IDENTIFICATION OF PLANT EXTRACTS AND PHENOLIC COMPOUNDS WITH THE POTENTIAL OF INHIBITING FISH-SPOILING BACTERIA

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by

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LIST OF ABBREVIATIONS

ABTS	2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ANOVA	analysis of variance
ATCC	American Type Culture Collection
BAW	butanol : acetic acid : water
cm	centimetre
DMSO	dimethyl sulphoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl-hydrate
EC_{50}	effective concentration at 50% activity
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalent
g	gram
M	molar
MIC	minimum inhibition concentration
µg	microgram (10^{-6} g)
mg	milligram (10^{-3} g)
ml	millilitre (10^{-3} litre)
mM	millimolar (10^{-3} M)
NA	nutrient agar
NB	nutrient broth
Pa	Pascal

PUFA	polyunsaturated fatty acids
R_f	retention factor
RNS	reactive nitrogen species
ROS	reactive oxygen species
SD	standard deviation
TPC	total phenolic content
UPLC	ultra-performance liquid chromatography
UV	ultraviolet
v	volume
w	weight

PENGENALPASTIAN EKSTRAK TUMBUHAN DAN SEBATIAN FENOLIK YANG BERPOTENSI UNTUK MERENCAT BAKTERIA PEROSAK IKAN

ABSTRAK

Tujuan projek ini ialah untuk mengenal pasti spesies tumbuhan dan sebatian fenolik yang menunjukkan aktiviti antibakteria dan antioksidan. Sampel yang dikaji terdiri daripada organ tumbuhan yang sering digunakan dalam penyediaan sajian ikan. Strain bakteria yang terlibat dalam kajian ini adalah *Pseudomonas fluorescens* (ATCC 13525), *Serratia liquefaciens* (ATCC 27592), *Aeromonas hydrophila* (ATCC 35654), *Staphylococcus xylosus* (ATCC 700404), *Listeria monocytogenes* (ATCC 19115) dan *Listeria innocua* (ATCC 33090) sementara assai antioksidan yang digunakan termasuk 1,1-difenyl-2-pikril-hidrazil-hidrat (DPPH), potensi antioksidan reduksi-ferik (FRAP), asid 2,2-azino-bis-3-etilbenztiazolin-6-sulfonik (ABTS) dan pengkelatan logam. Daripada saringan awal yang melibatkan 34 sampel yang diekstrak menggunakan metanol 80%, ekstrak rizom *Curcuma longa* telah menunjukkan sifat antibakteria yang tertinggi dengan nilai kepekatan perencatan minimum (MIC) berjulat daripada 15.6 kepada 62.5µg/ml terhadap keenam-enam bakteria. Ini diikuti oleh ekstrak daun *Murraya koenigii* yang mencatatkan nilai MIC antara 62.5 dan 125µg/ml apabila disaring terhadap *P. fluorescens, S. xylosus, L. monocytogenes* dan *L. innocua*. Assai-assai antioksidan mendedahkan bahawa ekstrak daun *Persicaria minor* mengandungi potensi yang tertinggi dalam assai DPPH (EC_{50} 19.2 ± 0.3µg/ml) dan FRAP (EC_{50} 40.0 ± 0.3µg/ml). Sampel ini juga mencatatkan kandungan fenolik yang paling tinggi antara sampel tumbuhan yang

diuji (59.1 ± 0.2µg GAE/mg sampel). Ketiga-tiga ekstrak ini kemudiannya difraksinasikan menggunakan kaedah kromatografi kertas untuk menghasilkan tujuh fraksi setiap satu untuk *P. minor* dan *M. koenigii* apabila pelarut BAW (butanol : asid asetik : air suling) digunakan dalam nisbah 4:1:5 dan lima fraksi untuk *C. longa* menggunakan kombinasi pelarut asetik asid 10% dan 50%. Fraksi-fraksi ini diperhatikan sebagai jalur yang jelas berbeza di bawah pencahayaan ultra-ungu dan diasingkan sebelum diuji menggunakan assai antibakteria serta antioksidan seperti ekstrak masing-masing. Analisis kromatografi cecair berprestasi-ultra (UPLC) dijalankan terhadap fraksi terbaik bagi setiap sampel yang telah disenarai pendekkan dan didapati bahawa kurkumin (*C. longa*) dan ρ-asid koumarik (*M. koenigii*) bertanggungjawab ke atas kesan antibakteria manakala kuersetin-3-*O*-rutinosida menyumbang kepada sifat antioksidan *P. minor*. Hasil kajian ini memberikan kita pengawet alternatif untuk melambatkan kerosakan produk ikan yang disebabkan oleh bakteria dan pengoksidaan.

