CHAPTER II

LITERATURE REVIEW

2.1. Honey

Man and bee have had a relation since the era of the Stone Age (Crane, 1983). The honey that bees produce is a viscous sweet liquid and comes from the nectar of flowers. Honey is not only a food, but a healing substance as well and there is even a reference to it in the Holy Quran in Surat El-Nahl (verses 98 and 69). The first written reference about honey mentioned its use as a drug and an ointment (Crane, 1975). There is ample evidence in the literature that mentions the use of honey in most cultures as a source of nutrition and for medical purposes, and using honey as a drug, nutrient and ointment has been carried into modern times. Since a long time ago honey has been a very important source of carbohydrates and used as a sweetener until industrial sugar was produced after 1800 (Crane, 1975). The practice of alternative medicine called apitherapy offers various treatments based on honey and other bee products against many diseases (Potschinkova, 1992; Cherbuliez & Domerego, 2003). The healing quality of honey can be seen in its many applications to treat wounds, burns and infections as reviewed by Molan (1999), Bogdanov *et al*. (2008) and Shakespeare (2011).

2.2. Chemical Composition of Honey

Honey is rich in many compounds including sugars, organic and amino acids, proteins, minerals, polyphenolics, aroma compounds and vitamins. It is suggested that the chemical composition of honey is related to its botanical origin (Persano & Piro, 2004).

2.2.1. Sugars

Carbohydrates are the main contents of honey, comprising about 95% of the honey's dry weight. The sugars composition in honey varies from one type to another depending on the source the honey comes from and the origin of the flower nectar (White & Maher, 1953; Fuente *et al*., 2011). The viscosity of honey comes from the high amount of sugar that is present in it and ranges from 80 to 85% (mainly glucose and fructose) (Buba *et al*., 2013). Honey samples from North-Central Nigeria were found to have sugar content ranging from 63.82 to 80.25% (James *et al*., 2009).

The monosaccharide fructose and glucose are the main sugars in honey and about 25 different oligosaccharides have been identified (Doner, 1977; Siddiqui, 1970). The main oligosaccharides in blossom honey (Golden aromatic honey) are the disaccharides sucrose, maltose, turanose and trehalose, and also some nutrition-relevant ones like panose, 1-kestose, palatinose and 6-kestose. In comparison with honeydew honey (Forest honey), honeydew contains more of the oligosaccharides melezitose and ranffinose. Following honey intake the primary carbohydrates fructose and glucose are transported rapidly into the blood to be used for energy requirements by the human body. A dose of 20 g honey every day could cover about three percent of the daily energy (Bogdanov *et al*., 2008).

Generally, honeys with high content of fructose are sweeter in comparison with high glucose concentration (Bogdanov *et al*., 2007).

2.2.2. Protein, Peptides and Amino Acids

Studies on the proteins of honey are very limited which may be due to the low concentration of proteins in honey (0.1 to 0.5%) (Chua *et al*., 2013). Several reports have extracted the proteins using ultrafiltration, ultracentrifugation or chemical precipitation, but they did not include identification of proteins which may be because of the low recovery of the instrument used nowadays (Chua *et al*., 2013). In recent times, some researchers have been attracted to the honey proteins that may be related to the potential applications of honey proteins in pharmaceuticals (Chua *et al*., 2015). For the extraction of honey proteins they have used chemical methods like ammonium sulphate precipitation and, the Bradford method and SDS-PAGE gels were used to determine protein content and profile (Chua & Adnan, 2014; Chua *et al*., 2015). These researchers concluded that most of royal jelly proteins were present in honey, and *Apis mellifera* bee proteins were more abundant especially in Acacia honeys. Other peptides such as bee defensin-1 was reported to be present in RS honey but not from other types of honey from different sources; while some honey contained proteinases (Mundo *et al*., 2004; Gallardo-Chacon *et al*., 2008).

Honey contains about 0.5% proteins, free amino acids and enzymes. The three major honey enzymes are amylase (decomposing starch into smaller sugar units), α-glucosidase (decomposing sucrose into fructose and glucose), as well as glucose oxidase (producing hydrogen peroxide and gluconic acid from glucose) (Bogdanov *et al*., 2008). The protein

content of 22 honey samples from Ceará State, Brazil varied between samples and from 178 to 1121 µg/g (da Conceição *et al*., 2013). Azeredo *et al*. (2003) found high protein content in Borreria verticillata honey (2236.00 µg/g) from Piauí, and the authors mentioned that the protein content of 1000 µg/g or more in honey is considered high. Chromatographic analysis of the amino acids profile in Malaysian honey was carried out by Chua and Adnan (2014) and found that 64.6 % of total amino acid was proline which was the most abundant in all tested honey samples.

