PHYTOCHEMICALS AND BIOLOGICAL ACTIVITIES OF WILD GRAPE (*AMPELOCISSUS MARTINII* PLANCH.) FRUITS

JENJIRA JIRUM

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Chemistry

Mahasarakham University

September 2013

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The examining committee has unanimously approved this thesis, submitted by Miss Jenjira Jirum, as a partial fulfillment of the requirements for the Master of Science degree in Chemistry, Mahasarakham University.

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Jenjira Jirum

ชื่อเรื่อง พฤกษเคมีและฤทธิ์ทางชีวภาพของผลอุ่นป่า (*Ampelocissus martinii* Planch.)
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บทคัดย่อ

งานวิจัยนี้ เตรียมสารสกัดจากผลอุ่นป่า (*Ampelocissus martinii* Planch.) ที่มีสีแตกต่างกันคือสีเขียว แดงและดำ แบบน้ำคั้นสดและจากผงอุ่นป่าซึ่งสกัดด้วยน้ำกลั่นและเมธานอล ก่อนนำไปตรวจสอบปริมาณสารพฤกษเคมี ได้แก่ ฟีนอลิกและฟลาโวนอยด์รวม โดยใช้วิธี Folin-Ciocalteu และ colorimetric aluminum chloride ตามลำดับ จากนั้น ทำการตรวจสอบฤทธิ์ในการต้านอนุมูลอิสระของสารสกัดด้วย 2,2'-diphenyl-1-picrylhydrazyl (DPPH) และวิธี ferric reducing antioxidant power (FRAP) นอกจากนี้ ยังทำการทดสอบฤทธิ์ในการยับยั้งแบคทีเรียก่อโรคของสารสกัดด้วยวิธี agar well diffusion ผลการทดลอง พบว่า น้ำคั้นสดจากผลอุ่นป่าสีเขียว สารสกัดจากผงอุ่นป่าด้วยน้ำสีแดงและดำมีปริมาณฟีนอลิกรวมสูงที่สุด ส่วนปริมาณฟลาโวนอยด์รวมจะพบปริมาณมากที่สุดในสารสกัดจากผงอุ่นป่าผลสีเขียวด้วยน้ำ อย่างไรก็ตาม ปริมาณฟลาโวนอยด์รวมที่พบมีค่าไม่แตกต่างกันมากนัก เมื่อตรวจสอบฤทธิ์ในการต้านอนุมูลอิสระพบว่า น้ำคั้นสดจากผลอุ่นป่าผลสีเขียวมีค่า IC_{50} ต่ำที่สุด แสดงว่ามีฤทธิ์สูงสุด แต่เมื่อพิจารณาจากค่า FRAB พบว่า สารสกัดผงอุ่นป่าผลสีเขียวด้วยน้ำ มีค่าสูงสุด เมื่อทดสอบฤทธิ์ในการต้านเชื้อแบคทีเรียก่อโรค พบว่า น้ำคั้นสดจากผลอุ่นป่าสีแดง มีฤทธิ์ในการต้านเชื้อได้ดี ในขณะที่สารสกัดจากผงอุ่นป่าผลสีเขียวด้วยน้ำและเมธานอลมีฤทธิ์ในการต้านเชื้อแบคทีเรียก่อโรคกว้างกว่าสารสกัดอื่น เมื่อหาความเข้มข้นต่ำสุดในการยั้งยั้ง (MIC) และฆ่า (MBC) เชื้อแบคทีเรีย พบว่ามีค่าอยู่ในช่วง 500-250 ไมโครกรัมต่อมิลลิลิตร จากผลการทดลองกล่าวได้ว่า ผลอุ่นป่าประกอบด้วยฟีนอลิกและฟลาโวนอยด์รวมปริมาณสูงและมีฤทธิ์ทางชีวภาพที่ดีซึ่งอาจสามารถนำไปใช้ประโยชน์ทั้งในด้านโภชนาการและเภสัชกรรมได้

คำสำคัญ: ฟีนอลิก ฟลาโวนอยด์ องุ่นป่า ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านแบคทีเรีย

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ABSTRACT

In this work, the extracts from wild grape (*Ampelocissus martinii* Planch.) in different fruit colors; green, red and black were prepared as wild grape juices and juice powder extracts using distilled water and methanolic. All samples were investigated for some phytochemical compositions such as total phenolic (TPC) and flavonoid (TFC) contents by using Folin-Ciocalteu and colorimetric aluminum chloride assays, respectively. They were then tested for antioxidant activity using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. In addition, they were also tested for their antibacterial activity against infective bacteria using agar well diffusion method. The results found that the juice from green color of wild grape fruit, water extracts of juice powder from red and black wild grape fruit color have colors of the highest of TPC while the water extract from juice powder fruit showed the highest of TFC. However, the TFC of all extracts found similar contents. With antioxidant activity investigation, the wild grape juice of green fruit color showed the lowest of IC_{50} values. This means it has the highest of antioxidant activity, but the water extract from juice powder of green color wild grape fruits powder showed the highest of FRAB value. The juice from red color of wild grape fruits showed the best of antibacterial activity whereas the water and methanolic extracts of juice powder of green fruit have the highest activity against infective bacteria. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of all extracts were arranged of 500-250 µg/mL. The results indicated that the wild grape fruits composed of high TPC and TFC which were also having excellent biological activity. This

suggested that the wild grape fruit extract may further use in nutritional and pharmaceutical applications.

Keywords: phenolic, flavonoid, wild grape, antioxidant activity, antibacterial activity

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CHAPTER 1

INTRODUCTION

1.1 Rationale and Background

Effect of toxic pollution is a problem that affect the body's biochemical balance, cause changes in chemical reactions and processes to disorders, radical or free radical increase in the body. Free radical is released when cell or the body are in oxidative stress, causing damage to living systems. Sometimes, this damage pattern has relationship with diseases and disorders such as cancer, multiple types of associated with cardiovascular diseases, action of immune diseases, and aging (Wu and Hansen, 2008). Many diseases are due to the "oxidative stress" that results from an imbalance between formation and neutralization of pro-oxidants. Oxidative stress is initiated by free radicals, causing protein and DNA damage along with lipid peroxidation and these changes contribute to various kinds of diseases (Poudel *et al*., 2008). It is well known that oxidation stress can be treated by substance called antioxidant. Several studies have shown that plants have antioxidant activities which could be therapeutically benefit. From the past, there are strong evidences of medicinal plants being used for treatment of diseases and revitalizing body system worldwide, especially in ancient civilizations (Zhou *et al*., 2003; Steiner, 1986). Until now, medicinal plants make an important part of traditional medicine (Atanassova *et al*., 2011). Generally, plants produce various secondary metabolites including phenols, flavonoids, quinines, tannins, alkaloids, saponins and sterols (Alghazeer *et al*., 2012). Those of metabolites are being used as pharmaceutical drugs (Liu and Zhang, 2004; Mares *et al*., 2005; Soylu *et al*., 2006; Yazaki *et al*., 2008). Over past decades, synthetic substances were increasingly denied; therefore, plant metabolites have been explored and applied (Suhaj, 2006).