IDENTIFICATION OF PLANT EXTRACTS AND PHENOLIC COMPOUNDS WITH THE POTENTIAL OF INHIBITING FISH-SPOILING BACTERIA

ABSTRACT

The aims of this project were to identify plant species and phenolic compounds that exhibited antioxidant and antibacterial activities. Samples studied were plant organs that are commonly used in the preparation of fish cuisines. The bacterial strains involved in this study were *Pseudomonas fluorescens* (ATCC 13525), *Serratia liquefaciens* (ATCC 27592), *Aeromonas hydrophila* (ATCC 35654), *Staphylococcus xylosus* (ATCC 700404), *Listeria monocytogenes* (ATCC 19115) and *Listeria innocua* (ATCC 33090) while the assays deployed to gauge antioxidant potentials were the 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH), ferric-reducing antioxidant power (FRAP), 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and metal-chelating assays. From the preliminary screenings in which 34 different samples extracted using 80% methanol were involved, the rhizome extract of *Curcuma longa* exhibited the highest antibacterial properties with minimum inhibitory concentration (MIC) values ranging from 15.6 to 62.5µg/ml against all six bacteria. This was followed by the leaf extract of *Murraya koenigii* which recorded MIC values between 62.5 and 125µg/ml when screened against *P. fluorescens*, *S. xylosus*, *L. monocytogenes* and *L. innocua*. The antioxidant assays revealed *Persicaria minor* to have the highest potential in the DPPH (EC_{50} of 19.2 ± 0.3µg/ml) and FRAP (EC_{50} of 40.0 ± 0.3µg/ml) assays. It also recorded the highest total phenolic content (59.1 ± 0.2µg GAE/mg sample) among the tested plant samples. These three extracts were subjected to fractionation using paper

chromatography to yield seven fractions each for both *P. minor* and *M. koenigii* using the BAW (butanol : acetic acid : distilled water) solvent in a ratio of 4:1:5 and five fractions for *C. longa* using a combination of 10% and 50% acetic acid solvents. The fractions were viewed as distinct bands under ultra-violet illumination and were collected separately before being subjected to antibacterial and antioxidant assays as their respective extracts. The ensuing ultra-performance liquid chromatography (UPLC) analysis conducted onto promising fractions of each shortlisted sample revealed that curcumin (*C. longa*) and ρ-coumaric acid (*M. koenigii*) were responsible for the antibacterial effects while quercetin-3-*O*-rutinoside contributed to the antioxidant property of *P. minor*. This outcome provides us with alternative preservatives to delay the spoilage of fish products caused by bacteria and oxidation.

CHAPTER 1:
INTRODUCTION

1.1 Background of study

Harvested fish constitutes a class of highly-perishable foods due to its rapid decline in freshness which is quicker than that of livestock and poultry meat. This is a result of the poikilothermic nature of living fish which, instead of deploying the homeostatic regulation of internal temperature, prefer to rely on the temperatures of surrounding waters (Gómez-Guillén & Montero, 2007). Such a characteristic promotes the growth of bacteria which survive well in a wide range of temperatures and this includes a host of psychrotrophs of both the Gram-positive and Gram-negative types (Gram & Dalgaard, 2002). Following the landing of fish from their waters, bacterial populations along with a number of other factors contribute to the deterioration and spoilage of the harvest (Ronsivalli & Charm, 1975). Spoilage here refers to the alterations in the sensory characteristics of a consumable product making it no longer suitable for consumption (Gram & Dalgaard, 2002).

Spoilage caused by oxidation is another prevalent problem especially among pelagic fish species which contain relatively high proportions of fats stored in their tissues (Fraser & Sumar, 1998). This type of spoilage is characterised by the oxidation of the double bonds in fatty acid chains when these come into contact with oxygen molecules which could decompose to produce radicals. Polyunsaturated fatty acids (PUFA) which make up most of the contents of fish lipids are rich in double bonds and are therefore highly susceptible to oxidative spoilage (Hultin, 1994).

In line with the exponential increase in the global population and the need for efficient storage and transportation of food from producers to consumers, sound food

preservation methods are essential so as not to compromise on quality and nutrition (Ghaly *et al.*, 2010). Microbial activity alone has been blamed for the loss of 30% of landed fish (Amos, 2007) not to mention the four to five million tonnes of trawled seafood products, especially fish and shrimp, which are lost annually to enzymatic and microbial activities due to unsatisfactory storage conditions (Unklesbay, 1992).

Goulas and Kontominas (2007) proposed that since the demand for fresh and minimally-processed fish products is growing, it is timely that more research is centred upon the identification of new methods for preservation. The objectives should be to guarantee fish products in the aspect of its microbiological safety as well as to enhance the shelf life of these products.

As of the moment, practised methods in fish preservation include low temperature storage. This approach however only serves to retard the metabolism of spoilage microorganisms instead of killing these outright (Ashie *et al.*, 1996). Neumeyer *et al.* (1997) reported that oxidative spoilage still persists in freezing conditions albeit at lower rates. Removal of the gut is another common preservation technique and is compulsory for lean species like cod (Huss, 1988). It is recommended to be done immediately upon the landing of the fish (Pedrosa-Menabrito & Regenstein, 1988) and with great care to prevent the spread of gut-inhabiting bacteria to other tissues as this could result in subsequent bacterial contamination (Erkan & Ozden, 2006).

The addition of sodium chloride has been shown by Siringan *et al.* (2006) to reduce oxidative spoilage. The use of other chemicals include acids which lower the pH making conditions unfavourable to spoilage bacteria (Martinez & Gildberg, 1988), ethylenediaminetetraacetic acid (EDTA) which removes pro-oxidant metals

via chelation (Shelef & Seiter, 2005) as well as nitrites and sulphites for their antimicrobial properties (Ray, 2004).

Mahmoud *et al.* (2006) reported that the currently favoured freezing approach is energy demanding and can therefore be substituted by the use of preservatives, both natural and synthetic, for the inhibition of microbial growth as well as the reduction of lipid oxidation in stored fish. The hand-in-hand application of refrigeration and preservatives will be able to cease the deterioration of fish quality due to spoilage (Bagamboula *et al.*, 2004).

