THE EFFECTS OF ENZYME INHIBITION ON THE MYRISTICIN CONTENT AND THE QUALITY PARAMETERS OF NUTMEG (*Myristica fragrans*) PICKLES

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LIST OF ABBREVIATIONS

TPC	Total Phenolic Content
DPPH	Free radical scavenging activity
AAT	Alcohol acyltransferase enzyme
MMDA	3-methoxy-4,5 methylenedioxyamphetamine
FDA	Food and Drugs Administration
SHS	Superheated steam blanching
USE	Ultrasonic extraction
ROS	Reactive oxygen spesies
HDL	High-density lipoprotein
SPE	Solid Phase extraction
GRAS	Generally Recognized as Safe
CSWG	Chemical Selection Working Group
NCI	National Clear Institute
TPCN	Texas Poison Center Network
CPCS	California Poison Control System
NBP	Non-blanch (traditional)
BP	Blanched (modified)
CP	Commercial pickle
BP5	Blanched nutmeg at 5 minutes
BP7	Blanched nutmeg at 7 minutes
BP9	Blanched nutmeg at 9 minutes

LOD	Limit of detection
BSA	Bovine Serum Albumin
SMB	Sodium metabisulfite
DTNB	5, 5’-dithiobis (nitrobenzoic acid)
PVPP	Polyvinylpolypyrorolidone
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PDA	Potato dextrose agar
ATP	Adenosine triphosphate
GC-FID	Gas Chromatographic Flame Ionization Detector
HPLC	High Performance Liquid Chromatography
FC	Follin-ciocalteu

LIST OF SYMBOLS

°C	Degree celcius
%	Percentage
w/w	Weight/weight
w/v	Weight/volume
mg	Milligram
g	Gram
kg	Kilogram
ml	Milileter
mm	Milimeter
µM	Micromolar
a_w	Water activity
kHz	Kilo Hertz
MHz	Mega Hertz
a*	Redness
b*	Yellowness
L*	Lightness
Log CFU/g	Log Colony forming unit per gram

KESAN PERENCATAN ENZIM PADA KANDUNGAN MYRISTICIN DAN PARAMETER KUALITI JERUK BUAH PALA (*Myristica Fragrans*)

ABSTRAK

Myristicin, merupakan kompaun utama yang terdapat di dalam isi buah pala (*myristica fragrans*), yang boleh menyebabkan mabuk dan kematian kepada manusia sekiranya diambil dalam dos yang berlebihan. Alkohol *acyltransferase* (AAT) adalah enzim yang bertanggungawab menghasilkan myristicin. Rendaman buah pala di dalam larutan pekat dilaporkan meningkatkan aktiviti enzim AAT dan kandungan *myristicin*. Sebanyak lima fasa kajian telah dijalankan untuk mengkaji kesan rawatan penjerukan terhadap sifat fizikokimia di dalam buah pala. Keluaran buah pala komersial yang terpilih, disaring untuk mendapatkan kandungan *myristicin*. Seterusnya, kesan rendaman di dalam larutan yang berbeza (NaCl dan Sukrosa) mengikut kepekatan dan masa yang berbeza pada aktiviti enzim AAT dan kandungan myristicin diperiksa. Kesan rawatan penceluran dan kesan kombinasi pada masa penceluran yang berbeza dan penjerukan (5, 7 dan 9 minit, masing-masing dilabelkan sebagai BP5, BP7 dan BP9) diperhatikan menggunakan parameter yang sama. Sifat fizikokimia daripada jeruk yang dicelur dan tidak dicelur juga dikaji. Hasil kajian menunjukkan jeruk buah pala komersial mengandungi myristicin yang tertinggi (2.18 mg/100g). Peningkatan peratus kepekatan dan masa rendaman untuk NaCl dan sukrosa telah meningkatkan aktiviti enzim AAT dan kandungan myristicin secara signifikan ($p < 0.05$), terutamanya lebih tinggi di dalam larutan NaCl berbanding larutan sukrosa. Peningkatan masa penceluran dari 0 ke 9 minit secara signifikan mengurangkan aktiviti enzim AAT dan kandungan myristicin di dalam buah pala segar dan buah pala celur, masing-masing 39.034 (pala segar) > 3.478 (pala celur)

u/mg protein dan *myristicin* 0.451 (pala segar) mg/100g kepada ND (pala celur). Aktiviti enzim AAT dan pengeluaran *myristicin* secara signifikan akan terhalang apabila proses penjerukan di dahului dengan penceluran. Analisis kandungan total fenol (TPC) dan antioksidan menunjukkan bahawa jeruk buah pala yang dicelur (BP) mempunyai sifat antioksidan yang rendah berbanding jeruk buah pala komersial ($p > 0.05$). Analisis warna tidak menunjukkan perbezaan antara CP, BP5, BP7 dan BP9 ($p > 0.05$) sementara analisis tekstur menunjukkan CP mengandungi tekstur lebih pejal berbanding BP5, BP7 dan BP9 ($p < 0.05$). Analisis mikrobiologi untuk yis dan kulat pula menunjukkan tiada perbezaan signifikan antara CP, BP5, BP7 dan BP9 ($p < 0.05$). Analisis sensori menunjukkan bahawa proses penjerukan meningkatkan kebolehterimaan keseluruhan untuk jeruk yang dicelur berbanding pala segar. Tiada perbezaan yang signifikan antara CP, BP5 dan BP7 untuk penampilan, rasa dan kerapuhan ($p > 0.05$). Kajian ini menyumbang pengetahuan terhadap rawatan penceluran yang berkesan untuk menghalang aktiviti enzim AAT, mengurangkan kandungan myristicin di bawah paras toksik dan meningkatkan kualiti jeruk dalam buah pala.