2.2.3. Phenolic Compounds, Flavonoids and Minerals

The composition of phenolic and flavonoids in honey is dependent on the original source of the honey; more than 8000 phenolic compounds and flavonoids are present in plants and floral. These compounds are transferred by the bee to honey and they contribute to honey flavour, colour, taste and other health benefits (Yao *et al.*, 2003; Al-Mamary *et al*., 2002; Estevinho *et al*., 2008). Phenolic compounds in honey are mostly divided into two sub-classes: benzoic acids and cinnamic acids, while flavonoids, which are present in honey are separated into three classes: flavanones, flavones, and flavonols (Yao *et al*., 2003; Al-Mamary *et al*., 2002). Different chemical substances such as aromatic acids, unknown compounds with different chemical properties (Bogdanov, 1997), phenolics and flavonoids (Cushnie & Lamb, 2005; Weston *et al*., 1999) have been detected in honey. Total phenolic composition of Tualang honeys was from 228.37 to 472.52 mg GAE/kg, and flavonoids content of Tualang honey samples from Malaysia ranged from 40.23 to 86.42 mg GAE/kg, however this content could decrease after storage (Khalil *et al*., 2012).

The colour of honey varies from one type to another, depending on the botanical origin (Crane, 1984). The honey aroma depends also on the content and kind of acids and amino acids (Bogdanov *et al*., 2007). Polyphenols are also important compounds for the appearance and functional properties of honey (Al-Mamary *et al*., 2002; Gheldof & Engeseth, 2002). Mostly flavonoids (kaempferol, luteolin, quercetin, apigenin, galangin, chrysin), phenolic acids and their derivatives (Tomás-Barberán *et al*., 2001) that are known for their antioxidant activity. Flavonoids content can vary between 60 and 460 µg/100 g of honey and this amount increases in dry seasons with high temperatures (Kenjeric *et al*., 2007).

It is known that varying unifloral honeys contain different levels of minerals and trace elements (Bogdanov *et al*., 2008). Nutritionally, honey contains manganese and selenium and the elements sulphur, boron, cobalt, fluoride, iodide, molybdenum and silicon which are important in human nutrition (Bogdanov *et al*., 2008). Honey contains 0.06 to 5 mg/kg acetylcholine and 0.3-25 mg/kg choline (Heitkamp, 1984).

2.3. Health Benefits of Honey

2.3.1. Oral Health

Caries are inhibited by honey because of its antimicrobial activity (Steinberg *et al*., 1996; Molan, 2001b) and could induce a carioprotective effect (Sela *et al*., 1998; Edgar & Jenkins, 1974). The possibility of honey functioning as food for oral health was studied by University of Illinois in Chicago, which concentrated on four varying areas including

the effects of the honey on the growth and cariogenic properties of plaque bacteria, the formation and acidity of human dental plaque, the use of honey treatment on caries formation and aphthous ulcers (Bogdanov *et al*., 2008). Inhibition of the growth and acid production of *Streptococcus mitis*, *Streptococcus sobrinus* and *Lactobacillus caseii* was reported from the oral intake of honey (Bogdanov *et al*., 2008). Similarly, manuka honey was reported to have antibacterial activity and positive effects against dental plaque development and gingivitis (English *et al*., 2004). From what has been discussed above, the conclusion is that honey, compared to other sugars is less cariogenic.

2.3.2. Wound and Burn Healing

In ancient times, honey was used to treat wounds and sores. Nowadays the wide-ranging effect of honey on the healing of wounds confirms the reputation of honey as an antimicrobial agent and promoter of healing (Molan, 2002; Seckam & Cooper, 2013). Many different types of wounds were successfully treated with honey dressings (Molan, 2001a). In wound healing therapeutic effects were observed, including fast healing, clearance of infection, reduction of inflammation, stimulated healing process, wound cleansing action, stimulated tissue regeneration, and comfortable dressings which did not adhere to the tissues (Molan, 2001a). In recent times, the wound healing quality of honey has been rediscovered (Molan, 2001b). Laboratory investigations have confirmed the effectiveness of honey against many infection causing pathogens such as some antibiotic resistant bacteria like MRSA (Methicillan resistant *Staphylococcus aureus*) and VRE (Vancomycin resistant *enterococci*) (Allen *et al*., 2000; Cooper *et al*., 2000a & b).