Grape, one of the fruit and widely cultivated throughout the world, contain large amounts of phytochemicals which offer health benefits (Pezzuto, 2008). Previous studies have been shown that grape composed of many types of phenolics such as monomeric flavanols, catechin and epicatechin, dimeric, trimeric and polymeric

procyanidins, phenolic acids (gallic acid) or anthocyanins (Yilmaz and Toledo, 2004). Polyphenolic antioxidants of grape are very effective in preventing cancer and cardiovascular diseases (Bianchinin and Vainio, 2003). Grape seed polyphenols are flavan-3-ol derivatives and only 4% exist in grape pulp. In grape skin, the polyphenol called anthocyanins are major phytochemical which usually have a purple color and have ~30% of total polyphenols in grapes. Phenolic compounds in grape seeds, skins and stem extracts have aslo found to be antimicrobial activity (Butkhup *et al*., 2010). Grapes extract are composed of antimutagenic, antineoplastic and reduce human low-density lipoprotein (LDL) oxidation and allergic inflammation (Nagendrappa *et al*., 2005). Procyanidins in grape seeds possess anti-inflammatory, antiarthritic, anti-allergic, anti cancer, prevents heart disease and skin aging, inhibits carrageenin- or dextran-induced hind paw edema, stabilizes the capillary wall and improves visual performance in humans (Gracenirmala and Narendhirannan, 2011). Wild grape (*Ampelocissus martinii* Planch.), the fruit found normally in the North and North-East of Thailand was chosen as subject for study in this work. Generally, it is very similar to the cultivated grape which suspected to be composed of same phytochemical substances. In addition, some parts of the wild grape have been used as medicinal herb ingredient. However, the information about activity of wild grape fruit extracts have rarely available.

1.2 Objective of the research

To investigate some phytochemical, antioxidant and antibacterial activities of wild grape *(Ampelocissus martinii* Planch.) fruit having different colors (green, red and black).

1.3 Expected results obtained from the research

This study will provide the expected as follows:

1.3.1 Obtain the total phenolic and total flavonoid contents of wild grape *(Ampelocissus martinii* Planch.) fruits having different colors (green, red and black).

1.3.2 Obtain the antioxidant and antibacterial activities of wild grape *(Ampelocissus martinii* Planch.) fruit having different colors (green, red and black).

1.3.3 The data obtained from this work might be used as basic information to use wild grape extracts in medicinal and pharmaceutical applications.

1.4 Scope of the research

The wild grape *(Ampelocissus martinii* Planch.) fruits were collected and extracted. The crude extracts were investigated for their total phenolics and total flavonoids using Folin-Ciocalteu and colorimetric aluminium chloride, respectively. The crude extracts were tested for their antioxidant activity using DPPH-scavenging assay and Ferric reducing antioxidant power assay (FRAP). Moreover, the crude extracts of wild grape fruits would be tested for their antibacterial activity using agar well diffusion and minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) value were obtained by agar two folds serial dilution assay.

1.5 Definition of terms

Antibacterial	an activities of compound or substance that kills or slow down the growth of bacteria.
Antioxidant	an activity of a molecule that inhibits the oxidation of other molecules
Flavonoids	are the largest of natural phenols
	Free radicals are atom, molecule or ion with unpaired electrons or an open shell configuration
IC_{50}	is the concentration of an inhibitor where the response is reduced by half
Natural phenols	are a class of natural organic compounds containing one or more phenolic group
Oxidation	is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent

Oxidation stress	represents an imbalance between the systemic manifestation of reactive oxygen species and a biological system
Reactive oxygen species	are species that associated with cell damage
Phytochemical	chemical compound that occur naturally in plants

CHAPTER 2

LITERATURE REVIEW

2.1 Free radicals

2.1.1 Introduction to free radical

A free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outermost shell (Denisov, 2005; Scott *et al*., 2004). It is easily formed when a covalent bond between entities is broken. Free radicals stay in the neutral and charge electrical state in both positive and negative charges. Until now free radical have been found in various kinds, almost belong to the oxygen and nitrogen reactive species (Pham-Huy *et al.*, 2008)

2.1.2 Types of free radicals

Most free radicals are coming from oxygen atoms and are called reactive oxygen species (ROS), such as superoxide ion, hydroxyl radical and singlet oxygen (Yang *et al*., 2010). Superoxide ion (or reactive oxygen species) is an oxygen molecule with an extra electron. This free radical can cause damage to mitochondria DNA and other molecules. Human body can neutralize superoxide ions by using superoxide dismutase (Kim *et al*., 2010). Hydroxyl radical is formed by the reduction of an oxygen molecule in the electron transport chain. It is a neutral (not charged) form of the hydroxide ion. Hydroxyl radical is highly reactive and form an important part of radical biochemistry. Unlike superoxide, the hydroxyl radical cannot be eliminated by an enzymatic reaction. It has a very short half-life and will only react with molecules its vicinity. Because of its high reactivity it will damage most organic molecules such as carbohydrates, DNA, lipids and proteins (Luo *et al*., 2009). Singlet oxygen is formed by immune system. Singlet oxygen causes oxidation of LDL cholesterol. Hydrogen peroxide is not a free radical but it is involved in the production of many reactive oxygen species. It is a by product of oxygen metabolism and is neutralized by peroxidases. Sometimes reactive nitrogen atoms are involved and these free radicals grouped under reactive nitrogen species (RNS). Nitric oxide is the most important RNS. Some transitional metals, such as iron and copper, have many numbers of unpaired

electrons and can also act as free radicals. These metals do not have strong electron affinity but can easily accept and donate electrons (Yabuta *et al*., 2010). The example of the free radicals are listed in Table 2.1.

Table 2.1 Radicals and related substances (Chattopadhyay *et al*., 2008).

Radicals	Related substance
Reactive oxygen species (ROS)	
Alkoxyl ($RO^{\bullet}$)	Organic peroxides (ROOH)
Carbon dioxide ($CO_2^{-\bullet}$)	Peroynitrous acid (ONOOH)
Carbonate ($CO_3^{-\bullet}$)	Peroxynitrite ($ONOO^-$)
Hydroperoxyl ($HO_2^{\bullet}$)	Hypochlorous acid (HOCl)
Hydroxyl ($HO^{\bullet}$)	Hypobromous acid (HOBr)
Peroxyl ($RO_2^{\bullet}$)	Singlet oxygen ($^1O_2$)
Superoxide, Superoxide anion ($O_2^{-\bullet}$)	H_2O_2, Ozone (O_3)
Reactive nitrogen species (RNS)	
Nitric oxide ($NO^{\bullet}$)	Nitrous acid (HNO_2)
Nitrogen dioxide ($NO_2^{\bullet}$), ($NO_2^{-\bullet}$)	Nitrosyl cation (NO^+), Nitroxyl anion (NO^-)
Dinitrogen tetroxide (N_2O_4)	Dinitrogen trioxide (N_2O_3)
	Peroxynitrite ($ONOO^-$)
	Peroynitrous acid (ONOOH)

2.1.3 Sources of free radicals

Free radical sources can be divided into 2 categories; exogenous or external and endogenous or internal sources. Exogenous sources of free radicals caused from food and drink, pollutants, organic solvents, anesthetics, environments, drugs, heavy metal, pesticides and radiation (Nagendrappa *et al*., 2005). The endogenous free radicals were formed in the body during metabolism of biomolecules. These can be enzymatic reactions, which included respiratory chain, phagocytosis, prostaglandin synthesis and cytochrome P450 system. Other internal sources of free radicals are mitochondria, xanthine oxidase, phagocytes, reactions involving iron and other transition metals,

peroxisomes, arachidonate pathways, exercise ischemia and inflammation (Nagendrappa *et al.*, 2005). On the other hand, mental status (stress and emotion) and disease conditions are also responsible for free radical formations (Valko *et al.*, 2006).

2.2 Antioxidants

Antioxidants are molecules that can inhibit the oxidation process. They are powerful substances even at small concentration. Nowadays antioxidants have been proved as important molecules in living cells and have diverse physiological roles in the body (Chattopadhyay *et al*., 2008). The main function of antioxidant is radical scavengers, and helps in converting the radicals to less reactive species. A variety of antioxidant is found in dietary sources like fruits, vegetables and tea, etc. (Mandal *et al*, 2009). According to literature, the definition of antioxidant is "substance that when present in low concentration compared to those of the oxidisable substrates significantly delay or inhibit the oxidation of that substance" (Cornish and Garbary, 2010). Antioxidants protect the body against oxidative damage from both enzymatic and non-enzymatic reactions. In general, antioxidant divided into 2 main groups: natural and synthetic antioxidants.