THE EFFECTS OF ENZYME INHIBITION ON THE MYRISTICIN CONTENT AND THE QUALITY PARAMETERS OF NUTMEG (*Myristica Fragrans*) PICKLES

ABSTRACT

Myristicin, a major compound found in the pericarp of nutmeg (*Myristica fragrans*) can cause chronic intoxication to fatality to human if consumed over the minimum dose. Alcohol acyltransferase (AAT) enzyme is said to be responsible in the production of myristicin. Soaking nutmeg in a concentrated solution to pickle has been reported to enhance AAT enzyme activities and myristicin content. A five-phase study was undertaken to examine the effects of different preservation treatments on physicochemical properties of pickled nutmeg. Selected commercial nutmeg products were first screened for myristicin content. Subsequently, the effects of soaking in different solutions (NaCl and sucrose) at different concentrations and soaking times on AAT enzyme activities and myristicin content of nutmeg were examined. The effects of blanching treatment and the combination effects of different blanching times and pickling process (5, 7 and 9 minutes, labelled as BP5, BP7 and BP9 respectively) on the same parameter were observed. Physicochemical properties of blanched and non-blanched nutmeg pickles were also studied. The results revealed that commercial nutmeg pickles contained the highest myristicin (2.18 mg/100g). Increased solutes concentrations and times in NaCl and sucrose significantly increased AAT enzyme activities and myristicin content ($p < 0.05$), notably higher in NaCl solution than in sucrose solution. Increase in blanching time from 0 to 9 minutes significantly reduced AAT enzyme activities and myristicin content in fresh and blanched pericarp, giving 39.034 (fresh) > 3.478 (blanched

pericarp) u/mg protein and myristicin 0.451 (fresh) mg/100g to ND (blanched pericarp) respectively. AAT enzyme activities and the production of myristicin were significantly inhibited when pickling process was preceded by blanching. Total phenol content (TPC) and antioxidants analyses showed that blanched pickle nutmeg (BP) had lower antioxidant properties compared to commercial pickle nutmeg (CP) ($p > 0.05$). Colour analyses indicated no significant difference between the colours of CP, BP5, BP7 and BP9 ($p < 0.05$) while texture analyses showed that CP had higher texture compared to BP5, BP7 and BP9 ($p < 0.05$). Microbiological analyses for yeast and mold showed no significant difference between CP, BP5, BP7 and BP9 ($p < 0.05$). Sensory analyses demonstrated that pickling process improved overall acceptability for blanched pickle as compared to fresh nutmeg. No significant differences were found between CP, BP5 and BP7 for appearance, taste and crunchiness ($p > 0.05$). The study suggests that blanching treatment was effective in inhibiting AAT enzyme activities, reducing myristicin content to below its toxic level, thus improving the qualities of nutmeg pickle.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nutmeg or its scientific name *Myristica fragrans* is an aromatic fruit belonging to Myristicaceae family that are commonly used as a household spice for many years, intentionally for improving the flavor of food and also for medicinal purposes (Carstairs & Cantrell, 2011). It posses high amount of antioxidants and other nutritional properties (vitamins, proteins, minerals, carbohydrates and dietary fibers) make it one of the most notorious fruits to be processed into food products (Agbogidi & Azabaekwe, 2013; Gupta & Rajpurohit, 2011; Malti et al., 2007). In Malaysia, it was processed before being commercialized locally and internationally by food industries into varieties of product, namely pickled, tea, liquorice, jam, sugar coated nutmeg, drink and candy (Sulaiman & Ooi, 2012). The demand for fresh nutmeg fruits and nutmeg products was high and keep increasing every year. To date, Malaysia is one of the biggest and active exporters for nutmeg fruits and nutmeg products together with India, Papua New Guinea and Sri Lanka to all over the world (Anon, 2016).

Even though, the demand for nutmeg was high, the production of the nutmeg fruit by the producing countries is still abundant (12000>9000 tonnes) every year (Rahman et al., 2015). For a long time, pickling have been proved to be one of the best, convenient and economical ways to preserve fruits by converting fresh fruits into pickled with stable shelf life by lowering water activity of the fruits using minimal process (Chavan & Amarowicz, 2012). Nutmeg pickled was well accepted

as a snack in Malaysia either to be eaten raw or combined it with other dishes. The process involves the immersion of fruit in the hypertonic solutions (salt and sugar solution) (Nurul & Asmah, 2012). However, according to Torres et al. (2006), the immersion of nutmeg pericarp in hypertonic solutions could triggered and increase the production of toxic compound called myristicin due to the osmotic stress that occur within the cell tissue of the fruit. Myristicin is the dominant volatile compounds in nutmeg and its intoxication is increasingly recognized as a serious, worldwide public health concern (Choo et al., 1999). Myristicin can be metabolized into amphetamine-like compounds (a type of drug) or also known as 3-methoxy-4, 5 methylenedioxyamphetamine (MMDA) during the ingestion process and the effects imitate the effects of illegal drugs (Ehrenpreis et al., 2014). MMDA is one of the prohibited substances, either to be consumed or sell due to its drug-like effects (Rahman et al., 2015).

Since for a long time, the issue of myristicin intoxication has received considerable critical attention among researchers and alarming cases in the emergency department (Sanggali & Chiang, 2000). Many previous and recent studies have reported the negative effects of high intakes and prolong consumptions of nutmeg due to the metabolism of myristicin in body which could cause traumatic brain damages and other serious neurological diseases (Rahman et al., 2015; Dawidowicz & Dybowski, 2013; Demetriades et al., 2005; Sanggali & Chiang, 2000; Quin et al., 1998; Abernethy & Becker, 1992). The psychoactive effects of myristicin intoxication usually can be observed within five hours after ingestion. Two fatal cases and thousands of intoxication cases regarding nutmeg have been reported by researchers in previous reports (Nowak et al., 2016; Ehrenpreis et al., 2014; Carstairs & Cantrell, 2011; Barceloux, 2009; Forrester, 2005; Demetriades et al.,

2005; Stein et al., 2001; Sanggali & Chiang, 2000). Alcohol acyltransferase (AAT) was recognized as a key enzyme that responsibility in the production of esters or volatile compound including myristicin in nutmeg through the esterification process (Beekwilder et al., 2004). Many factors could trigger the activities of AAT; however, one of the main factors is due to osmotic stress. Other factors that can influence the activity of AAT is climate, type of soil, time of harvest and processing (Tang et al., 2008; Shalit et al., 2001).