Honey can cure burns more effectively than traditional dressings (Postmes *et al*., 1997). Several studies from India have reported that dressing with pure unprocessed, undiluted honey obtained from hives showed positive impact over conventional medical treatments such as OpSite® (Subrahmanyam, 1993a), Silver sulfadiazine (Subrahmanyam, 1997) and traditional, low-cost treatments such as boiled potato peels (Subrahmanyam, 1996). A comparison of various dressings elaborated that honey is an excellent dressing which hastens healing, improves the formation of tissue, reducing inflammation and scaring (Cowley, 1990). Other advantages are the low coast of honey and easy dressing. On top of that, it was found that significant curing of skin grafts occurred at room temperature in honey (Subrahmanyam, 1993b).

Honey can be effective in curing infecting non-healing skin wounds (Mclnerney, 1990; Somerfield, 1991). Honey was evaluated on Fournier's gangrene with topical unprocessed honey and exhibited fast healing (Efem, 1991; Hajase *et al*., 1996). Ampicillin and nitrofuazone were compared with honey in animal study using buffalo and, honey was more effective than tested antibiotics (Gupta *et al*., 1992; Kumar *et al*., 1992). Rozaini *et al*. (2004) reported that topical application of honey on burn wounds can improve healing with regard to the tensile strength property. Nasir *et al*. (2010) noted that Malaysian Tualang honey has a bactericidal and bacteriostatic effect against wound and burn organisms, it is beneficial as a dressing due to its ease of application and reduced stickiness in comparison with Manuka honey. On the other hand, in the case of Gram positive bacteria, Tualang honey loses out in effectiveness compared to silver-based dressing or medical grade honey dressing.

Honey was evaluated in surgical dressing for vulvectomies due to its bactericidal properties and it was also successfully used to treat ulcerations after radical surgery for breast carcinoma and varicose veins (Bulman, 1955). Other researchers have been reported to use undiluted honey after radical operations for vulva carcinoma and the result showed no infections, minor debridement and shorter hospital stay (Cavanagh *et al*., 1970). Honey is recommended to be applied on wounds especially surgical wounds because of the low cost of honey (Ustunol, 2000).

2.4. Antimicrobial Activity

Honey was reported to inhibit the growth of *Rubella* virus *in vitro* (Zeina *et al*., 1996), and three species of the *Leishmania* parasite (Zeina *et al*., 1997) and *Echinococcus* (Kilicoglu *et al*., 2006). Mundo *et al.* (2004) collected honey samples from different sources and evaluated them against food spoilage organisms and pathogens namely, *Alcaligenes faecalis*, *Aspergillus niger*, *Geotrichum candidum*, *Penicillium expansum*, *Lactobacillus acidophilus*, *Pseudomonas fluorescens*, *Bacillus cereus*, *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica Ser.* Typhimurium, *S. aureus*, *S. aureus* 9144 and *B. stearothermophilus*. The results showed that *B. stearothermophilus* was highly sensitive to honey, *Alcaligenes faecalis* and *L. acidophilus* were less sensitive, and the fungi *A. niger*, *P. expansum*, *G. candidum* and *S. aureus* were unaffected by honey. Taormina *et al*. (2001) observed that honey from six different sources in USA showed inhibitory activity against *E. coli* O157:H7, *Shigella sonnei*, *S. typhimurium*, *L. monocytogenes*, *B. cereus* and *S. aureus;* the activity was related to hydrogen peroxide

and non-peroxide components. The antibacterial agent hydrogen peroxide is produced by honey glucose oxidase (White *et al*., 1963).

Malaysian honey has been reported to have antibacterial activity (Aljadi & Yusoff, 2003; Tan *et al*., 2009). Tan *et al*. (2009) evaluated the antimicrobial activity of Malaysian Tualang honey from different aromatic and medicinal plants against multidrug resistant bacteria isolated from human; all honey samples showed strong activity against *E. coli, S. aureus, B. subtilis* and *P. aeruginosa*. Recently, Abd-Rahman (2011) observed that Tualang honey obtained from seven different sources in Malaysia showed good inhibitory activity against *E. coil*, *S.* Typhimurium, *Klebsiella pneumonia*, *B. cereus*, slight inhibitory activity towards *S. aureus*, and no activity against *S. epidermidis*. Voidarou *et al*. (2011) observed that almost all of the honey samples of coniferous, thyme, citrus, and polyfloral honey showed antibacterial activity on clinical bacteria such as *S. aureus, S.* Typhimurium, *Streptococcus pyogenes, B. subtilis* and *B. cereus*. Similarly, Manuka honey from New Zealand showed similar antibacterial effects against those bacteria (Mandal & Mandal, 2011). Aween *et al*. (2012a and b) isolated *Lactobacillus acidophilus* from Malaysian, Libyan, New Zealand and Saudi Arabian honey and found good inhibitory activity against Gram-positive and Gram-negative pathogenic bacteria.