Over the centuries, various herbs and spices have been used for culinary as well as medicinal purposes (Opara & Chohan, 2014). In Southeast Asia, the incorporation of these ingredients as well as their mixtures has become a common practice not only for the purpose of enhancing the aroma and flavours but also for other reasons (Chomchalow, 2002). Herbs and spices are still used extensively today for marinating and seasoning in local fish cuisine.

Brandi *et al.* (2006) reported that some herbs and spices contain antimicrobial properties which are effective in controlling human and plant pathogens. To date, however, only temperate and Mediterranean herbs and spices have been widely studied. Examples include rosemary (Sebranek *et al.*, 2005), cranberry (Lin *et al.*, 2004), thyme (Abdollahzadeh *et al.*, 2014), garlic (Millet *et al.*, 2011), basil (Bagamboula *et al.*, 2004), parsley and oregano (Iturriaga *et al.*, 2012).

The leaf extracts of *Clitoria ternatea* was shown to contain antimicrobial properties against fish pathogens (Ponnusamy *et al.*, 2010). The method used however, was the well diffusion approach and the bacterial selection was different, except for *Aeromonas hydrophila*. The potential of the juices of bilimbi (*Averrhoa*

bilimbi) and tamarind (*Tamarindus indica*) in reducing spoilage in raw shrimps caused by two bacterial species, namely *Listeria monocytogenes* and *Salmonella typhimurium* were studied by Wan Norhana *et al.* (2009). The parameters used in this study included the preparation methods of the juices, their concentrations, the washing methods and the duration of shrimp storage. Unlike the slight reduction in the population of *S. typhimurium* on the seventh day after treatment, *L. monocytogenes* increased significantly in population size during refrigeration.

1.2 Problem statement

As described by Ronsivalli and Charm (1975), the deterioration in quality of fish is initiated immediately upon the removal from its waters and this is largely due to the action of bacteria. The high compositions of polyunsaturated fatty acids in its flesh also render landed fish susceptible to oxidative spoilage (Hultin, 1994). Although there are methods which are currently being practised to prolong the shelf-life of fish and fish products, options like freezing are not economical in terms of energy (Mahmoud *et al.*, 2006) and cost. Even with freezing, microbial and oxidative spoilages are not completely prevented (Ashie *et al.*, 1996; Neumeyer *et al.*, 1997).

The use of synthetic and chemical preservatives is often questionable besides being a focus of public scrutiny as well as a cause for health concerns (Tajkarimi *et al.*, 2010; Goon *et al.*, 2014). This gives prominence to the application of plant-based samples with antimicrobial and antioxidant properties to be used in the preservation of fish.

There is the need to identify the necessary concentrations required of these herbs and spices to work effectively. Any less would render the inclusion of these as pointless and an excess in application may compromise the organoleptic properties of food items. This alteration is a problem often encountered when the essential oils of herbs and spices are used, even at low concentrations. The avoidance of this phenomenon as well as the relatively lower cost of extraction make phenolic compounds preferred over essential oils for this application (Shan *et al.*, 2005).

Furthermore, there is a lack of comparative studies which are centred upon the use of natural preservatives in the inhibition of fish spoilage bacteria and fish preservation. This is especially the case in Malaysia in the application of seafood preservation. To our knowledge, conducted studies using herbs and spices as natural preservatives in this country are limited to the preservation of sensory properties of raw shrimps (Wan Norhana *et al.*, 2009), the reduction in microbial count on spoilt fish using plant-based solutions (Selvam *et al.*, 2013) and the anti-microbial activity of Zingiberaceae extracts against four strains of bacteria (Tg Kamazeri *et al.*, 2012).

The first study involved only the juices of bilimbi and tamarind as well as only *L. monocytogenes* and *S. typhimurium*. The second study was more of a survey and did not involve any known bacteria. Only the bacterial count and the palatability of treated fish mattered. The third study involved only three plant samples and four bacteria non-specific to fish spoilage. To our knowledge, this current study is considered to be one of the first comparative studies of its kind in Malaysia.

Most studies conducted abroad deployed the agar-well diffusion or the disc-diffusion methods which both require more materials as well as larger volumes of samples unlike the broth micro-dilution approach utilised in this study which is more

cost-effective. The relatively larger selection of fish spoilage bacteria as well as plant samples constituting mainly local herbs and spices as well as mixtures of these enables this comparative study to achieve a more comprehensive outcome. The results of this study could be useful for a multitude of future discourses.

1.3 Objectives

This study therefore aims to achieve the following objectives.

i) To determine the comparative inhibition potentials of individual plant extracts and their mixtures that are used in the preparation of local fish cuisine towards bacteria, which are causal agents of fish spoilage.

ii) To explore the antioxidant properties of the same samples and the possible links between high-performing samples and their respective antibacterial potentials.

iii) To identify phenolic constituents in plants shortlisted for promising antibacterial and antioxidant potentials.

CHAPTER 2:
LITERATURE REVIEW

2.1. Microbial spoilage of fish

The fisheries industry is a very old and established industry in Malaysia which serves as a source of protein nourishment to about two thirds of the local population (Saharuddin, 1995). Over the ages, the industry has also been a provider of employment and income to many Malaysians (Raduan *et al.*, 2007). At the same time, the global population is expanding at an exponential rate and in line with this, the importance of food preservation is paramount so as to conserve its quality as well as nutritional contents throughout the duration of transportation and storage (Ghaly *et al.*, 2010). The United Nations Food and Agriculture Organisation (FAO) report in 2014 cited that fish processing establishments in most developing countries can be regarded as small or medium-scale and are therefore, ill-equipped in terms of human resources, finance and infrastructure. About 68% of the global fishery production is currently from Asia and this figure is expected to increase to 71% in 2022. An estimated 20% to 75% of fish, by region, are lost to post-harvest spoilage with Asia and Africa being the main contributor to the statistics (FAO, 2014).