The investigation of techniques to reduce myristicin in nutmeg was run earlier by Nguyen et al. (2011) by using alcohol. However, there are certain drawbacks associated with the use of alcohol as the main chemical to reduce myristicin in nutmeg. It has a high risk to contribute to the other diseases, including liver and heart diseases (Gronbaek, 2009). The uses of alcohol in food products also are forbidden for Muslim due to the religious principles (Michalak & Trocki, 2007). Blanching seems to be a promising and more 'green' technique to be applied in nutmeg processing. So far, there has been no discussion about the application of blanching treatment to reduce the myristicin content in nutmeg was reported. Blanching is a thermal pre-treatment with the aim to inactivate the enzyme activities and prevent further formation of myristicin (Deylami et al., 2016). According to Escriche et al. (2000), the application of blanching at the pre-treatment stage managed to inhibit further formation of volatile esters in strawberry. Similarly, Kalua et al. (2007) reported the decreasing of volatile compound of olive fruit after being subjected to heat treatment.

As stated earlier, myristicin can produce adverse effects and even fatality when consumed in excess. However, Malaysia has various food products made from nutmeg (pickled, liquorice, sugar coated and tea). Previously, most of the studies in

the field of the nutmeg fruit are mainly focus on the effects of its toxicity to human health. To the best of our knowledge, far too little attention has been paid in reducing the myristicin content in nutmeg and process it into a palatable product. As research in this area is very limited, the effects of various treatments and processing on the myristicin content in the nutmeg pericarp are not fully understood.

Generally, this study involved five stages of phase. The first phase is the analysis of myristicin content in the selected commercial nutmeg pericarp products in Malaysia. In the second phase, the individual effects of different solutes (sodium chloride and sucrose) on the enzyme activity and myristicin content of nutmeg were investigated. The effects of blanching treatment time on enzyme activity and myristicin content were determined on the third phase. The results from phase two and phase three was manipulated and apply in phase four, where the effects of combination technique (blanching-pickling) and the production of pickled was investigated. The physicochemical and sensory analysis of blanched nutmeg pickled was carried out in the phase five in which fresh nutmeg and commercial nutmeg pickled was used as the control.

1.2 Objectives of Research

The primary objectives of this project are to observe the effects of treatments on physicochemical properties and quality parameters of the nutmeg pericarp. The specific aims of the study are:

1. To determine the amount of myristicin content in selected commercial nutmeg pericarp products (pickled, liquorice, sugar coated and tea).

2. To investigate the individual effects of different solutes of sodium chloride (NaCl) and sucrose ($C_{12}H_{22}O_{11}$) in pickling process on the AAT activity and myristicin content of nutmeg pericarp.

3. To determine the effects of blanching treatment at different duration on AAT activity and myristicin content.

4. To analyse the combined effects of blanching and pickling process on AAT activity and myristicin content.

5. To evaluate the physicochemical and sensory properties of blanched nutmeg pickled and commercial nutmeg pickled.

CHAPTER 2

LITERATURE REVIEW

2.1 Nutmeg (Myristica fragrans)

Myristica fragrans houtt or ‘nutmeg’ is an aromatic fruit that originally comes from a Myristicaceae family (Hayfaa et al., 2013). The word ‘*Myristica*’ is believed to be originated from ‘myron’ a Greek word meaning ‘a sweet distilled liquid from the plant’ (Latha et al., 2005). Nutmeg tree (Figure 2.1) was originated from Eastern Indonesia (Banda Island) or also known as ‘Spice Islands’ (Calliste et al., 2010). As reported by Nagore et al. (2013), nutmeg fruit contains a high therapeutic value that can be used to cure a wide range of diseases. The therapeutic potential of nutmeg was initially discovered a few decades ago by the Portuguese in 1512 in which it has been used to treat a severe fatal epidemic known as plague faced by European at that time (Gupta & Rajpurohit, 2011). Since then, nutmeg has become popular and has been widely utilized in Europe and Asia for medicinal purpose. Besides, due to its strong aromatic properties, nutmeg also has been added in cooking as a flavour enhancer (Olaleye et al., 2006).

Figure 2.1: Nutmeg tree (References: Anon, 2017)

Due to its advantages, nutmegs become one of the main commodities that generated high economic values for its producing countries. As of the year 2015, it was reported that about 80% the production of nutmeg come from Indonesia. Another 20% production of nutmeg comes from other countries including Malaysia, Grenada, Sri Lanka, Papua New Guinea and Carribean Island with the average production of 10,000-12,000 tonnes per year. These tropical countries are among the top ten exporters of nutmeg fruits in the world (Rodianawati et al., 2015; Assa et al., 2014). Most of the nutmeg fruits were exported to Europe countries as the main user for nutmeg where it has been widely utilized in different industries including food, pharmaceutical and cosmetics industries (Asika et al., 2016).