2.5. Compounds Responsible for the Antimicrobial Activity in Honey

Honey contains a mixture of compounds, some of which are known while others remain unidentified or uncharacterized; most of these compounds may contribute to the

antimicrobial activity of honey. Some researchers divided the antimicrobial activity of honey into two types, the first one is hydrogen peroxide activity and the second one is non-hydrogen peroxide i.e. other compounds present in honey (Bogdanov, 1997; Kwakman & Zaat, 2012; Aggad & Guemour, 2014). It has been reviewed by Kwakman and Zaat (2012) that honey contains antimicrobial compounds like methylglyoxal (MGO) and hydrogen peroxide (H_2O_2). These compounds were neutralized and tested against *S. aureus* and found that neutralization of one of these compounds does not affect the antibacterial activity of honey, while neutralization of both resulted in 2 log. reduction in the antibacterial activity of honey. Substances of botanical origin of honey could contribute to the antimicrobial effects of honey (Molan, 1997; Bogdanov, 1997). From these observations, it can be said that the antimicrobial activity of honey may not be from one specific single compound which is hydrogen peroxide or methylglyoxal, but the result of various combinations of compounds and most of them are still unknown. Low pH of honey can also contribute to the antibacterial activity as observed by Yatsunami and Echigo, (1984). Some of the known compounds with antibacterial activity are discussed in next section.

2.5.1. Hydrogen Peroxide

One of the carbohydrate-metabolizing enzymes is glucose oxidase. This enzyme is transferred to the nectar of the flowers by bees, and glucose oxidase converts glucose to gluconic acid and hydrogen peroxide (H_2O_2) (White *et al*., 1963; Bang *et al*., 2003; Kwakman & Zaat, 2012). The assumed function of hydrogen peroxide is to prevent the spoilage of unripe honey when the sugar levels do not reach the amounts that will not

allow the microbes to grow (Kwakman & Zaat, 2012). H_2O_2 is inactivated in the ripe honey, and once honey gets diluted it becomes activated for antibacterial effect. H_2O_2 reaches its highest levels when honey diluted in the range of 30 to 50% and decreases very quickly below 30% honey due to the relatively poor affinity of honey bee glucose oxidase for its substrate glucose to produce hydrogen peroxide (Schepartz *et al.*, 1964). Hydrogen peroxide can be affected and destroyed by heat or light (Kwakman & Zaat, 2012). It was reported that coconut honey and gelam honey produced in Malaysia contained non-peroxide factors of antimicrobial activity; these types of honey were shown to inhibit the growth of *S. aureus* and *E. coli* (Aljadi & Yusoff, 2003).

2.5.2. Methylglyoxal

Methylglyoxal is formed by sugars after heat or storage effects and it is one of the compounds present in honey especially Manuka honey (Weigel *et al.*, 2004). Manuka honey is a type of honey that comes from the Manuka tree (*Leptospermum scoparium*) and its origin is from New Zealand (Kwakman & Zaat, 2012). Methylglyoxal is reported to be found in some trees in low amounts, while the nectar of the Manuka tree flowers contains high amounts of methylglyoxal (Adams *et al.*, 2009). Methylglyoxal found in other foods in levels of 3 to 47 mg/kg, and in other honeys with amounts of 24 mg/kg but, in Manuka honey the level is still much higher ranging from 38 to 1,541 mg/kg (Adams *et al.*, 2008; Mavric *et al.*, 2008; Kwakman *et al.*, 2010; Kwakman & Zaat, 2012). Methylglyoxal from Manuka honey showed antimicrobial activity and that has been proven many of studies (Kwakman & Zaat, 2012). In addition, the amount of methylglyoxal in honey is found to be correlated to the antimicrobial activity of honey

which indicates that methylglyoxal is the compound responsible for the antimicrobial activity of that honey. Manuka honey contributes non-peroxide antimicrobial activity that is mostly related to the presence of methylglyoxal (Adams *et al*., 2008; Kwakman & Zaat, 2012).