2.2.1 Natural antioxidants

Sources of natural antioxidant are mainly found in plant (fruits, vegetables), animal or microorganism (Van Acker *et al*., 2000). Various types of the antioxidant have been reported. Plants have been proposed as main natural antioxidant sources (Hwang *et al*., 2006). During plant growth, different systems were developed including defense strategies or antioxidant system to protect the plant from oxidative stress. The natural antioxidants occurred in all parts of plant (Rastogi *et al*., 2010). Previous works showed that many kinds of plant derived antioxidants were found in both non-enzymatic system such as vitamin C, α- tocopherol, tannin, gallic acid, catechin etc. (Jiménez *et al*., 2010) and enzymatic system including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) , ascorbate peroxidase (APX), glutathione reductase (GA) and polyphenol oxidase ect. (Nagayama *et al*., 2002). In current, the study on medicinal plant was considerly focused. This was due to the medicinal plants having been suggested about their phytochemical components. Until now, some plant derived

antioxidants have been used for treatment of viral, bacterial, amoeboid (Luo *et al*., 2009) and fungal (Aisa *et al*., 2005) diseases. The natural antioxidants can be divided in to 2 groups. First are enzymes that involved converting the toxic radicals to less reactive species. Second are low molecular weight antioxidants which are subdivided into lipid-soluble antioxidants (α-tocopherol, carotenoids, quinones, biliubin, and some polyphenols) and water-soluble antioxidants (ascorbic acid, uric acid and polyphenols). The lipid-soluble antioxidants act as highly efficient scavengers of free radical produced from lipid, while the water soluble antioxidants are less efficient to lyophilic radicals. However, they may act in a synergistic power with lipophilic antioxidant (Veena *et al*., 2007). The structures of some natural antioxidants are shown in Figure 2.1 and sources of them are listed in Table 2.2.

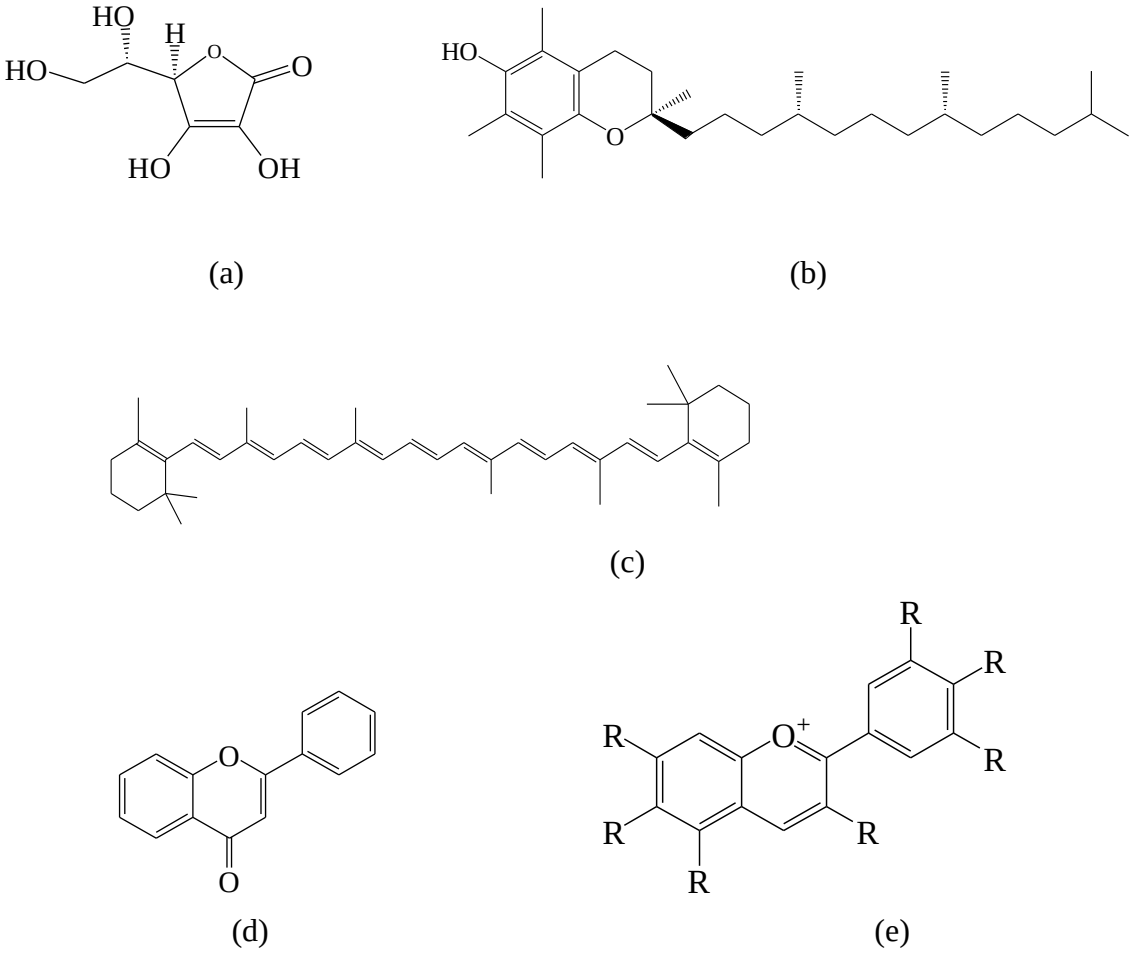

Figure 2.1 Chemical structures of natural antioxidants: (a) ascorbic acid, (b) α-tocopherol, (c) beta-carotene, (d) flavonoid, (e) anthocyanins.

Table 2.2 Natural antioxidants and their sources.

Natural Antioxidant	Source In
Vitamin C (ascorbic acid)	most fruits (particularly citrus fruits) (McGhie *et al.*, 2007), some vegetables, tomatoes (Urquiaga *et al.*, 2000)
Vitamin E/Tocopherols	cereal grains, broccoli, Brussels sprouts, cauliflower (Croft 1998), cookingoils (olive, sunflower,safflower), almonds, hazelnuts (Urquiaga *et al.*, 2000)
Beta-Carotene	vegetables such as kale, red paprika, spinach, parsley, and tomatoes (Croft, 1998), carrots, sweet potatoes, apricots, papayas (Urquiaga *et al.*, 2000)
Flavonoids (a type of polyphenol)	potatoes, tomatoes, lettuce,onions, wheat, dark chocolate, concord grapes, red wine, black tea (Urquiaga *et al.*, 2000)
Anthocyanins (a type of flavonoid)	high content in red wines, some in whiskey, sa-ke´ (Carr *et al.*, 2000)
Various polyphenols	teas (mainly green, some rooibos), as well as many red/purple hued fruits or vegetables, such as concord grapes, red cabbage, blueberries, blackberries, etc. (Carr *et al.*, 2000)
Lycopene	tomatoes, papaya, watermelon, pink grapefruit, guava, the skin of red grapes (Urquiaga *et al.*, 2000)
CoQ10	wheat bran, fish, organ meats (eg. chicken liver), (Urquiaga *et al.*, 2000)

2.2.2 Chemical pathways for natural antioxidants

All antioxidants undergo certain chemical reactions in order to protect other compounds from oxidation. Natural antioxidants donate electrons from two major electron-rich sources via hydroxyl groups and double bonds (Figure 2.2). After donating electrons, they undergo broken down or recycled by physiological systems in order to re-use them for their antioxidant capacities. Most natural antioxidant can be broken down into metabolites for excretion (McGhie *et al.*, 2007).