In Malaysia, most of the nutmeg tree was widely planted in Penang which is near the coastal area and the fruit was locally known as ‘buah pala’ (Rahman et al., 2015; Shafiei et al., 2012). The characteristics of nutmeg fruit are succulent,

pendulous with one seeded fleshy drupe, wide with six to nine centimeters long (Barceloux, 2009). It will achieve maturity after 206 to 237 days the fruit was set (Thangaselvabai, et al 2011). Upon maturity, the outer layer of nutmeg fruit will change colour from green to soft yellow and it will split into two halves and exposed the seed inside (Al-Jumaily & Al-Amiry, 2012). To the best of our knowledge, there are no publish reports regarding the maturity index of the nutmeg fruits. Maturity index of the fruits is usually was determined by it's colour, firmness, soluble solid content, soluble sugar and organic acid component (Zhang et al., 2017). However, according to the Thangaselvabai et al. (2011), nutmeg fruits growth and maturity followed the single sigmoid growth pattern. There are three phases involve in single sigmoid growth pattern which will effect the physical and chemical compound of the fruits including; first growth due to cell division, hardening of mesocarp and endocarp and second growth due to cell enlargement (Seifi et al., 2015).

The nutmeg fruit can be divided into three distinct parts which are the pericarp, mace and seed (Tan, 2013). The pericarp is the soft yellow flesh of the nutmeg fruit with the thickness approximately one to two 8lavor8d8rs (Choo et al., 1999). Mace is the red aril (net-like skin) that covering the seed kernel (Gupta & Rajpurohit, 2011). The seed is a hard shell nut (egg-shaped) that located in the middle of the fruit and will turn black upon ripening. The size of the nutmeg seed are roughly 20 to 30 mm long and 15 to 18 mm wide with weight approximately five to ten gram after drying (Assa et al., 2014). Figure 2.2 shows the whole nutmeg and the different parts of nutmeg fruits; pericarp, mace and seed.

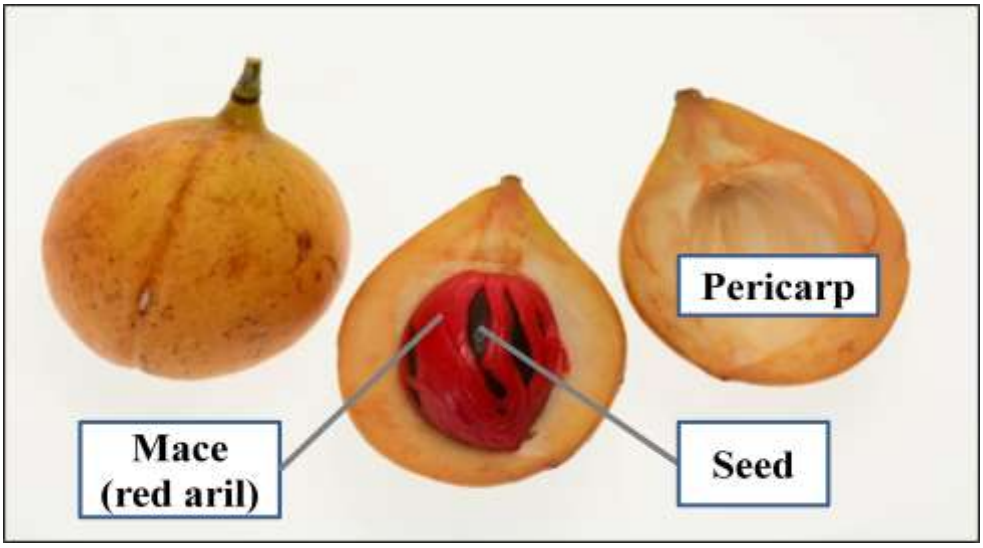


Figure 2.2: Fresh nutmeg fruit (References: Anon, 2010)

In the food industry, the nutmeg pericarp was fully 9lavor9d by processing it into various products such as nutmeg pickles, flavorful fruit candy that covers with thick sugar syrup or honey, refreshing drink, juice, syrup, jam, tea and jellies (Hewage & Vithana, 2013; Choo et al., 1999). Besides, unripe nutmeg pericarp also was harvested and distilled into aromatic essential oil (Assa et al., 2014). The essential oil was applied and served as the major flavourings ingredients for cola drinks (Martins et al., 2014). Another part of nutmeg which is the red aril or mace is usually processed into spice by drying it under the sun before being ground into powder. It is usually used as food seasoning in cooking (Muchtaridi et al., 2010). Apart from that, it also was used as a hair dye and was applied in folk medicine (Orabi et al., 1991). Similar to mace, nutmeg seed was also used as a spice and commonly applied as a food seasoning. Having the similar function, both spices (mace and seed) usually been added in various foods as a food 9lavor such as soups, gravies, milk products, confectioneries, fruit juices, sauces, gelatin, snacks, cereals and seasoning of meat and vegetables (Akinboro et al., 2011; Muchtaridi et al., 2010). Furthermore, the spices are usually being added in the sugary or sweet food to

give them a delicate and smooth flavour such as; cakes, cookies, doughnuts, fruit pies, eggnog (egg milk punch) and puddings (Hewage & Vithana, 2013).

The application of nutmeg in the food products not only beneficial as a food flavouring, but it also nourishes the food with adequate nutrients. According to Juwita & Tsuchida (2017), the demand for nutmeg based products keeps increasing every year due to the fact that nutmeg contains high antioxidant properties that could contribute into better health-promoting. As reported by Tan (2013), there is growing interest and awareness among the consumers on consuming the products that contain therapeutic and antioxidant properties that could help in preventing from severe diseases as well as maintaining a healthy body.

2.1.1 Nutritional benefits of nutmeg

Nutmeg fruit is known to be composed of various phytochemicals and nutrient compounds that beneficial to human health. As reported by Assa et al. (2014), nutmeg fruit is packaged together with energy (525 Kcal/100g), carbohydrates (49.29 g/100g), proteins (5.84 g/100g), fats (36.31 g/100g), dietary fibres (20.80 g/100g), vitamins and minerals that essential for human body. According to Asgarpanah & Kazemivash (2012), regular consumptions of nutmeg can help in reducing hypertension, cardiovascular and diabetic problems. Moreover, it was reported could help against a cough and very effective in recovering respiratory problems such as asthma (Agbogidi & Azabaekwe, 2013).