2.5.3. Phenolic Compounds and Flavonoids

Phenolic compounds with antibacterial activity were isolated from Manuka honey and identified using a thin layer of chromatography (TLC) (Russell *et al*., 1990). Weston *et al*. (1999) reported that Manuka honey contains phenylactic acids, benzoic acids, cinnamic acids and flavonoids and also that these compounds showed inhibitory activity against phatogenic bacteria. A total of 23 samples of honey produced in northwest Spain were analysed for their antibacterial phenolic content, and phenolic compounds were extracted and showed antibacterial activity against *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Escherichia coli*, and *Proteus mirabilis*. The antibacterial phenolic extracts were analysed using solid-phase extraction (SPE) and HPLC/DAD and the result was that p-coumaric, caffeic, pinocembrin, ellagic acids, galangin, chrysin, kaempferol and tectochrysin were present in all fractions (Escuredo *et al*., 2012).

It was reported that Malaysian honeys (coconut and gelam honey) had antibacterial activity against *S. aureus* and *E. coli* and believed to be attributed to components such as phenolic acids (Aljadi & Yusoff, 2003). Phenolic compounds were extracted from clear and dark honeys from Portugal and tested for their antimicrobial properties against a

range of Gram-positive and Gram-negative bacteria; all extracts showed inhibition activity against the target bacteria, but *S. aureus* was more sensitive than other strains (Estevinho *et al*., 2008). These phenolic extracts were analysed using HPLC and it was found that naringenin, phydroxibenzoic acid, cinnamic acid, chrysin and pinocembrin were present in all extracts (Estevinho *et al*., 2008). Phenylactic acids and syringic acid are the major components found in Manuka honey and both are present in types of European honeys (Wilkins *et al*., 1993; Steeg & Montag, 1987); these acids are well known to have antimicrobial activity and they could be part of the responsible compounds for the non-peroxide antimicrobial activity of honey (Kim *et al*., 2004; Zhu *et al*., 2011). In contrast, Weston *et al*. (2000) concluded that the non-peroxide antibacterial activity of Manuka honey was not correlated to the phenolic compounds in honey.

2.5.4. Peptides

Reports on the antimicrobial peptides from honey are very limited. Bee defensin-1 (Royalisin) is one of the main honeybee peptides and it was discovered in antimicrobial medical-grade honey. When bee defensin-1 was neutralized and tested against *B. subtilis* the activity of honey decreased compared to the presence of bee defensin-1 (Kwakman *et al*., 2010). Bee defensin-1 has antibacterial activity only against Gram-positive bacteria including *S. aureus*, *Paenibacillus larvae*, and *B. subtilis* (Kwakman *et al*., 2010; Bachanova *et al*., 2002). There are some proteinases with antibacterial activity from 6 of 26 honeys (Mundo *et al*., 2004; Gallardo-Chacon *et al*., 2008). Proteins from four of those honeys showed antibacterial activity against *Bacillus* spp., but not against *S. aureus*, similar to what has been observed with bee defensin-1. Therefore, the active proteomic

compounds in these honey samples can be bee defensin-1. In contrast, Weston *et al*. (2000) suggested that the non-peroxide antibacterial activity of Manuka honey was not from peptides like royalisin and lysozyme which were not detected in this honey.

2.5.5. Sugars

Honey consists of high amounts of sugar (about 80%) and it is one of the well-known antibacterial factors in honey (Molan, 1992). Basson and Grobler, (2008) reported that honeys from South African plants possess antimicrobial activity against bacteria and yeasts and suggested that 25 % of this activity is related to the sugar concentrations in honeys. In contrast, Weston and Brocklebank, (1999) reported that the non-peroxide antibacterial activity of Manuka honey was not related to the presence of oligosaccharide, but mostly from other components.

2.6. Honeybee

A member of the genus *Apis* has the following characteristics: it produces and stores honey and constructs perennial, colonial wax nests. Honey bees are the only existent members of the tribe *Apini*, all belonging to the genus *Apis*. Nowadays, there are only seven types of honey bees that are recognised, with a total of 44 subspecies, compared to historically, only six to 11 species were recognised. However, honey bees are representative of only a small fraction of the roughly 20,000 known bee species. Although there are related types of bees that also produce and store honey, it is only the

members of the genus *Apis* that are true honey bees. The science of the study of honey bees is known as apiology (Engel, 1999).

A high number of studies exist on the proteins and peptides from different types of honey bees. The antimicrobial peptides are some of the compounds present in honey bees and they are very important to the immune system of the honey bee, helping it to treat any infection (Hoffmann *et al*., 1999). Types of antimicrobial peptides were found in honey bees: apidaecin present in 12 isoforms (Casteels *et al*., 1989), abaecin (Casteels *et al*., 1990), hymenoptaecin (Casteels *et al*., 1993) and defensin present two isoforms, defensin 1 and 2 (Casteels *et al*., 1993; Casteels-Josson *et al*., 1994; Qu *et al*., 2008; Ilyasov *et al*., 2012). Antimicrobial peptides from families of abaecin (11 peptides), defensin (29 peptides), apidaecin (13 peptides), and hymenoptaecin (34 peptides) were identified from the Asiatic honeybee, *Apis cerana* (Xu *et al*., 2009).