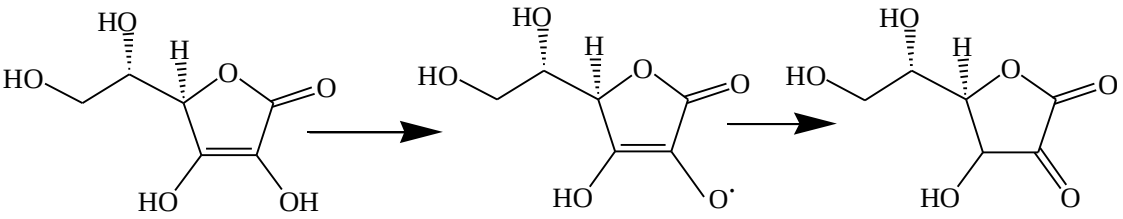

Figure 2.2 Oxidation of vitamin C via the donation of an electron from a hydroxyl group and the generation of a double bond.

Hydroxyl (-OH) groups are a good source of electron density. In general various type of substances act as an antioxidant by donating hydrogen atoms from their's hydroxyl groups in order to quench reactive radical species and generating double bonds. The other strategy which antioxidants prevent oxidation is to use double bonds to donate electron density (Figure 2.3).

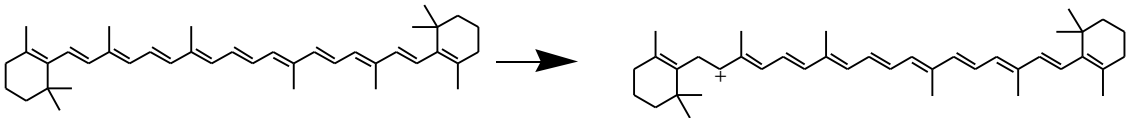

Figure 2.3 Oxidation of β-carotene via the donation of an electron from a double bond.

2.2.3 Synthetic antioxidants

Most synthetic antioxidants are chemically synthesized since they do not found in nature. These antioxidants divided into 2 categories depending on their mode of action: primary and secondary antioxidants. The primary antioxidants act as main substances to prevent the formation of free radicals during oxidation via radical

terminators, oxygen scavengers, and chelating agent (Yang *et al*., 2000; Pokorny *et al*., 2001). Those of radical terminators are butylated hydroxyanisole (BHA), butylated hydroxytoluene *(*BHT), tertiary butyl hydroquinone (TBHQ), and gallates such as propyl gallate (PG), dodecyl gallate (DG) and octyl gallate (OG). The examples of oxygen scavengers are glucose oxidase and ascorbyl palmitate. Polyphosphatases and ethylene diamine tetra acetic acid (EDTA) are chelating agent. These agents prevent oxidation of lipids by binding the lipid oxidation catalysts such as heavy metals (iron, copper, etc). In addition, they also act as precipitating the metal or by occupying all its coordination sites (Sangeetha *et al*., 2009). The example of secondary antioxidant is thiodipropionic acid and dilauryl thiodipropionate. They act by breaking down hydrogen peroxide which formed during lipid oxidation into stable products (Yabuta *et al*., 2001). The structures of some synthetic antioxidants are shown in Figure 2.4.

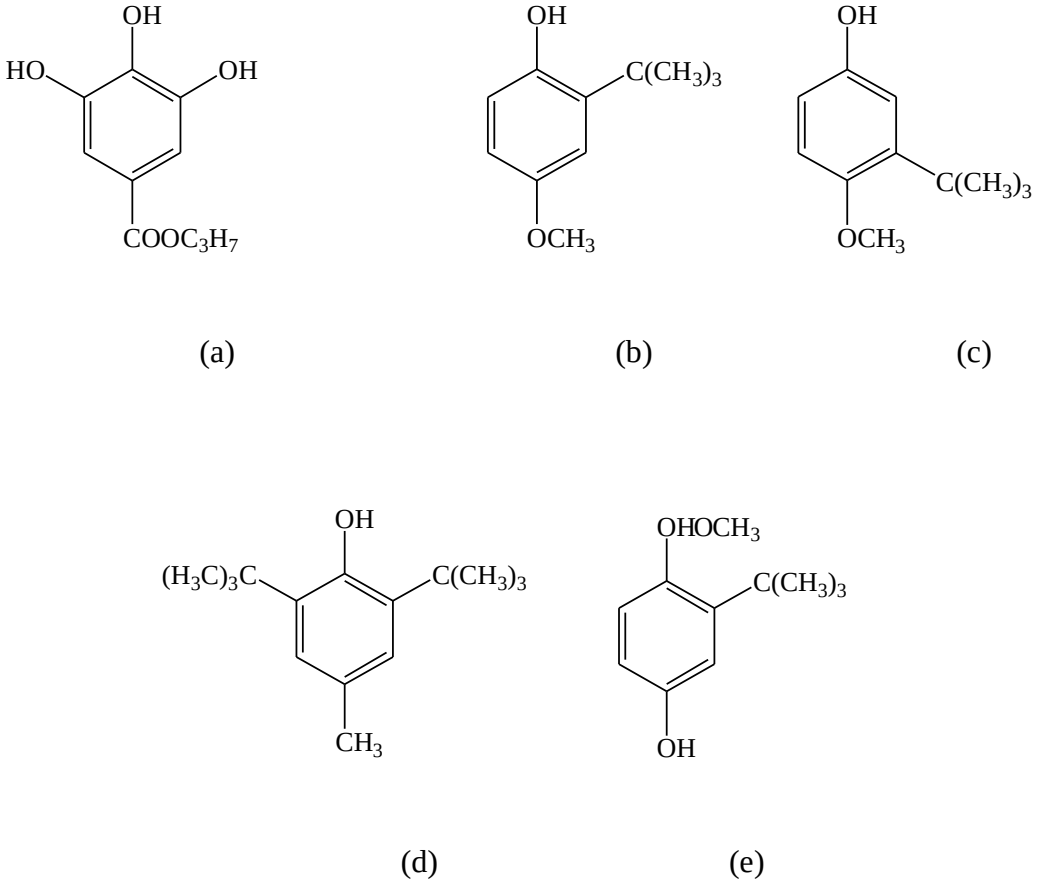

Figure 2.4 Chemical structure of synthetic antioxidants; (a) propyl gallate, (b) 3-butylated hydroxyanisole, (c) 2-butylated hydroxyanisole, (d) butylated hydroxytoluene, (e) tertiary butyl hydroquinone.

2.2.4 Chemical action of synthetic antioxidants

The major class of synthetic antioxidants is phenols. Their action mechanism involves transferring hydrogen of the phenolic hydroxyl group to a lipid free radical in order to form stable free radicals as shown in Figure 2.5.

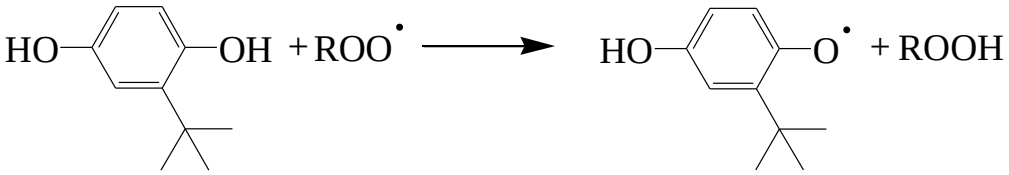

Figure 2.5 Antioxidant mechanism of a radical terminator via the donation of H atom from a phenolic hydroxyl group.