As reported by Gupta et al. (2013), major vitamin in nutmeg fruits is vitamin C (3 mg/100g). An adequate amount of vitamin C is essential in the human body as it

plays a vital role in helping a bone formation, wound healing and maintenance of healthy gums (Chambial et al., 2013). In addition, regular consumption of food that contains high amount of vitamin C could prevent the scurvy problem by improving the skin collagen. Besides, it also involved in the enhancement of brain cell which will consequently improve the memory and learning process (Figueroa-méndez & Rivas-arancibia, 2015). Apart from that, nutmeg also was reported to contain folates (76 µg/ 100g), niacin (1.30 mg/100g), pyridoxine (0.16 mg/100g), riboflavin (0.06 mg/100g) and thiamin (0.35 mg/100g). Previous studies by Chan et al. (2013) have reported the importance of folates in the production of nucleotides (purines and thymidine) as well as the involvement in the synthesis and repair of DNA. Niacin plays a key role in the reduction of cholesterol in the body by increasing the high-density lipoprotein (HDL) cholesterol or known as 'good cholesterol'. Thus, it reduces the risk of cardiovascular diseases (Zeman et al., 2015). Pyridoxine plays a vital role as an enzyme cofactor for enzyme-catalyzed reactions. It helps in assisting and play essential roles in amino acid metabolism, glycolysis, gluconeogenesis, glycogenolysis, transsulfuration and polyamine biosynthesis (Stover & Field, 2015). Riboflavin is a vitamin that assists in the iron absorption by the body. The deficiency and lack of riboflavin will cause anaemia problem to the human (Powers, 2003). Thiamine is important in energy metabolism by assisting the conversion of glucose into energy form (Lonsdale, 2006).

Nutmeg also been reported to contain a lot of essential minerals such as calcium (184 mg/100g), copper (1.03 mg/100g), iron (3.04 mg/100g), zinc (2.15 mg/100g), magnesium (183 mg/100g), manganese (290 mg/100g) and phosphorus (213 mg/100g) (Zakaria et al., 2015; Agbogidi & Azabaekwe, 2013). According to Balick & Paul (2000), having a diet that rich with minerals could improve the blood

circulation and stimulate the cardiovascular system. As reported by Peacock (2010), minerals are essential for facilitating various body functions such as skeletal mineralization (calcium), building red blood cells (iron, zinc and copper) and assist the metabolic process by changing the food to energy sources for the body. Magnesium is essential in the body as it plays various roles in the body including nerve transmission, cardiac excitability, neuromuscular conduction, muscular contraction, vasomotor tone, blood pressure, and glucose and insulin metabolism (Volpe, 2013). Manganese is important in cellular adaptation to oxidative stress as it can prevent oxidative damage without any deleterious effects (Aguirre & Culotta, 2012). Phosphorus plays key functions in adenosine triphosphate (ATP) process, structure and strengthen the bones, synthesis of cell walls and in the formation of RNA and DNA (Zhang et al., 2016).

Phytochemical studies on nutmeg also showed nutmeg fruits contain high in flavonoids, anthocyanins, carotenoids, terpenoids, alkaloids and phenolic compounds that beneficial for the human body (Jaiswal et al., 2009). Besides, as reported by Hou et al. (2012) nutmeg is one of the best natural sources of antioxidants as it contains a powerful antioxidant compound known as Malabaricone C. It is a compound from acylphenol group which contribute to antimicrobial and cytotoxic properties of the fruits (Chong et al., 2011). As reported by Hou et al. (2012), malabaricone C exhibit a strong antioxidant activity compare to synthetic antioxidant butylated hydroxytoluene (BHT). Apart from that, it also contains lignan from the phenolic compound that also contributes to its antioxidative properties (Tan, 2013). Nowadays, it is important to have a diet containing high antioxidant content as it could help in inhibits damage of protein, nucleic acid and lipid caused by reactive oxygen species (ROS) (Francis et al., 2014). On top of that, having antioxidants in

the diet are reported to help delay or stop the progression of acute diseases such as arthritis, atherosclerosis, cancer, heart diseases, inflammation and brain dysfunction (Pham-Huy et al., 2008).

Apart from that, a number of pharmacological studies also showed nutmeg exhibit anti-inflammatory activity (Latha et al., 2005), anti-parasitic activity (Pillai et al., 2012), anti-cancer (Akinboro et al., 2011), anti-microbial activity (Takikawa et al., 2002), analgesic properties, anticonvulsant activity (Muchtaridi et al., 2010) antifungal properties (Latha et al., 2005), anti-diabetic (Lee et al., 2003), anti-depressant (Asgarpanah & Kazemivash, 2012), anti-cancer properties (Zakaria et al., 2015) and hepatoprotective (Olajide et al., 1999).

2.1.2 Traditional uses and benefits of nutmeg

Nutmeg is well known as the herb that can cure a wide range of diseases. The interest of using nutmeg as the herbal medicine has been reported as early as 6th century where it started being used as a stimulant for muscles in India (Agbogidi & Azagbaekwe, 2013). During that time, most of the local population was mainly relying on natural plants as the main medicines to treat different types of diseases as it was considered cheap and easy to be obtained (Bahmani et al., 2014). Nutmeg is one of the best herbal medicines that are very popular to treat various diseases. In South-east Asians, nutmeg has been used to treat fevers, headaches and bronchial problems (Morsy, 2016). The sedative effect or tranquil effect due to nutmeg is also used to treat nervous problems and to promote better sleep in Malaysia and India (Ratsch, 2005). In Western and Chinese traditional medicine, nutmeg was very popular to cure diseases such as arthritis, diarrhea, flatulence and gastrointestinal problems (Smith, 2014; Bahmani et al., 2014). As reported by Al-Jumaily & Al-

Amiry (2012), nutmeg has been proven to treat human digestive related problems such as food indigestion by removing gas and relaxes the muscles in the digestive system and also helps in inhibiting nausea, vomiting, kidney disorders, diarrhea, inflammation, abdominal pain and liver disease. The essential oil of nutmeg has been used as an external dental cream and as a local massage ointment to reduce muscular pain for sprains and rheumatism pain of joints. Besides, the aromatic essential oil of nutmeg also being used in an aromatherapy practice to help reduce stress, anxiety and depression (Al-Rawi et al., 2013).