While food spoilage may be the conduct of chemical, enzymatic or microbial activities, it is reported that chemical spoilage and quality deterioration by microbial activity is responsible for the loss of at least a quarter of gross primary fishery and agricultural produces annually (Baird-Parker, 2000; FAO, 2013). Amos (2007) reported that as much as 30% of all landed fish are lost to microbes.

In the tropics, spoilage of landed fish usually commence within twelve hours following the onset of *rigor mortis* or stiffening of the muscles (Berkel *et al.*, 2004).

Digestive enzymes and lipases, oxidation as well as microbial activity of surface-dwelling bacteria account for this degradation of fish flesh (AMEC, 2003).

Levin (1968) reported that not much was known with regard to the identities of fish spoilage bacteria down to the species level. A lot of progress has been made in this field since that time. The composition of microorganisms on landed fish is a function of the microbial populations in the waters from which the fish originates (Ghaly *et al.*, 2010). Despite this, Huss (1995) reports that not all bacteria present on landed fish actually contribute to its spoilage. The presence of spoilage microorganisms depends on their type, the species of fish and its history, the fishing method as well as the processing methods (Ashie *et al.,* 1996). To an extent, the rate of microbial spoilage is dependent on the bacterial count. The type of bacteria also influences this since some bacteria are more damaging than others (Adams *et al.*, 1964; Lerke *et al.*, 1965; Licciardello *et al.*, 1967).

There are a number of specific intrinsic factors in fish that would contribute towards the microbiology population present in it as well as towards the incidence of spoilage. These include the poikilothermic nature of fish, the high pH conditions within the flesh of landed fish (usually exceeding 6.0) as well as the presence of relatively high compositions of non-protein-nitrogen (NPN) of which trimethylamine oxide (TMAO) is a component (Gram & Huss, 1996).

Fishes, being poikilothermic, allow for the growth of a host of bacteria with broad temperature requirements. On living fishes, these survive on the external and internal surfaces such as gills, gastro-intestinal tract as well as skin. The muscle tissues of most species of fish contain very minimal carbohydrate content, often less than 0.5%. This leads to the post-mortem production of very minute volumes of

lactic acid causing the pH value to remain high (usually above 6.0). Such a condition would permit the spread of pH-sensitive spoilage bacteria such as *Shewanella putrefaciens* which was once synonymous to *Pseudomonas putrefaciens* (Long & Hammer, 1941) until it was reassigned to the newly-established genus *Shewanella* and family Vibrionaceae (MacDonell & Colwell, 1985).

Free amino acids and nucleotides which are growth substrates of bacteria are made readily available within the non-protein-nitrogen (NPN) present in fish flesh (Gram & Huss, 1996). Decomposition of cysteine and methionine in particular contributes to the production of hydrogen sulphide and methylmercaptane which respectively, cause off-odours and off-flavours of fish flesh (Herbert & Shewan, 1975).

Trimethylamineoxide (TMAO) is found in all marine fish as well as in several species of fresh-water fish (Gram *et al.*, 1989; Anthoni *et al.*, 1990). In marine fishes, it functions as an osmoregulant to prevent dehydration while in fresh water species, this compound serves to prevent tissues from becoming waterlogged. Several species of established anaerobic spoilage bacteria such as *Shewanella putrefaciens*, *Photobacterium phosphoreum* as well as members of the genus *Vibrio* use TMAO during respiration as the terminal acceptor of electrons and this results in the formation of trimethylamine (TMA) which is characterised by off-odours and off-flavours (Gram *et al.*, 1987; Gram *et al.*, 1990; Dalgaard *et al.*, 1993). TMA therefore, is used universally as an indicator of fish spoilage by microbial activity (Ghaly *et al.*, 2010).

Usually, the microorganism count is directly related to the degree of spoilage especially in cases of visible evaluation such as the formation of moulds,

pigmentations and slime. Exceptions to this would be incidences due to bacterial metabolism which includes the production of off-odours and off-flavours since only a fraction of the bacteria present are responsible (Castell *et al.*, 1948; Huss *et al.*, 1974). Often times, the durations taken for fish to spoil depend upon factors like temperature, origin and species of the fish (Gram & Huss, 1996). Typically, the spoilage of marine fish is described as the development of an offensive hydrogen sulphide related odour and flavour while the encounter with tropical fish and fresh water fish undergoing spoilage is characterised by off-flavours and fruity sulphydryl off-odours (Lima dos Santos, 1978; Gram *et al.*, 1989).

While autolytic enzymes are also recognised as contributors to fish spoilage, these are only responsible for the reduction of textural quality and do not result in the production of off-flavours and off-odours (Hansen *et al.*, 1996). Fraser and Sumar (1998) however added that the autolysis of muscle proteins of fish would release free amino acids and peptides which then promote the growth of microorganisms.

2.1.1 Antimicrobial agents from plants

Atlas (1997) defined an antimicrobial agent as one which inhibits the growth or kills off completely specific microbe strains. This definition has its application in many fields and across a diverse host of agents and target microbes. Burt (2004) went further by describing that the purpose of incorporating antimicrobial agents in food is mainly to achieve two objectives; to control food spoilage and also, to control the growth of microorganisms.