Phenolic antioxidants including BHA, BHT, TBHQ and PG act as excellent antioxidants, according to 2 reasons. Firstly, their hydrogen bond dissociation energy is low making them efficient hydrogen or electron donors. Secondly, the phenoxy radicals generated after the donation of electrons to the lipid free radicals inhibit themselves generate other free radicals. They are relative stable due to resonance stabilization by delocalization of electrons across the aromatic ring and the lack of suitable sites for attacking by molecular oxygen. The action mechanism was shown in Figure 2.6.

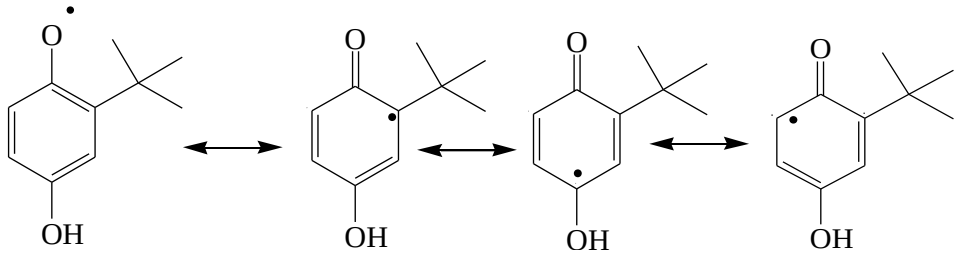

Figure 2.6 Resonance stabilization of a phenolic antioxidant.

Furthermore, the efficiency of these phenolic antioxidants varies depending on the absence or presence of certain other groups on the aromatic ring, especially at the main positions: *ortho* and *para* positions. One example for such antioxidant is 2,6 ditertiary

butyl, 4- methoxyphenol group at the *ovtho* position in butylated hydroxyanisole (BHA) as shown in Figure 2.7.

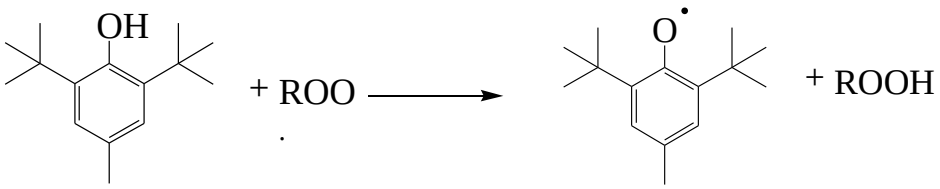

Figure 2.7 Oxidation of BHA via donation of H-atom from a phenolic hydroxyl group.

The presence of bulky group enhances steric hindrance in the region of radicals, which decreased the reaction rate of further propagation. On the other hand, the antioxidant activity is also affected by other factors such as concentration and prooxidant substances (Pravst *et al*., 2010). Moreover, presence of an extra hydroxyl group at the *ortho* or *para* position of phenol illustrates the increasing of antioxidant activity via the formation of intramolecular hydrogen bond as shown in the Figure 2.8.

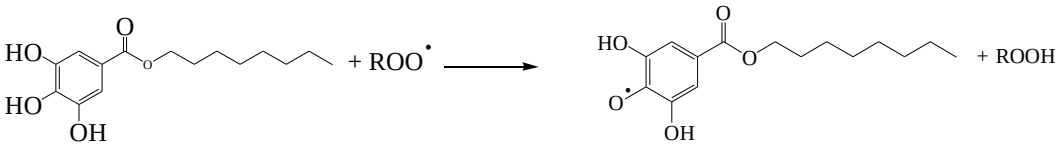

Figure 2.8 Generation of a phenoxy radical, with the intramolecular hydrogen bond.

2.2.5 Biological effects of synthetic antioxidants

The synthetic antioxidants have several common biological effects on the molecular, cellular and organ levels. Those of effects are modulation growth, macromolecule synthesis and differentiation, modulation of immune response, interference with oxygen activation and miscellaneous antioxidant (Cha *et al*., 2006). Both BHA and BHT influence cellular metabolism through induction of various drug metabolizing enzymes. BHA has been shown to cause increase in organ weight, whereas BHT has been shown to cause cell proliferation (Yuan *et al*., 2005). PG may induce drug metabolizing enzymes and might inhibit mitosis in human cells, lipid peroxidation and bacterial growth and toxin formation. In addition, it may enhance

microsomal H_2O_2 formation and decrease smooth and heart muscle contractility. Furthermore, PG has been shown to have local anesthetic action and anti-inflammatory action (Vichitphan *et al.,* 2007; Lai *et al*., 2007).

2.3 Indroduction of bacteria

Bacteria are single-celled microorganisms which lacking a true nucleus and organelles such as mitochondria, chloroplasts, and lysosomes. Bacteria are very small of a few micrometers (μm, 10^{-6} meters) in length. Bacteria come in a wide variety of shapes and size. The most common shapes are rod-like (bacillus). or spherical (coccus).

2.3.1 Bacterial cell wall

One of the most important elements of bacterial is a cell wall, which is responsible for cell shape maintenance and protecting against osmotic lysis. The strength and rigidity of the cell wall results from a layer of peptidoglycan, which is a covalent macromolecular structure consisting of strong glycan chains that are crosslinked by flexible peptide bridges (Cabeen el al., 2005). The glycan strands are made up of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) residues linked by β-1→4 bonds. The D-lactoyl group of each NAM residue is substituted by a side peptide chain whose composition is most often L-Ala-D-Glu-L-Lys-D-Ala (Vollmer *el al*., 2008). Neighbouring glycan strands are connected by crosslinking peptide chains between two side peptide chains. Crosslinking chains are the most variable components of peptidoglycan, and their composition is species-specific. Peptidoglycan layer has some unique biophysical properties, but flexible allowing reversible expansion of the cell wall (Vollmer el al., 2008).

2.3.2 Classification of bacteria

Bacteria can be divided into two basic groups; Gram-positive and Gram-negative. Formerly, this differentiation was based on the result of Gram staining. In the case of Gram-negative bacteria, there are three principal layers in the cell envelope; the inner (cytoplasmic) phospholipid membrane, the peptidoglycan cell wall, and the outer membrane made of lipopolysaccharides, phospholipids, lipoproteins and porins. In Gram-positive bacteria the outer membrane is absent but the peptidoglycan layer is much thicker than is found in Gram-negative bacteria (Parisien el al., 2008). Moreover,

Gram-positive cell wall contains one more important component, teichoic acids. These anionic polymers occur in two distinct forms depending on whether they are linked to the peptidoglycan (teichoic acid) or to the head groups of cytoplasmic membrane lipids (lipoteichoic acid) (Schirner el al., 2009).

2.4 Research related on antioxidant and antibacterial of phytochemicals

Almeida *et al*, (2011) compared the contents of phenolics, vitamin C, anthocyanin and antioxidant activity of 11 fresh exotic fruits, cultivated in the northeastern part of Brazil. The antioxidant activities were evaluated using two antioxidant systems 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), expressed as TEAC (Trolox Equivalent Antioxidant Capacity) and VCEAC (Vitamin C Equivalent Antioxidant Capacity) values. The results indicated that murici and mangaba, fruits were good sources of antioxidants. The phenolic contents showed positive correlations with total antioxidant by ABTS ($R = 0.94$, $P \leq 0.001$) and DPPH ($R = 0.88$, $P \leq 0.001$) assays. However, this correlation was not noticed when examining vitamin C and anthocyanins contents. The 11 fruits studied had comparable antioxidant activity in both ABTS and DPPH assays.

