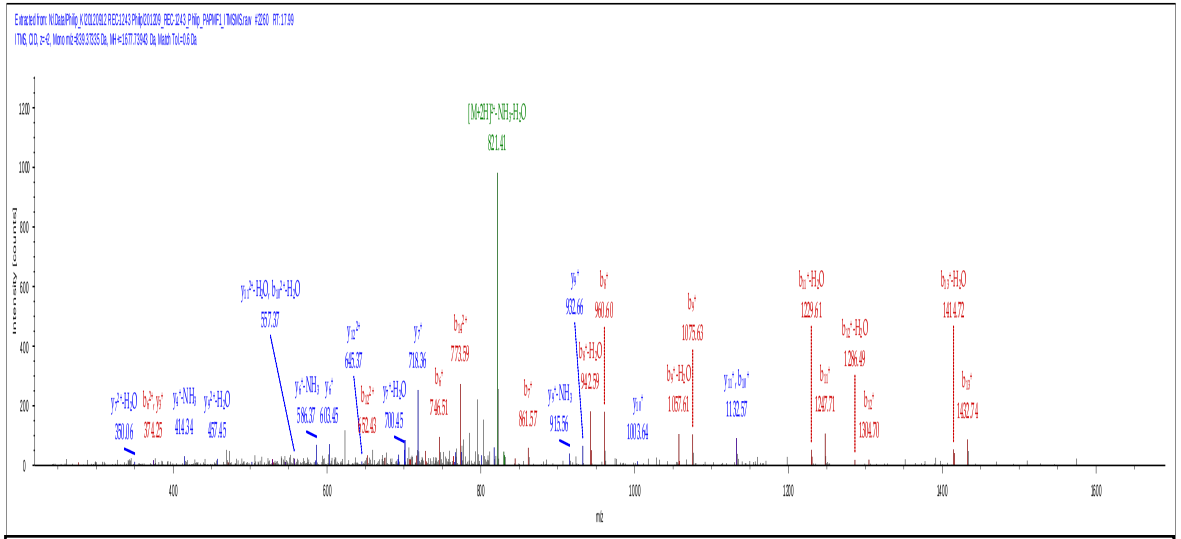

| Peptide accession | Sequence | Score | $MH^+$ [Da] | Charge |
|---|---|---|---|---|
| P25986 | IVEGNGGPGTIK | 48 | 1142.60442 | 2 |

**Appendix 65.** MS/MS spectrum of active fraction of APM with amino acid sequences. The spectrum was deduced to being part of pathogenesis-related protein 2 of *Phaseolus vulgaris* with sequence of (IVEGNGGPGTIK), peptide accession P25986, score 48, $MH^+$ at *m/z* .1142.60442 and charge 2.



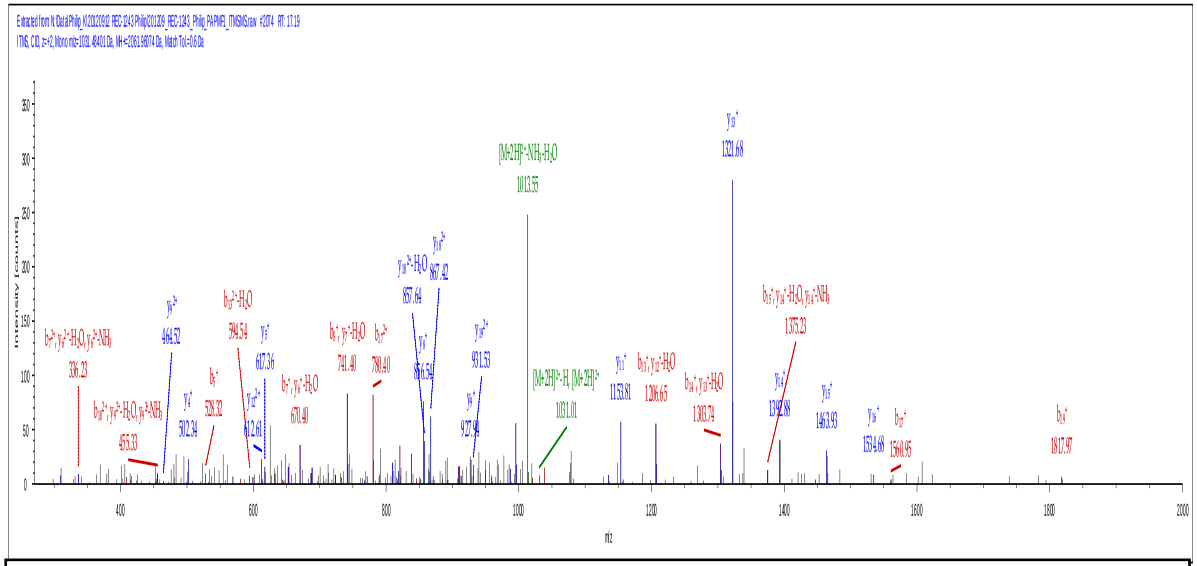

| Peptide accession | Sequence | Score | $MH^+$ [Da] | Charge |
|---|---|---|---|---|
| Q00434 | GGKTDSEGGFESD | 71 | 1285.51738 | 2 |

**Appendix 66.** MS/MS spectrum of fraction 2 of APM with amino acid sequences. The spectrum was deduced to being part of oxygen-evolving enhancer protein 2 from APM with sequence of (GGKTDSEGGFESD), peptide accession Q00434, score 40, $MH^+$ at *m/z* 1285.51738 and charge 2.




| Peptide accession | Sequence | Score | $MH^+$ [Da] | Charge |
|---|---|---|---|---|
| Q39752 | EMIREADVDGDGQIN | 44 | 1677.73943 | 2 |

**Appendix 67.** MS/MS spectrum of fraction 3 of APM with amino acid sequences. The Spectrum was deduced to being part of calmodulin of *Fagus sylvatica* from APM with sequence of (EMIREADVDGDGQIN), peptide accession Q39752, score 44, $MH^+$ at *m/z* 1677.73943 and charge 2.



| Peptide accession | Sequence | Score | $MH^+$ [Da] | Charge |
|---|---|---|---|---|
| P13194 | AEEPTAAAPAEPAPAADEKPE | 32 | 2061.96074 | 2 |

**Appendix 68.** MS/MS spectrum of fraction 4 of APM with amino acid sequences. The spectrum was deduced to being part of photosystem I reaction center subunit IV, chloroplastic protein**)** of APM with sequence of (AEEPTAAAPAEPAPAADEKPE), peptide accession P13194, score 32, $MH^+$ at *m/z* 2061.96074 and charge 2.