Over the years, plants which are aromatic in nature or those with medicinal values have been shown to contain bioactive compounds which are able to participate in reactions with target organisms and ultimately lead to their inhibition (Sengul *et al.*, 2009). Brandi *et al.* (2006) quipped that other than their contribution to flavour, some herbs and spices are also able to inhibit pathogens of humans and plants.

The five mechanisms which are often deployed by antimicrobial agents as reported by Cushnie and Lamb (2005), Prescott *et al.* (2005) and Tenover (2006) are as listed in the following.

i) Interference with the cell wall synthesis of microbes through the activation of autolysins, preventing peptidoglycan chains from forming cross-links and bringing about a compromise of the cell wall strength and ultimately, resulting in lysis of the cells.

ii) Blocking the syntheses of important microbial proteins by binding to the ribosomes of microbes, bacteria in particular, impeding the process of protein translation.

iii) Preventing the syntheses of microbial DNA or RNA by interfering with the production of nucleic acids.

iv) Compromising the structure of the cytoplasmic membrane of bacteria by gaining entry across the membrane and sabotaging the function of the selective barrier.

v) Disrupting the metabolic pathways of microbes by causing a starvation of oxygen.

With respect to the application of herbs and spices in the preservation of the quality of fish products, extracts of these herbs and spices may be applied during the steps of marinating and seasoning prior to cooking (Rakshit & Ramalingam, 2013). At the same time, plant extracts are also incorporated into fish feed in aquaculture establishments with the objective of reducing bacterial populations in living fishes which might later contribute to post-harvest spoilage. Pachanawan *et al.* (2008) reported on the effectiveness of *Psidium guajava* leaf extracts supplemented into the feed of tilapia (*Oreochromis niloticus*) in the inhibition of *Aeromonas hydrophila*.

2.1.2 Phenolic compounds as antimicrobial agents

Phenolic compounds are produced via the shikimic acid and the malonic acid pathways (Manach *et al.*, 2004) and are involved generally in the adaptations of plants towards conditions of environmental stress among which includes the defence against pathogens (Farah & Donangelo, 2006). Examples of defence-related phenolic compounds include flavonoids, anthocyanins, phytoalexins, tannins, lignin and furanocoumarins.

According to Erdemoglu *et al.* (2007), the antimicrobial potential of phenolic compounds has been well-documented. Among others, Aziz *et al.* (1998) exhibited in a study that phenolic compounds are potent as antiviral, antifungal and antibacterial agents. A more specific study reported on the efficacy of phenolic compounds, applied individually or in combinations, against multiple strains of bacteria (Tafesh *et al.*, 2011). All these make phenols a choice disinfectant in hospitals and clinics where sterility is of paramount importance.

The phenolic constituents in herbs and spices are often credited for the bactericidal and bacteriostatic properties of plant extracts when tested against bacterial strains. This is due to the high compositions of eugenol, carvacrol and thymol which are often found in plant extracts with high antibacterial potentials (Mandalari *et al.*, 2007; Gutierrez *et al.*, 2008).

Generally, the phenolic compounds would adsorb onto the surface of microbes and disrupt the structure of its membrane, react with enzymes and render them non-functional or cause the deprivation of metal ions (Fattouch *et al.*, 2007). Phenolic compounds like thymol, eugenol and carvacrol have been proven to disrupt the membrane of several bacterial species as well as inhibit the activity of ATPase enzymes and the release of ATP and other metabolic products (Lambert *et al.*, 2001). Carvacrol, in specific, was found to inhibit the synthesis of flagellin in *Escherichia coli* as well as increase the production of heat shock protein 60 HSP (GroEL) (Burt *et al.*, 2007).

Other compounds which are also phenolic like vanillin and gallic acid are able to damage the cytoplasmic membrane of bacteria by upsetting the ion gradient, causing morphological changes. Respiratory processes of microbes are also often compromised with (Fitzgerald *et al.*, 2004).

In food systems, herbs and spices are usually present in the range of 500-1000mg/l. Compared to others, some spices are more potent in their antimicrobial ability and work at 1000mg/l. Others, however, require higher concentrations in order to successfully inhibit bacterial strains (Ceylan & Fung, 2004).

2.2 Oxidative spoilage of fish

A free radical is defined by Halliwell and Gutteridge (1999) as a molecule or a fragment of a molecule which contains one or more than one unpaired electrons in its most outer orbital but is capable of existing independently. Usually formed through the homolytic cleavage of chemical bonds or through redox reactions, radicals are highly reactive and are capable of triggering chain reactions (Bahorum *et al.*, 2006). These radicals may have beneficial contributions in low concentrations especially in the aspects of cell growth, cell defence and production of energy (Poli *et al.*, 2004). In high concentrations however, oxidative stress might be resulted, which leads to the weakening of enzymes responsible for protection, as well as other detrimental effects (Valko *et al.*, 2007).

Fraser and Sumar (1998) reported that spoilage due to the oxidation of lipids account for the spoilage of most pelagic fish species which contain high compositions of oil and fat stored in their flesh. The oxidation of lipids involves a step-by-step mechanism of which free radicals are a part of. These stages are, by order; the initiation, propagation and termination steps (Khayat & Schwall, 1983; Frankel, 1985). In the initiation phase, lipid-free radicals are formed and facilitated by catalysts such as heat, irradiation and metal ions. Peroxyl radicals are then formed when the resultant radicals react with oxygen (Fraser & Sumar, 1998). In the propagation phase, these peroxyl radicals would participate in reactions with other lipid molecules which are present within fish flesh to produce hydroperoxides and numerous new free radicals (Hultin, 1994). The latter group would interact during the termination phase to produce non-radical products.