Wootton-Beard *et al*. (2011) analyzed the total antioxidant capacity of 23 commercially available vegetable juices [via Ferric reducing antioxidant power (FRAP), $DPPH^{\bullet}$, 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) and Folin–Ciocalteu reagent (FCR) for total polyphenols] and determined the stability of the antioxidant capacity following an in vitro digestion procedure using the same methods. All 23 juices were significant sources of antioxidants both in terms of total antioxidant capacity and total polyphenols, although results varied considerably between the juices [1369–9500 μmol/L (FRAP), 57.8–100% inhibition of $DPPH^{\bullet}$, 10.9-90.7% inhibition of $ABTS^{\bullet+}$and 449-3025 μg ferulic acid equivalents/mL for FCR]. Beet root juice displayed the highest level of total antioxidants and total polyphenols compared to the other juices which were analyzed (tomato, carrot, mixed vegetable, mixed fruit and vegetable). The antioxidant capacity of the juices remained high throughout the *in vitro* digestion.

Costa *et al*. (2012) analyzed total phenolics, total flavonoids and ascorbic acid contents, as well as DPPH scavenging activity of several commercial samples, namely green tea and other herbal infusions, dietary supplements, and fruit juices, available in the Portuguese market. In general, beverages containing green tea and hibiscus showed higher phenolics contents (including flavonoids) and antioxidant activity than those without these ingredients. A borututu infusion presented the lowest concentrations of bioactive compounds and scavenging activity, due to the low recommended amount of plant to prepare the beverage.

Dutta *et al*. (2012) evaluated the total phenolic content and antioxidant properties of 80% methanol extracts of ten high yielding rice varieties, five each from two different seasons namely aman and boro of Bangladesh by Folin-Ciocalteau method while DPPH radical scavenging, hydroxyl ion scavenging, ferric reducing antioxidant power (Ferric reducing antioxidant power), and total antioxidant capacity (TAC) by ammonium molybdate. The results found that rice variety BR5 of aman and BRRI dhan 28 of boro season comparatively showed higher TPC and Antioxidant properties than the other rice varieties. BR22 of aman season showed the highest hydroxyl ion scavenging activity although it displayed the lowest TPC. Except for hydroxyl ion scavenging activity, aman rice varieties displayed comparatively higher total phenolic content and antioxidant property than the boro rice varieties.

Dutta *et al*. (2012) studied the effect of bioaccumulated Cu on the antioxidant properties of medicinal plants grown in Cu rich soil. The total phenolic and flavonoid content, DPPH scavenging activity, reducing power, metal chelating activity and inhibition of lipid peroxidation were assayed in methanol MeOH extracts of eight medicinal plants grown in the vicinity of copper mining impact site and compared with control samples. Corresponding IC_{50} values of DPPH scavenging ability and metal chelating ability were found to be significantly ($P<0.05$) higher in mining impact samples e.g., *Withania somnifera*, *Azadirachta indica*, *Andrographics peniculata* and *Ocimum sanctum*. The IC_{50} of inhibition of lipid peroxidation of all the mining impact samples were significantly ($P<0.05$) higher than the control samples, indicating lower inhibition capacity of lipid peroxidation by the mining impact samples.

Kaur *et al*. (2012) analyzed the ten commercial and three exotic/wild cultivars (cvs) grown under Indian conditions for variations in lycopene, β-carotene, total phenolics, quercetin, ascorbic acid and antioxidant activity (AOX). AOX was measured using three in vitro assays namely FRAP, DPPH and TEAC assays. The lycopene content in tomato cvs ranged from 4.31 to 5.97 mg/100 g fw. The wild/exotic cvs had exceptionally high total phenolic content (141.98 mg/100 g fw), quercetin (56 mg/g fw) and total AOX (5.39 mmol TE/g fw). *Solanum pimpinellifolium*, with nearly six times lycopene content than commercial cvs, may serve as the most desirable gene pool in breeding programmes to develop functional tomatoes. Results suggest that TEAC may be more useful than DPPH assay for detecting total AOX in tomatoes.

Khanam *et al*. (2012) determined the phenolic compounds and total antioxidant capacity of eight leafy vegetables, namely Komatsuna, Mizuna, Pok choi, Mitsuba, Salad spinach, Lettuce, Red amaranth and Green amaranth. The phenolic compounds were characterized as hydroxybenzoic acids, hydroxycinnamic acids and flavonoids. Salicylic acid was the most common hydroxybenzoic acid, ranging from 4.40 to 117.36 mg/g fresh frozen weight (ffw). Vanilic acid, gallic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid andm-coumaric acids were commonly found in all of these vegetables. Isoquercetin and rutin, the most common flavonoids, ranged from 3.70 to 19.26 and 1.60 to 7.89 mg/g ffw, respectively, and hyperoside was highest (38.72 mg/g ffw) in Mizuna. Total antioxidant capacity values varied widely between $ABTS^{+}$ and DPPH assay methods, with values reported as equivalents to trolox, quercetin and ascorbic acid. Among these vegetables, total antioxidant capacity was found in the following order: Pok choi > Komatsuna > Mizuna > Mitsuba > Red amaranth > Lettuce > Green amaranth > Salad spinach.

Serpen *et al*. (2012) investigated the effects of solvent composition of different radicals (ABTS, DPPH) on measured total antioxidant capacity (TAC) of foods determined by the QUENCHER procedure. The working solutions of ABTS radical were prepared in the mixture of water-ethanol with different volume ratios (0:100, 25:75, 50:50, 75:25, 100:0). The solvent composition had a significant effect on the measured antioxidant capacity of various food matrices including cereals, fruits and vegetables, pulses and nuts ($p<0.05$). The use of ethanol alone gave the lowest values during measurement while introducing water to ethanol significantly improved the

levels of antioxidant capacity. These results suggested that the mixture of water-ethanol (50:50, v/v) may be the most appropriate solution for standardizing the TAC database of foods tested by the QUENCHER procedure. The need of water is due to its ability to open the structure enabling better access of radicals to functional ends of the food matrices.

Sulaiman *et al*. (2012) compared for their total phenolic content, antioxidant and anti food-borne bacterial capacities of *Myristica fragrans* (leaf and different parts of fruit (pericarp, aril, seedkernel and shell). The 80% methanol extracts of aril, seed-kernel and shell shared the highest total phenolic content with shell extract acted as the greatest primary antioxidant, by having the highest ferric reducing antioxidant power (FRAP) activity (EC_{50} = 9.7 ± 0.1 mg/mL), β-carotene-bleaching activity (EC_{50} = 21.5 ±2.7 mg/mL) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity (EC_{50} = 160.9 ± 13.9 mg/mL), whereas the pericarp extract exhibited highest secondary antioxidant activity as a metal chelator (EC_{50} = 75.6 ± 14.4 mg/mL). Only the aril and seed-kernel extracts were found to inhibit the food-borne bacteria with the lowest minimum inhibition concentration of 50 mg/mL, against *Staphylococcus aureus* (ATCC12600) and *Bacillus cereus* (ATCC10876). The results suggest the possibility of using the aril and seed-kernel extracts as natural food preservative and other parts as a new source of natural antioxidant for food and pharmaceutical industries.

Tenore *et al*. (2012) quantified the polyphenols from flesh and peels of red pitaya (*Hylocereus polyrhizus*) fruit by employing an extract sub-fractionation procedure applied for the first time to this fruit. Higher polyphenolic contents than those from the literature were measured by using analogous spectroscopic techniques to those reported in previous works. Betacyanin fractions exhibited the highest reducing and radical-scavenging capacities among the extracts and fractions tested by FRAP and DPPH assays, respectively. Finally, polyphenolic fractions showed a broad antimicrobial spectrum by inhibiting the growth of all of food-borne pathogens tested, while the non-fractionated extracts revealed a very low or no activity. Results indicated flesh as a good source of antioxidants with healthy benefits for human diet and peels as a valuable manufacture by-product to be exploited for the formulation of nutraceuticals and food applications.