The defensins of honey bees have a high level of polymorphism and are present as two peptides-defensin 1 and 2. Defensin 1 is synthesized in the salivary glands and is responsible for social immunity, while the synthesis of defensin 2 is by cells of the fat body and hemolymph is responsible for individual immunity (Ilyasov *et al*., 2012). Defensin from honeybee exhibited antibacterial activity against Gram-positive and Gram-negative bee pathogens as confirmed in several studies (Mandrioli *et al*., 2003; Bulet & Stocklin, 2005; Ilyasov *et al*., 2012). Of all antimicrobial peptides of honeybees, only defensins exhibit cytotoxic activity against *Aspergillus flavus*, *Ascosphaera apis*, *Aspergillus niger* Tieghem, *Aurobasidium pullulans* and *Candida albicans* (Aronstein *et al*., 2010; Chernysh *et al*., 1999; Ilyasov *et al*., 2012).

2.7. Proteins and Peptides from other Sources and their Antimicrobial Activity

Antimicrobial peptides are one of the natural solutions that could be used to replace commercial antibiotics and preservatives which are based on old wisdoms. Additionally, most of the known antimicrobial peptides were extracted from different sources such as plants, trees, milk, milk fermentation, dairy products, bacteria metabolite, fish and animals (James *et al*., 1996; Nes & Holo, 2000; Douglas *et al*., 2003; Hayes *et al*., 2006; Broekaerta *et al*., 2010; Mukesh, 2011; Vriens *et al*., 2014; Fadaei, 2012; Wilsona *et al*., 2012; Rybal'chenko *et al*., 2013). The following are some of the previous reports of peptides with antimicrobial activity against pathogens and fungi including food spoilage and harmful bacteria which were extracted and purified from various origins.

Host defensin peptides are antimicrobial peptides that are present naturally in skin of animals and plants to protect and support their immune systems against any attacks from pathogens, viruses or parasites (Wilsona *et al*., 2012). Plant defensin is antimicrobial peptide which belongs to a cationic family; defensin was detected in plant pathogenic bacteria infections and one of the main defensive weapons of the plants (Mukesh, 2011). Plant defensins are peptides which contribute to antifungal activity especially against plant pathogens; those peptides are non-toxic and can be used in medical fields because of their ability to inhibit the growth of different pathogens. Most previous reports on defensin were based on *in vitro* studies, while *in vivo* also should be considered, as reviewed by Vriens *et al*. (2014).

Fadaei (2012) extracted peptides from milk and found that the peptides attribute to the antimicrobial activity against pathogenic microbes and suggested that the peptides can be

used as preservatives and health enhancing. The peptides were α_{S2}-casein, α_{S1}-casein, κ-casein, lactoferrin, α-lactalbumin, lysozyme, and β-lactoglobulin. Host defensin peptide acts as a natural antibiotic in milk to protect the animal against neonatal diarrheal disease (Wilsona *et al*., 2012). Bioactive peptides were extracted from native milk and found to have the inhibitory ability against pathogens and the bioactive peptides were sequenced and found to contain from 2 to 20 amino acid residues (Jabbari *et al*., 2012).

Lactic acid bacteria (LAB) have been well known for their antimicrobial activity for a long time, and most LAB activity is related to the peptides that are produced by this bacteria (Piard & Desmazeaud, 1991; Klaenhammer, 1993; Nes & Holo, 2000; Hayes *et al*., 2006; Aly *et al*.; 2006; Rybal'chenko *et al*.; 2013; Muhialdin *et al*., 2015). In addition, in the last few years more than 50 peptides with antimicrobial activity were extracted from the metabolites produced by lactic acid bacteria; and most of those peptides contained 20 to 60 amino acid residues. These peptides were more effective against Gram-positive bacteria, which cause food spoilage and also including pathogens; it was concluded that lactic acid bacteria peptides can be very useful as preservatives or in the medical field (Nes & Holo, 2000). Three peptides of SDIPNPIGSENSEK, VLNENLLR, and IKHQGLPQE were produced by *Lactobacillus acidophilus* DPC6026 fermentation of sodium caseinate and these three peptides contribute to antibacterial activity against *Escherichia coli* DPC5063 and *Enterobacter sakazakii* ATCC 12868 (Hayes *et al*., 2006). Rybal'chenko *et al*. (2013) suggested that the antimicrobial activity of lactobacilli except reuterin is related to peptides with low molecular weight ranging from 4 to 6 kDa. Novel peptide with Antifungal activity against *Aspergillus niger* was produced by *Leuconostoc mesenteroides*, reported by Muhialdin *et al*. (2015).