The ability of the nutmeg fruit to cure certain diseases has always been related to its volatile compound that available within the nutmeg fruits (Leela, 2008). Previously, during the folk's time, the consumption of nutmeg was done indiscriminately without having any idea or enough information on the safety and the toxicity of the plants (Khan, 2012). Even though nutmeg has been used widely as a traditional medicine to cure certain diseases for a long time, however, the Food and Drug Administration (FDA) does not approve the medical usage for nutmeg in any form due to certain drawbacks associated with the uses of nutmeg in the folk medicines without control dosage. This is due to the psychoactive effects caused by volatile compounds that available within the nutmeg fruits (Smith, 2014). As reported by Rahman et al. (2015) and Jukic et al. (2006), consumption of nutmeg above five gram was reported to be psychoactive and can cause hallucinogenic to human.

2.2 Chemical composition in nutmeg

2.2.1 Major volatiles compound in nutmeg

Nutmeg fruits contain 5 to 15% of essential oils which consists about 40 elements of volatile compounds (Muchtaridi et al., 2010; Choo et al., 1999). The volatile compounds in the essential oil of nutmeg comprise of 80% monoterpene hydrocarbon, 5% monoterpene alcohols, 5% aromatic ether fraction and other minor components (Jukic et al., 2006). As reported by Ogunwande et al. (2003), the monoterpene hydrocarbon includes of α-pinenes, limonene, sabinene, 1, 8-*p*-methadiene, β-pinene, 1, 4-*p*-menthadiene and camphene. The monoterpene alcohol includes linalool, geraniol, terpineol (Jaiswal et al., 2009). The aromatic ether fractions or known as alkyl benzene group comprise of myristicin, elemicin, safrole, methyl eugenol and isoeugenol (Barceloux, 2009).

According to Parimala and Amerjothy (2013), volatile compounds within nutmeg fruit are the main factor that contributes to its hallucinogenic and psychoactive effects. The consumption of nutmeg is always related to its toxicity problems (Smith, 2014). As reported by Martins et al. (2014) and Muchtaridi et al., (2010) alkyl benzene group which comprises of myristicin, elemicin, safrole, methyl eugenol and isoeugenol is the dominant group that's responsible and contributed to its toxic effects. Myristicin is the major compound in alkyl benzene group is often implicated as a chemical that contributed to the hallucinogenic effects and intoxication problems of the nutmeg (Krishnamoorthy & Rema, 2001). The summary of alkyl benzene compounds in nutmeg is shown in Table 2.1.

Table 2.1: Summary of alkyl benzene compound in nutmeg (Myristica fragrans Houtt).

Compounds	Percentage (%)
Myristicin	**16.2**
Safrole	3.9
Methyl eugenol	1.8
Elemicin	1.1
Methyl isoeugenol	0.4
Eugenol	0.4

References: **(**Muchtaridi et al., 2010; Jukic et al., 2006).

2.2.2 Metabolism of myristicin in body

Myristicin (5-allyl-1-methoxy-2, 3-methylenedioxybenzene) is a flavouring plant constituent and has been known to produce significant psychopharmacological responses as well as insecticidal activity (Lee et al., 2005). Apart from natural sources, myristicin can be produced synthetically, where it has been considered as a cheap drug due to its hallucination effects that resulted in being considered as hallucinogen agent (Dawidowicz & Dybowski, 2013). The hallucinogen agent is basically a psychoactive substance that forcefully alters perception and mood of human in a negative way by disturbing the neuron functions in the brain (Nichols, 2004). Due to its negative effects, the Chemical Selection Working Group (CSWG) by National Cancer Institute (NCI) of the United States (US) which is responsible for carcinogenic and toxicology testing monitors the use of myristicin closely to prevent its excessive presence in foods and beverages that could trigger various adverse effects on health (Sangalli & Chiang, 2000). The chemical structure of myristicin is presented in Figure 2.3.

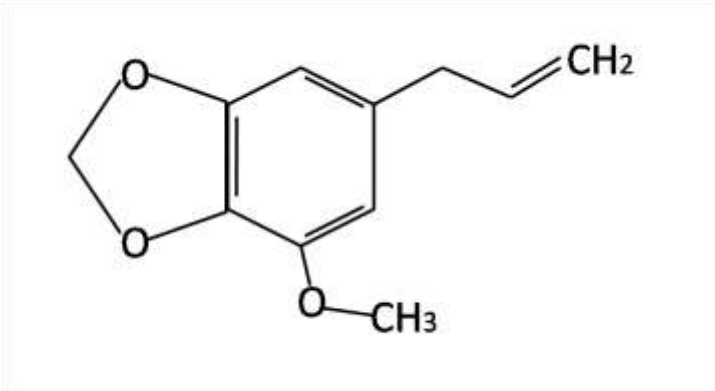
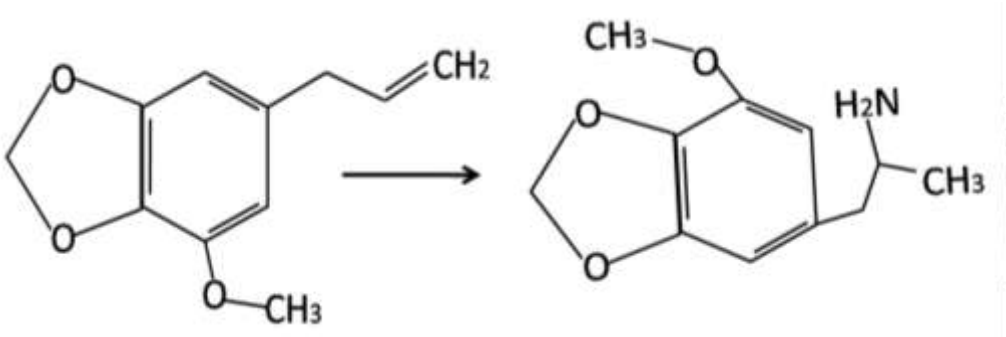


Figure 2.3: Chemical structure of myristicin ($C_{11}H_{12}O_3$).