Thaipong *et al*. (2012) analyzed the guava fruit extracts for antioxidant activity in methanol extract (AOAM), antioxidant activity measured in dichloromethane extract (AOAD), ascorbic acid, total phenolics, and total carotenoids contents. The ABTS, DPPH, and FRAP assays were used for determining both AOAM and AOAD, whereas the ORAC was used for determining only AOAM. Averaged AOAM [mM Trolox equivalent (TE)/g fresh mass (FM)] were 31.1, 25.2, 26.1, and 21.3 as determined by the ABTS, DPPH, FRAP, and ORAC assays, respectively. Averaged AOAD (mM TE/g FM) were 0.44, 0.27, and 0.16 as determined by the ABTS, DPPH, and FRAP assays, respectively. AOAM determined by all assays were well correlated with ascorbic acid ($0.61 \leq r \leq 0.92$) and total phenolics ($0.81 \leq r \leq 0.97$) and also among themselves ($0.68 \leq r \leq 0.97$) but had negative correlation with total carotenoids ($0.67 \leq r \leq 0.81$).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials

Wild grape (*Ampelocissus martinii* Planch.) fruits having different colors; green, red and black were collected from Roi-Et province during August-October in early winter.

3.2 Chemicals

All of chemicals used in this research were listed in Table 3.1.

Table 3.1 Chemicals used in this research.

Name	Formula	Grade	Company

2, 2- diphenyl-1-Picrylhydrazyl	$C_{18}H_{12}N_5O_6$	AR	Sigma-Aldrich
3, 4, 5-hydroxyl-benzoic acid	$C_7H_6O_5$	AR	Acros organics
Acetic acid glacial	CH_3COOH	AR	Carlo Erba Reagents
Aluminium-chloride	$AlCl_3$	AR	Merck
Ethanol	C_2H_5OH	AR	Merck
Ferric chloride-hexahydrate	$FeCl_3 \cdot 6H_2O$	AR	Carlo Erba
n-Hexane	$CH_3(CH_2)_4CH_3$	AR	Carlo Erba
Folin-Ciocalteu's	-	AR	Carlo Erba
Hydrochloric acid-fuming 37%	HCl	AR	Merck
Methanol	CH_3OH	AR	Merck
Potassium chloride	KCl	AR	Carlo Erba
Sodium acetate trihydrate	$CH_3COONa \cdot 3H_2O$	AR	Carlo Erba
Sodium carbonates	Na_2CO_3	AR	Carlo Erba
Sodium hydroxide	NaOH	AR	Merck

Name	Formula	Grade	Company
2, 4, 6-Tri (2-pyridyl)-s-triazine	$C_{18}H_{12}N_6$	AR	.... Acros organics
Butyliertes-hydroxyanisol	$C_{16}H_{16}O_2$	AR	.... Acros organics
(±)-Catechin hydrate	$C_{15}H_{14}O_6$	AR	.... Univar
Ferrous sulphate-heptahydrate	$FeSO_4 \cdot 7H_2O$	AR	.... Univar
L-Ascorbic acid	$C_6H_8O_6$	AR	.... Univar

3.3 Instruments

All of instruments used in this research were listed in Table 3.2.

Table 3.2 Instruments used in this research.

Instruments	Model
Auto pipette	-
Shaker, PSU-20	Platform Shaker
Vacuum rotary evaporator	Buchi Rotavapor R-210
Visible spectrophotometer,	Perkin Elmer
UV-Vis spectrophotometer	Perkin Elmer

3.4 Methods

3.4.1 Juice of wild grape fruits preparation

The wild grape juice of each fruit colors (green, red and black) were prepared by fingers forcing. The juices were then filtrated through Whatman No.1 filter paper to exclude all of debris. The filtered extracts were collected and kept at 4˚C temperature until use.

3.4.2 Solvent extraction of wild grape juice powder

The wild grape juice was freez-dried into powder and then extracted with distilled water and methanol by weighing 1 g of the powder before adding 10 mL of solvents. The mixture was stirred and stands for 3h, then filtrated through Whatman No.1 paper to exclude all of debris. The extracts were collected and kept at 4˚C temperature until use.

3.4.3 Total phenolic content

The amount of total phenolic content in the wild grape fruit extracts were determined using the Folin-Ciocalteu reagent according to the method of Bonli *et al.,* (2004) using gallic acid as a standard. For the modified procedure, fifty microliters of the extract were mixed with 3 mL of 10% Folin-Ciocalteu reagent (diluted 10 folds with

distilled water). The solution mixture was allowed to stand at room temperature for 15 min. After that 1.5 mL of 10% (w/v) sodium carbonate solution was added to the mixture and then left in room temperature for 15 min. The absorbance of all samples was measured at 750 nm using an UV-Vis spectrophptometer. This experiment was carried out in triplicate and averages of values content. The total phenolic content was analyzed against gallic acid calibration curve standard and expressed as milligrams of gallic acid equivalents (mg GAE) per gram of fresh weight (g of FW).

3.4.4 Total flavonoid content

The total flavonoid content of the extracts were determined according to the modified method of Yang *et al.,* (2009). The two hundred and fifty microliters of each extract were mixed with solution which contained of 1.25 mL of deionized water and 75 µL of 5% sodium nitrite ($NaNO_2$) solution and then the mixture solution was allowed to stand for 5 min at room temperature. One hundred and fifty microliters of 10% (w/v) aluminium chloride ($AlCl_3$) solution was added to the mixture solution and allowed to react for 6 min at room temperature. Five hundred microliters of 1M sodium hydroxide (NaOH) and 775 µL of distilled water were added to the mixture and immediately measured at 510 nm. Total flavonoid content was calculated using the standard curve of (±)-catechin, and expressed as milligrams of catechin equivalents (mgCE) per gram of fresh weight (g of F 3.4.5 Determination of antioxidant activity

3.4.5.1 Free- radical scavenging activity

Free radical scavenging activity of the extract was determined by using a stable 2, 2´-diphenyl-1-picrylhydrazyl (DPPH) following a modified method of Chan *et al.,* (2007). A total of 1.0 mL of extract was added to 2.0 mL of 0.1 mM DPPH solution. The solution mixture was incubated at room temperature in a dark room for 30 min. Absorbance of all samples was measured at 517 nm using an UV-Vis spectrophotometer. The percentage of inhibition was calculated using the following equation;

$$\text{Inhibition (\%)} = [(A_{517} \text{ of control} - A_{517} \text{ of sample}) / A_{517} \text{ of control}] \times 100$$

BHA dissolved in methanol was also be analyzed as control. DPPH radical scavenging activity was expressed as IC_{50} value, which represented the amount of antioxidant in the

aqueous extract necessary to reduce the initial DPPH concentration by 50%. The experiments were performed in triplicates.

3.4.5.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was conducted with a modified method described by Benzie and Strain (1996). The 100 µL of methanolic extract was mixed with 3.0 mL of FRAP solution (300 mM acetate buffer (pH 3.6): 10 mM tripyridyltriazine solution: 20 mM ferric chloride solution (in 40 mM HCl) of 10: 1: 1 by volume) and 300 µL of deionized water, then incubated at 37 ˚C for 4 min. The absorbance was then measured at 593 nm. The results were expressed as millimolar ferrous sulphate per gram of fresh weight (mM Fe (II) g^{-1} of FW).