Oxidation spoilage therefore occurs when the double bonds of fatty acids are involved in the propagation phase. Hultin (1994) added that this fact promotes the occurrence of high rates of oxidative spoilage in fish species with relatively high compositions of polyunsaturated fatty acids (PUFA). The presence of transition metals spurs this process as these may act as activators of molecular oxygen.

Lipid oxidation in fish may occur enzymatically (lipolysis) or non-enzymatically. In the former, lipolytic enzymes are derived either from within the food product or from psychrotrophic microorganisms (FAO, 1986; Huis in't Veld, 1996). The latter category is resulted by compounds containing the haem group such as cytochrome, myoglobin and haemoglobin which undergoes catalysis to produce hydroperoxides (Fraser & Sumar, 1998). Undeland *et al.* (2005) supported this by reporting that highly pro-oxidative haemoglobin promotes lipid oxidation in fish muscles. The denaturation of fish flesh would then occur when the fatty acids produced during the lipid hydrolysis interacts with proteins of the myofibrillar and sarcoplasmic types (Anderson & Ravesi, 1969).

An antioxidant is therefore, a species that limits or ceases altogether, the oxidation of substrates when these antioxidants are present in low proportions as compared to the substrate (Halliwell & Gutteridge, 1999). The two classes of antioxidant agents would be the endogenous and the exogenous types; the former being produced by the body while the latter is obtained from the environment (Hu *et al.*, 2000).

2.2.1 Antioxidant agents from plants

Over the past two decades, a lot of attention has been generated by antioxidant agents of natural origins as well as their contribution to the health of humans (Arnous *et al.*, 2001). One large bank of natural antioxidants is plants. These have developed survival characteristics over the millennia in such a way that various antioxidant agents are naturally produced in order to counter the threat posed by reactive oxygen species (ROS) (Lu & Foo, 1995). These ROS species which are constituted of free radicals (superoxide anion and hydroxyl) and non-free radicals (hydrogen peroxide and singled oxygen) are the many forms of activated oxygen that are detrimental to the process of ageing as well as injuries to cells (Gulcin *et al.*, 2003).

With respect to food items, ROS species may result in lipid peroxidation which may then lead to the destruction in quality of food (Miller & Rice-Evans, 1997). The oxidative spoilage of food is characterised by the production of rancid odours and off-flavours which may be encountered during the stages of processing or storage. This is potentially harmful because it may lead to the production of secondary compounds which are potentially poisonous to consumers. Such a scenario justifies the incorporation of antioxidant agents into food items as a measure to preserve these (Cook & Samman, 1996).

Wang *et al*. (1999) reported that many fruits and vegetables owe their ability to serve as sources of antioxidant agents to the presence of phenolic compounds which are low in molecular weight. According to the report, these compounds are highly able as antioxidant agents. Several studies have successfully co-related the antioxidant properties of plant extracts to their phenolic contents. These include the

extracts of ‘Du-Zhong’ or eucomnia bark (*Eucomnia ulmoides*), ear mushrooms (*Auricularia polytricha*) and aniseed (*Pimpenella anisum*) (Yen & Hsieh, 1998; Chao, 2001; Gulcin *et al.*, 2003).

Focusing on local plant species, good antioxidant properties have been reported in turmeric (*Curcuma longa*), betel leaf (*Piper betel*), pandan leaf (*Pandanus odorus*), asam gelugur (*Garcinia atroviridis*), mengkudu (*Morinda citrifolia*), pegaga (*Centella asiatica*), ginger (*Zingiber officinale*), cassava shoots (*Manihot esculenta*), kesum (*Polygonum minus*) and selom (*Oenathe javanica*) (Jayamalar & Suhaila, 1998; Mohd. Zin *et al.*, 2002; Zainol *et al.*, 2003; Noriham *et al.*, 2004; Huda-Faujan *et al.*, 2007).

2.2.2 Phenolic compounds as antioxidant agents

Phenolic compounds are found commonly in edible plants as well as non-edible plants. A number of biological activities are reported to be present in plant phenolic compounds. Among these include the ability to reduce the oxidative spoilage of foods. Other than being originally present in the ingredients that go into the preparation of food items, phenolic compounds are also commonly incorporated into foods as well as beverages for its antioxidant potentials and to a lesser extent, for colouring purposes (O’Connell & Fox, 2001).

Phenolic compounds have been shown to be able to cause a retardation to the oxidative degeneration of lipid components in food and ultimately, preserving the nutritional content and quality of food (Kahkonen *et al.*, 1999).This potential, along with the ability to prevent diseases often associated with oxidative pressure has

resulted in phenolic compounds being the focus of many studies (Gülçin *et al.*, 2011).