The main possible cause of the psychoactive and hallucinogenic effects of nutmeg could be due to the metabolic conversion of myristicin into amphetamine-like compounds during the metabolization process (Beyer et al., 2006). As reported by Gupta & Rajpurohit (2011), myristicin was observed to be metabolized into 3-methoxy-4, 5-methylenedioxyamphetamine (MMDA), which is amphetamine derivatives (types of drug). Figure 2.4 represents the chemical structure of myristicin before and after metabolisation in the human body.



Myristicin MMDA

Figure 2.4: Metabolism of myristicin into MMDA after ingestion (Gupta & Rajpurohit, 2011).

The metabolization of myristicin into MMDA was reported can cause serious intoxication to human (Beyer et al., 2006). According to Stein et al. (2001), MMDA is a type of drugs that will attack neuron cell of the brain and gave cerebral stimulations as well as the main cause for intoxication in the body. The intoxication effect can be detected by a few clear symptoms after a few hours of consumptions and could last days (Forrester, 2005).

2.2.3 Symptoms of myristicin intoxication

The symptoms of myristicin intoxication usually begin about 3 to 6 hours after ingestion of myristicin containing foodstuffs and resolve by 24 to 72 hours (Lee et al., 1998). Numerous previous studies had reported the consumption of nutmeg in large quantity could to lead to psychopharmacological symptoms and health problems such as facial flushing, tachycardia, hypertension, dry mouth, blurred vision, palpitation, psychoactive hallucinations, feelings of euphoria (unreality), general body pain and delirium (Dawidowicz & Dybowski, 2013; Gupta & Rajpurohit, 2011; Demetriades et al., 2005). According to Stein et al. (2001), consumption of nutmeg as low as 5 g is considered as a toxic dose. Table 2.2 represents the summary of nutmeg ingestion cases recorded by the Poison Information Center Erfurt (An establish centre in Germany that did research on drugs and toxin).

Table 2.2: Summary of nutmeg ingestion cases recorded by the Poison Information Center Erfurt (An establish centre in Germany that involves in drugs and toxins research).

Age/Sex	Dose of nutmeg	Ingestion Time	Symptoms
24 years/ female	20 g	5 h	Weaknesses
19 years/male	80 g	5 h	Decelaration, clouding of consciousness, slight dysarthria; tachycardia (135/min), BP 150/100 mmHg; mydriasis; redness of skin
35 years/male	19 nutmeg	5 h	Drowsiness, nausea, epigastric pain
Adult/male	40 g	11 h	Restlessness, bad feeling, tachycardia (130/min), normal BP, repeated vomiting
Adult/female	Approx. 21-28 g	20 h	Insomnia, dizziness, tachycardia
16 years/female	Approx. 14-21 g	6 h	Drowsiness; tachycardia (130/min), mydriasis, dry mouth, hot skin, blood level 2µg/ml 8 h after ingestion
71 years/female	½ nutmeg	unknown	Prevalent gastroenteritis; no toxic symptoms

References: (Stein et al., 2001)

Most of the symptoms of nutmeg intoxication shows were related to the central nervous system which can lead to the fatal cases (El-Alfy et al., 2009). However, nutmeg intoxication also might attack another part of the body, such as in central nervous, cardiovascular, gastrointestinal and peripheral. Early actions by observing the symptoms might save the patient's life (Forrester, 2005).

2.2.4 Nutmeg intoxication reports

A considerable amount of literature has been published on the hazardous effects of myristicin in the human body. The side effect of myristicin seems to be serious and frightening. Moreover, a person with myristicin intoxication needs a detail and frequent medical treatments in order to save their life. Therefore, a firm legislation on the usage of myristicin based products must be established and legal actions on the misused of nutmeg must be taken.

A lot of cases have been reports regarding nutmeg intoxication over the past decades. Most of the cases reported caused by the overused of nutmeg as food flavouring in cooking, meanwhile part of the case is due to the misused of nutmeg as a cheap drug (Ehrenpreis et al., 2014). Nutmeg intoxication could lead to the negative impact, including serious shock, coma, acidosis and death (Forrester, 2005). Table 2.4 shows the list of nutmeg intoxication reports.

Table 2.4: Nutmeg intoxication reports

Cases	Authors/Years
23 years old student was sent to the emergency department after consumed nutmeg.	Abernethy & Becker (1992)
55 years old woman was found dead, and the postmortem found myristicin in her blood and directly confirm that she undergoes nutmeg intoxication.	Stein et al. (2001)
17 cases of nutmeg poisoning were reported to Texas Poison Center Network (TPCN).	Forrester (2005)
Nutmeg poisoning occurs when a woman was reported to consume 10 to 12 nutmeg.	Stein et al. (2011)
Two young adults were reported to undergo intoxication and hallucinogenic effects after consuming nutmeg.	Carstairs & Cantrell (2011)
119 cases of nutmeg exposure were reported to the California Poison Control System (CPCS).	Carstairs & Cantrell (2011)
Reported nutmeg was used as cheap recreational drugs and also as a substitute for marijuana.	Asgarpanah & Kazemivash, (2012)
Nutmeg was being misused by combining with other drugs including cannabis, amphetamines, lisdexamfetamine, benzodiazepines, diphenhydramine, duloxetine, clonazepam, benzodiazepines, acetaminophen, K2, cough syrup and antihistamine.	Ehrenpreis et al., (2014)