The antimicrobial peptides defensins and thionins were isolated from plants (Broekaerta *et al*., 2010). Five hepcidin-like peptides with antimicrobial activity were isolated from bony fish of *Atlantic salmon*, *Salmo salar* (L.) and *Pseudopleuronectes americanus* (Walbaum) and with amino acids length of 24 to 40 residues (Douglas *et al*., 2003). Boland and Separovic (2006) reviewed antimicrobial peptides with positive charge and residues of 13 to 21 were isolated from tree frog; the peptides were amphipathic α-helices. Novel protein with molecular weight of 190 kDa with antibacterial activity was extracted from biofilm-forming marine bacterium D2, which was isolated from the surface of tunicate *Ciona intestinalis* (James *et al*., 1996).

2.8. Mechanisms of Killing by Antimicrobial Peptides (AMP)

2.8.1. Membrane Dysfunction

The membrane is in charge for mediating many necessary functions in pathogens, such as cellular energetics, synthesis and cross-linking of peptidoglycan, motility, chitin, other biopolymers, and processing or display of adhesins (Yeaman &Yount, 2003). The effect of antimicrobial peptide on the membrane of Gram-negative bacteria differs from Gram-positive bacteria. For Gram-negative bacteria the antimicrobial peptides interact independently on the inner and outer membranes (Yeaman &Yount, 2003). For example, it was verified that human defensins sequentially permeabilize the outer following by inner membrane (Lehrer *et al*., 1989). In Gram-positive cells exposure with antimicrobial peptides results in direct increases in water and ion flow, swelling and osmotic

dysregulation (Juretic *et al*., 1989; Ohta *et al*., 1992; Matsuzaki *et al*., 1997). These notions relate to the fast and generalized membrane effects by antimicrobial peptides (Kagan *et al*., 1994; Lehrer & Ganz, 1996; Yeaman &Yount, 2003).

2.8.2. Inhibition of Extracellular Biopolymer Synthesis

The antimicrobial peptides could inhibit chitin, peptidoglycan and other macromolecular synthesis, which are considered an important mechanism. For example, peptidoglycan biosynthesis is naturally related to membrane function (Yeaman &Yount, 2003). Peptidoglycan precursors are activated and transported across the cytoplasmic membrane, and crosslinking occurs in the immediate proximity of this setting. Cationic or other peptides perturb membrane and peptidoglycan synthesis integrity, such that direct or indirect inhibition of peptidoglycan precursor synthesis, translocation, and/or cross-linking may result, all these could end to the kill of the bacteria cell (Yeaman &Yount, 2003).

2.8.3. Inhibition of Intracellular Functions

Membrane perturbation certainly contributes to antimicrobial peptide mechanisms of action, some studies suggest that disruption of key intracellular processes may contribute to cell death (Lehrer *et al*., 1989; Park *et al*., 1998; Sharma *et al*., 1999; Yeaman &Yount, 2003). These concepts imply a temporal and functional dissociation of membrane permeabilization, depolarization, and target cell viability. In some cases, microorganisms may survive for extended periods of time following membrane permeabilization, suggesting that non-membranolytic mechanisms are responsible for cell death (Yeaman &Yount, 2003).

2.9. Antimicrobial Evaluation Methods

There are several methods used to detect the antimicrobial activity, and some of these methods are considered as qualitative such as disk diffusion method and well diffusion method, while others as quantitative like microtiter plates and plate count assay method. The most common methods for detection of the antibacterial activity are disk and well diffusion methods, both of which are based on the ability of the sample's active compounds to diffuse into the agar (Bauer *et al*., 1966; Perez *et al*., 1990; Aween *et al*., 2014). The differences between disk and well diffusion methods are in the sample inoculation. In the disk diffusion method, an unknown amount of sample is poured into the disks, while in the well method a known amount of sample is poured into the agar wells. The results from both methods are taken as the diameter of zones around the disks or the wells (Bauer *et al*., 1966; Perez *et al*., 1990; Aween *et al*., 2014).