Boudet (2007) defined polyphenols as plant metabolites with one or usually more than one aromatic rings and several hydroxyl groups. Phenolic compounds in general, promote antioxidation through a number of approaches. This includes the scavenging of free radicals (Antolovich *et al.,* 2002), deactivation of ROS, inhibition of enzymes that promote oxidation (Edenharder & Grunhage, 2003) as well as via interactions with biological membranes (Liao & Yin, 2000). In polyphenols, the hydroxyl groups are especially reactive in neutralising free radicals (-R) through the donation of a hydrogen atom (-RH) or an electron ($-R^-$). The antioxidant property of plant phenolic compounds is mainly attributed to their oxidation-reduction ability which allows these compounds to fulfil roles as reducing agents, donors of hydrogen and removal of singlet oxygen (Rice-Evans *et al.*, 1995). The high antioxidant properties is also purportedly linked to the total phenolic content of the plant sample as illustrated in the studies of Mongkolsilp *et al.* (2004) and Qusti *et al.* (2010) where rhizome extracts of *Curcuma longa* and seed extracts of *Vitis vinifera* were studied, respectively.

On top of those, plant phenolic compounds have the potential for metal-chelation. Leopaldini *et al.* (2006) reported that this chelation of metal ions was possible in aqueous solutions. As suggested by Michalak (2006), the ability of phenolic compounds to chelate metals is a contribution of the high nucleophilic character of the aromatic rings of the phenolic compounds. Its prominence in this application has received a boost in recent years due to the strict regulation and potential hazards of synthetic antioxidants which have been proven to promote carcinogenesis (Park *et al.*, 2001).

One of the most extensively studied classes of polyphenols due to their antioxidant potentials is flavonoids. *In vitro,* these are able to scavenge a vast range of ROS and reactive nitrogen species (RNS) (Hernandez *et al.*, 2009). Flavonoids are also able to chelate metal ions through the complexation of redox-catalytic metal ions (Mira *et al.*, 2002). Quercetin which happens to be a common flavonol has been proven time and again as an effective antioxidant through a number of *in vitro* systems. This includes the oxygen radical absorbance capacity (ORAC) assay, the ferric reducing antioxidant power (FRAP) assay and the 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH) radical scavenging assay (Kemertelidze *et al.*, 2000); Pulido *et al.*, 2000; Ou *et al.*, 2001).

2.3 Spoilage prevention methods

Temperature regulation is important because it has a direct role in the rate of bacterial population growth (Duncan & Nickerson, 1961). Storage of seafood at low temperatures have been practised since the mid-19th century despite the fact that it does not completely kill off spoilage microbes but merely reduces the *in vitro* metabolism (Ashie *et al.*, 1996). Berkel *et al.* (2004) however, brought to attention two possibilities of fish storage by freezing; the first being storage between -1ºC and 4ºC which inhibits the growth of microorganisms and the second being storage between the temperatures of -18ºC and -30ºC where bacterial growth ceases completely.

Johnston *et al.* (1994) emphasised that while freezing fish is an efficient method of preservation, it does very little to enhance or preserve its quality. The

FAO (1973) added that it is important to store fish below 0ºC as soon as they are removed from their waters since spoilage occurs very quickly.

Despite freezing at temperatures as low as -25ºC, only 90-95% of water freezes while the remaining 5-10% moisture, which is typically composed of water molecules chemically bound to specific sites, remain unfrozen (Garthwaite, 1997). At times, the quality of fish is compromised by the rate of freezing. Fast freezing would naturally result in fish products of a more superior quality as compared to slow freezing because in the case of the latter, larger ice crystals are more likely to form and these are detrimental to quality since these can cause damages to the walls of fish cells as well as denature proteins (Rahman, 1999).

Even though the recommended temperature for managing spoilage caused by microbial action is between -9ºC and -12ºC, spoilage may still occur and this is attributed to the actions of enzymes (Graham, 1982; Cassens, 1994). Spoilage due to oxidation still occurred albeit at lower rates (Neumeyer *et al.*, 1997). At these temperatures, approximately 10-60% of the microbial population which is considered viable would perish. However, the survivors would gradually proliferate and increase in numbers (Rahman, 1999).

Mahmoud *et al.* (2006) suggested that the cost and energy-demanding freeze storage can be modified by including the applications of both, natural and synthetic preservatives to keep under control spoilage due to oxidation and microbial activity. This view was also shared by Bagamboula *et al.* (2004) who promoted the idea that the process of spoilage can be drastically reduced by combining the applications of preservatives with low-temperature refrigeration.

Abbas *et al.* (2009) suggested that fish preservation can be improved by lowering the water activity (a_w) which refers to molecules of water which are not chemically bound to other molecules, rendering them available for use by spoilage organisms. In fish, water activity can be controlled by dehydration or the application of chemicals or usually, a combination of both. Often, sodium chloride and sugars have been used to displace free water molecules and to create an osmotic imbalance which then leads to the inhibition of microbial growth (Ray, 2004). Chirife (1994) concurred by reporting that the inhibition of *Staphylococcus aureus* by sucrose and sodium chloride were a result of lowered water activity. His report suggested the trials of other solutes which might render antibacterial effects partly due to interactions with the enzymes of the bacterial membranes which serve in the synthesis of peptidoglycan.

Gutting of caught fishes is a common practice among some fishermen, especially when fish are caught after feeding as the increased contents within the gut and stomach may lead to increased levels of digestive enzymes (Pedrosa-Menabrito & Regenstein, 1988). Care has to be taken to avoid contact between the enzymes and other fish tissues as cross-contaminations may occur (Erkan & Ozden, 2006). If gutting is not carried out, there is the possibility of the belly walls to burst and for the digestive enzymes to leak out and degrade other tissues (Gildberg & Raa, 1980). The products of the ensuing hydrolysis could then support the growth and spread of spoilage microflora.