2.2.5 Legislation on myristicin

Even though myristicin in nutmeg can cause serious intoxication to human, however, to date, there are no regulations or specific guidelines on the usage of myristicin based products by the Food and Drugs Administration (FDA) (FDA, 2017). Most of the naturally occurring toxic compounds were labeled as generally recognized as safe (GRAS) by the FDA even though it is potentially harmful to human (Dolan et al., 2010). Nevertheless, in Oman and Saudi Arabia nutmeg is illegal and being banned from being imported as well as to buy, sell or processes due to the hallucinogenic effects by myristicin within the nutmeg (Anon, 2002). Even though the legislation by FDA on myristicin compounds was light, it is the role of public health policy maker to provide the public with sufficient information about the toxic food and the amount of 'dose' that can cause harm to human in order to protect the public from any health problems (Dolan et al., 2010).

2.3 Key enzymes in myristicin synthesis: Alcohol acyltransferase (AAT)

Alcohol acyltransferase (AAT) is well known as a key enzyme that played an important role in the formation of myristicin as well as other volatile compounds in nutmeg fruits (Chedgy et al., 2015). The formation of volatile compounds is a crucial stage where it is not only contributed to the aroma but as well as to the 22lavor of the fruits. This natural occurrence in fruits helps to improve the attractions and sensory qualities of the fruits (Perez et al., 1996). The AAT enzyme is responsible for the production and synthesizing of myristicin compound as well as other volatile compounds through an esterification process in the nutmeg (Beekwilder et al., 2004).

2.3.1 Esterification process by AAT enzyme

The AAT enzyme can be considered as a versatile enzyme because it can produce a wide range of volatile compounds by the combination of acetyl-CoA with different alcohols (El-Sharkawy et al., 2005). During the esterification process, the AAT enzyme plays the role by facilitating the last transcylation process by transfer acetyl-CoA to an appropriate alcohol to form a specific volatile compound (Beekwilder et al., 2004). For myristicin synthesis, further esterification process after the combination of acetyl- and alcohol will produce myristicin compound via shikimate pathway (Tan & Nishida, 2012). The formation of volatile esters by acylation of alcohols and acetyl-CoA is shown in figure 2.5.

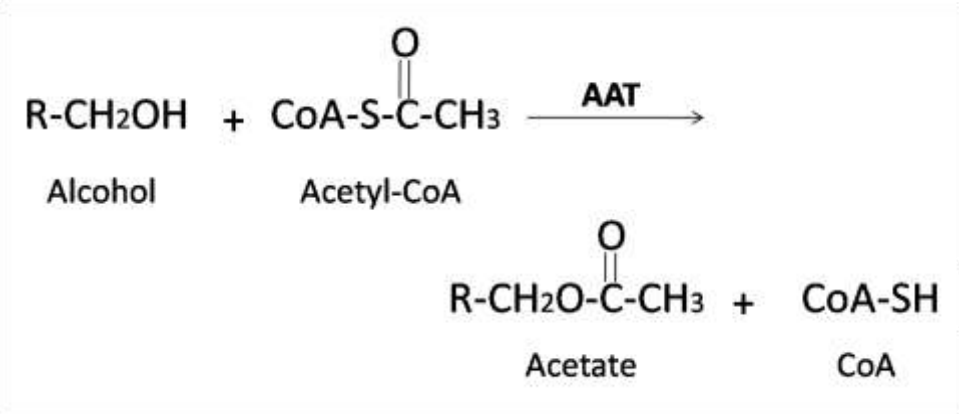


Figure 2.5: The acylation process of alcohols and acetyl-CoA which catalyze by AAT Enzyme to produce volatile esters and coenzyme A (Shalit et al., 2001).

According to Smith (2014), even though the production and synthesizing of myristicin and other volatile compounds were naturally occurring within nutmeg fruits, however, the amount of the volatile compounds could vary due to different factors including source, quality, storage, overall freshness and types of processing. These factors could alter the AAT enzyme activity as well as affecting the production of volatile compound.

2.3.2 Factors that influence AAT enzyme activity

As reported by D'Auria et al. (2007), the main factors that will influence the activity of AAT enzyme are osmotic stress and heat treatment. Osmotic stress occurs when the fruits are immersed in hypertonic or high concentration solution, causing water leaching out from the fruits and the solutes from the hypertonic solution transfer into the fruits. This resulted in an increase in osmotic pressure within the cell membrane of the fruits and resulted in the increasing of osmotic stress (Chavan & Amarowicz, 2012). According to Phisut et al. (2013), osmotic stress occurs within the cell tissue of the fruit due to the breakdown of cellular tissue. These physiological and physicochemical alterations of the fruits enhance AAT enzyme activity and thus increase volatiles compound. The AAT enzyme within the fruits was reported to have a stress-related gene, which will react and act as a defense response when the plant was in stress conditions (Torres et al., 2006). Therefore, in this situation, the plant will automatically elevate the formation of volatile compounds as a defense response (Engelberth et al., 2004). Even though the production of volatile compounds is important in sensory qualities of the fruits, however according to Yamada et al. (2015), in certain fruits the increasing of volatile compounds contributes to its toxicity effects. Therefore, the application of heat treatment to the fruits is essential to reduce the activity of the enzyme as well as reduce the production of volatile compound (Mizobutsi et al., 2010). As reported by Peterson et al. (2007), the application of heat to the fruits will retard the activity of the enzyme. In the industry, pickled is one of the examples of products that involves soaking of the fruits in the high concentration solutions (Singh et al., 2008).