Microtiter plates assay is one of the most successful methods used for the quantitative antimicrobial activity (Magnusson and Schnurer, 2001; Aween *et al*., 2014; Muhialdin *et al*., 2015). This method can be used for all types of microorganisms and it is not complicated when a small amount of sample is used. In this method, the sample and the microbe culture are poured into the universal microtiter 96 well plates and after the incubation time the results can be read by the Elisa reader. The reading from the Elisa reader is based on the turbidity of the microbial growth and it is compared with the positive and negative controls after converting the reading to numbers (Magnusson and Schnurer, 2001; Aween *et al*., 2014; Muhialdin *et al*., 2015).

Plate count assay is a very successful quantitative antibacterial method and used only for bacteria, because it is based on bacteria cells count. This method is usually used to confirm the ability of the tested sample to inhibit the target bacteria by counting the survival from the bacteria after spreading them on the agar plate (Aween *et al*., 2012; Aween *et al*., 2014). All the previous methods should be confirmed by plate count assay, because it is the only way to determine survival bacteria from the tested sample.

2.10. Proteins and Peptides Purification and Identification Methods

There are several methods used in the purification of proteins and peptides. These include capillary electrophoresis, reverse-phase high-performance liquid chromatography (RP-HPLC) and column chromatography or size exclusion chromatography (Gel filtration).

2.10.1. Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

High-performance liquid chromatography (HPLC) was introduced more than three decades ago to enable a sensitive and rapid analysis of proteins and peptides. Nowadays, HPLC plays the main role in the characterization of peptides and proteins, which is needed in most pharmaceutical industries. HPLC is not only a method of analytical experiment, but it is also developed to isolate the targeted compounds based on the method followed and that instrument is called reverse-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC is one of the most common methods used to

purify peptides and depends on the biological recovery of the peptides (Espitia *et al*., 2012). RP-HPLC separate the peptides based on hydrophobicity, the first peptides are more hydrophilic, followed by the low hydrophilic and finally the peptides which are more hydrophobic (Aguilar & Hearn, 1996). Two mobile phases can be used to elute the mixture of peptide sample (A and B), for example: mobile phase A contains 0.1 trifluoroacetic acid (TFA) and deionized water to elute the hydrophilic peptides and mobile phase B consists of 0.1 TFA and acetonitrile for eluting the hydrophobic peptides. RP-HPLC as reported previously could affect the nature of peptides or proteins by its condition (Muhialdin *et al*., 2015).

2.10.2. Sephadex G-50 Fine Column Chromatography

Sephadex column chromatography or gel filtration method is used to separat the proteins or peptides based on their molecular weight. This method is not complicated as well and it is cost efficient compared to other methods. Sephadex column allows the use of a higher amount of sample in injection; it also allows the use of any buffer that peptide samples are eluted in such as phosphate buffer or deionized water. Proteins and peptides with high and low molecular weight can be separated using Sephadex column chromatography, because there are several sizes of Sephadex including G-10, G-15, G-25, G-50, G-75 and G-200, fine or superfine (Porath & Flodin, 1959; Janson, 1987). After pouring a mixture of protein or peptide sample into the column, the high molecular weight proteins or peptides will travel and come out faster as the first fractions followed by the lower molecular weight proteins and peptides. This method does not denature the proteins or peptides (Porath & Flodin, 1959; Janson, 1987; Zhao *et al*., 2013).

2.10.3. Peptide Identification Methods

Several methods are used in identifying peptides and proteins. Such methods include: gas chromatography (GC), liquid chromatography tandem mass spectrometry (LC/MS-MS), and nuclear magnetic resonance (NMR), all of which are the most significant methods used in this field. Gas chromatography is the most suitable instrument for volatile compounds, while nuclear magnetic resonance is used more to identify the structure of the low molecular weight compounds. On the other hand, LC/MS-MS is the most reliable instrument for proteomic studies and it can separate a wide high range of compounds with low and high molecular weight including organic acids, proteins and peptides. However, tandem mass spectra analysis is complicated and needs to be handled by human experts, but nowadays analysis software has been developed to interpret the data in order to have an accurate result. Some of the most common software in analysing and identifying the peptides from tandem mass spectra are Lutefisk, PEAKS6, PepNovo, Profound, Mascut, Swiss-Prot and Pepsea (Perkins *et al*., 1999; Ma & Johnson, 2012). The previous programmes were used to identify the fragments of the peptides and were compared to recorded data base. However, some peptides cannot be found by these software and the result will come out as no data found or de novo, which means that this peptide is unknown or unidentified (He *et al*., 2013; Muhialdin *et al*., 2015).