Oxidative spoilage may be controlled by eliminating the presence of catalysts for free radical mechanisms which includes molecular oxygen and transition metals. This removal may be achieved through the inclusion of antioxidant and chelating agents into the picture (Ghaly *et al.*, 2010). Among potential additives to inhibit lipid

oxidation includes phenolic derivatives (Davidson, 1993) and ethylenediaminetetraacetic acid (EDTA). As a bonus, established phenolic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) contain antibacterial properties, particularly against Gram-negative types (Branen *et al.*, 1980). The efficacy of phenolic antioxidants is thought to be a contribution of the hydroxyl groups available for the purpose of scavenging free radicals. If this is the case, then it would make sense that TBHQ which has two hydroxyl groups fared better than BHA and BHT which each contain only one hydroxyl group (Ghaly *et al.*, 2010).

EDTA is a polyaminocarboxylic acid which is able to fulfil chelating, sequestering and metal complexing roles (Shelef & Seiter, 2005). With regards to fish preservation, it can be added to remove trace metals by chelating itself to these. The removal of these catalysts would qualify EDTA as an inhibitor of lipid oxidation. It has also been proven to be an antibacterial agent through the binding of divalent cations which are present within the cell walls of bacteria (Shelef & Seiter, 2005).

The addition of nitrites in the form of their salts such as sodium nitrite and potassium nitrite is to harness its antimicrobial properties as it has been reported to exhibit exceptional antibacterial effects against *Yersinia enterocolitica*, *S. aureus* and *Clostridium botulinum*. These are also reportedly effective in regulating lipid oxidation (Roberts, 1975; De Giusti & De Vito, 1992; Archer, 2002; Lovenklev *et al.*, 2004; Sindelar & Houser, 2009). The antibacterial mechanisms of nitrites are still not properly understood but nitrites are believed to be involved in the functioning of enzymes within germinating spores and vegetative cells. These also restrict the utilising of iron within bacteria and limits cross-membrane transport by interfering

with its permeability (Ray, 2004). There is a certain degree of concern among the consumer class as to the formation of nitrosamines compounds, which are carcinogenic, upon prolonged exposures (Ghaly *et al.*, 2010)

Sulphides, especially sodium sulphide, are common preservatives of meat products due to its ability to retard the growth of undesirable microorganisms. Sodium sulphide is particularly effective against Gram-negative bacteria where the dissociated sulphurous acid crosses into the bacterial cell and reacts with thiol groups present within, specifically those of proteins, enzymes and cofactors (Ray, 2004). Currently, sulphides are not allowed in Canada for its application as additives in meat or fish products (DJC, 2009) due to possible health concerns.

The addition of lactic acid relies on the lowering of pH values to inhospitable conditions for bacterial growth (Doores, 2005). In fact, in nature, lactic acid bacteria gain an edge over the survival of other competing bacteria by producing lactic acid and other organic acids (Matamoros *et al.*, 2009). Using several species of lactic acid bacteria, Tome *et al.* (2008) was successful in regulating the growth of *Listeria monocytogenes* on cold smoked salmon.

Ascorbic acid is commonly applied onto fish products together with nitrites and sulphites as it is shown to enhance the antibacterial potential of both groups of chemical preservatives (Baird-Parker & Baillie, 1974). Tompkin *et al.* (2007) reported that this enhancement is present in two forms; in the antioxidant potential as well as in the ability to sequester iron. According to Kelleher *et al.* (1992), ascorbic acid is able to oxidise reactive oxygen species to yield water.

Benzoic acid and sodium benzoate are used as preservatives in fish products which are naturally acidic. However, the focus of inhibition is centred more upon

yeasts and fungi rather than bacteria (Chipley, 2005). In a test however, Dabrowski *et al.* (2002) exhibited that sodium benzoate was able to reduce the diversity of bacteria and yeasts in low-salt slices of herring but had no impact onto the total number of microflora. This implies that vacant niches were created through the elimination of selected species and the survivors filled these spots.

Lightly preserved fish products are defined as products preserved in low salt concentrations (usually less than 6% sodium chloride (w/w) solution). It also includes products preserved with the addition of preservatives like benzoates, sorbates, nitrates or by smoking. The pH values of these products are considerably high at values of above 5.0 and these are usually packed in vacuum and stored at temperatures of below 5ºC. Spoilage of lightly preserved fish products still occur and it is dominated by species of lactic acid bacteria (Magnusson & Traustadottir, 1982; Leisner *et al.*, 1994; Civera *et al.*, 1995; Truelstrup Hansen *et al.*, 1995). The most common bacterium was reported by Truelstrup Hansen (1995) to be *Lactobacillus curvatus*.

Salting is another approach deployed to preserve fish products and it can be divided into wet-salting or barrel salting for fatty fish species such as herring and anchovy while dry-salting is applied for non-fatty fish species. Spoilage in wet-salting occurs in the form of (i) a sweet and sour off-odour and off-flavour resulted by Gram-negative obligate anaerobic halophilic rod bacteria which usually grows only one year after storage (Knøchel & Huss, 1984a); (ii) fruity off-odours caused by high levels of osmotolerant yeasts (Knøchel & Huss, 1984b); and (iii) activity by a Gram-negative, aerobic, halophilic bacteria (Magnusson & Møller, 1985). Spoilage encountered in dry-salting on the other hand, is either the growth of extremely halophilic bacteria which are strongly proteolytic and produce off-flavours and off-