3.4.6 Antibacterial activity investigation

3.4.6.1 Preparation of bacterial for determination

The different 17 types of bacterial were chosen for determination of antibacterial activity of the wild grape (*Ampelocissus martinii* Planch.) fruits extracts. All of bacterial strains including *Salmonella typhi* DMST 5784 (*S. typhi* DMST 5784), *Shigella flexneri* DMST 17569 (*S. flexneri* DMST 17569), *Enterobacter cloacae* (*E. cloacae*), *Staphylococcus aureus* ATCC 25293 (*S. aureus* ATCC 25293), *Salmonella typhi Group* B (*S. typhi* gr. D), *Salmonella paratyphi* ATCC 14028, (*S. paratyphi* ATCC 14028), *Salmonella typhi* DMST 16122 (*S. typhi* DMST 16122), *Shigella flexneri* DMST 4423(*S. flexneri* DMST 4423), *Escherichia coli* ATCC 25922 (*E. coli* ATCC 25922), *Salmonella typhimurium* ATCC 14028 (*S. typhimurium* ATCC 14028), *Enterobacter* sp, *Bacillus cereus* ATCC 11778 (*B. cereus* ATCC 11778), *Escherichia coli* 0157: H7 DMST 12733 (*E. coli* 0157: H7 DMST 12733), *Psuedomonas aeruginosa* (*Ps.aeruginosa*), *Staphylococcus aureus* MRSA DMST 20625 (*S. aureus* MRSA DMST 20625), *Klebsiella pneumoniae* (*K. pneumonia*e) and *Shigella dysenteriae* (*S. dysenteriae*) was cultured in Mueller-Hinton broth at 37˚C for 48 h. The cultured bacterial were diluted with 0.85% normal saline by adjusting the turbidity of bacterial suspension as equal to McFarland No. 0.5 for obtaining bacterial density of about 1.5×10^{8} cells/mL.

3.4.6.2 Investigation of antibacterial activity of the extracts

The bacterial inhibition activity of the wild grape fruit extract was tested using Agar well diffusion method. The 1 mL of cultured bacterial with turbidity at equal of McFarland No.0.5 was pipetted. All bacterials were swab and placed into the surface of Mueller-Hinton Agar. The medium was punctured into 3 holes per each culture plate using 0.5 cm diameter of cork borer. Twenty five microliters of the aqueous extracts were poured into 2 holes of agar medium. Another well of medium was used as control which poured with the solution without the aqueous extract. The cultured plates were incubated at 37 ˚C for 24 h. Finally, the diameters of inhibition zones (DIZ) were measured in millimeter (mm) and results were recorded as the mean of triplicate experiments.

3.4.6.3 Determination of minimal inhibitory concentration (MIC)

Minimal inhibitory concentration (MIC) of the wild grape fruits extracts was carried out using two folds serial dilution assay. The concentration of the extract (mg/ml) was prepared and started at 5 mL. They was added into 5 mL of Mueller-Hinton Broth, and diluted into500, 250 and 125 µg/mL of an initial concentration, respectively. The 10 µL of selected bacterial with turbidity at equal to McFarland No. 0.5 would be added into the medium broth. They were incubated at 37 ˚C for 24 h. Reference antibiotics and the solvent was also assayed. The MIC was recorded as the lowest concentration of the sample that inhibition after incubation for 24 h. In addition, minimal bactericidal concentration (MBC) was also performed.

3.5 Statistical analysis

Data was expressed as means ± standard deviations (SD) of triplicate experiments and then analyzed by SPSS V.11 (Statistical Program for social sciences, SPSS Corporation, Chicago, IL) using one way analysis of variance (ANOVA). The significant difference would be considered of p=0.05. The correlation analysis was tested between antioxidant activity, total phenolic and total flavonoid contents

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Total phenolic and flavonoid contents

The wild grape juice and extracts of wild grape (*Ampelocissus martinii* Planch.) fruits having different colors (green, red and black) were analyzed for their some phytochemical compositions. The results showed that the wild grape has rich in total phenolic **(**TPC**)** and flavonoid contents **(**TFC**)**. The highest of TPC in the wild grape juice was found in black fruit color (11.37 ± 0.25 mg GAE/g FW), secondly was found in green fruit color (3.63 ± 0.03 mg GAE/g FW), and the last was found in red fruit color (2.51 ± 0.12 mg GAE/g FW) with gallic acid as standard. The wild grape juice showed a good source of TPC and the results was indicated as mg cathecin equivalent per g of FW. The TFC in all extracts were arranged in higher than 10 mg CE/g FW. The highest value was found in the juice of black color of wild grape fruit (19.41 ± 0.30 mg CE/g FW), then green fruit (16.83 ± 0.04 mg CE/g FW), and red fruit (12.97 ± 0.03 mg CE/g FW) as shown in Table 4.1.

The TPC and TFC of the wild grape juice powder extracts using distilled water and methanol were also evaluated and summarized in Table 4.1. The results found that the water extracts of red and black colors of wild grape fruit showed similar content of TPC (3.201 ± 0.032 and 3.087 ± 0.047 mg GAE/g FW) which were higher than that of green fruit color (1.498 ± 0.068 mg GAE/g FW). The methanolic extract showed the lowest of TPC compared to other extracts. In this group, the extract of red fruit showed the highest of TPC (2.946 ± 0.118 mg GAE/g FW), then black and green fruits color (2.209 ± 0.643, 1.576 ± 0.109 mg GAE/g FW) respectively. On the other hand, the water extract showed slightly higher TFC content (~11-12 mg CE/g FW) than methanolic extract (~10-11 mg CE/g FW) and both of the extracts were similar content in all of fruit colors.

4.2 Antioxidant activity

The antioxidant activity of the wild grape juice was analyzed using DPPH scavenging assay and ferric reducing antioxidant power (FRAP) assay. The DPPH assays are reported in Table 4.2. The IC_{50} was calculated and expressed as the concentration of antioxidant exists in the extracts that able to decrease the DPPH amount by 50%. The juice of wild grape fruits seems to be good free radical scavenging. The juices of green and black fruit colors indicated IC_{50} of 0.97 ± 0.88 µg/mL and 1.38 ± 0.07 µg/mL, respectively while red fruit color has 39.62 ± 2.56 µg/mL. The IC_{50} of the water extract showed the highest values of 160.280 ± 5.921, 234.670 ± 5.341 and 52.723 ± 2.842 µg/mL for green, red and black fruit colors, respectively. However, the IC_{50} value not be detect (ND) from the methanolic extracts of green and red fruit colors, except in the methanolic extract of black fruit color (2.842 ± 0.075 µg/mL).

The antioxidant power of the extracts was also tested for reducing ability. The results found that wild grape juice showed the reducing ability of 304.740 ± 0.555, 365.000 ± 0.962 and 612.120 ± 1.923 mM $FeSO_4$/100g FW for green, red and black fruit colors, respectively. For the green fruit color, water extract showed the highest of FRAB value (729.100 ± 1.469 mM $FeSO_4$/100g FW), then methanolic extract (382.950 ± 2.937 mM $FeSO_4$/100g FW). The FRAB value of the red fruit color has the highest in the water extract (525.900 ± 1.256 mM $FeSO_4$/100g FW), followed by juice (365.000 ± 0.962 mM $FeSO_4$/100g FW) and methanolic extract (242.240 ± 2.002 mM $FeSO_4$/100g FW). The extracts of black fruit color showed the highest of FRAB value in juice (612.120 ± 1.923 mM $FeSO_4$/100g FW), followed by water extract (490.000 ± 2.885 mM $FeSO_4$/100g FW) and methanolic extract (43.850 ± 0.962 mM $FeSO_4$/100g FW), of juice powder, respectively.

Table 4.1 show the content of total phenolic (TPC) and total flavonoids (TFC) of wild grape extracts from different fruit colors (green, red and black).

Fruit colors	TPC (mg GAE/g FW) ± SD			TFC (mg CE/g FW) ± SD		
		Juice powder			Juice powder	
	Juice	Water	Methanol	Juice	Water	Methanol
Green	3.632 ± 0.031	1.498 ± 0.068	1.576 ± 0.109	16.832 ± 0.045	11.715 ± 0.026	11.625 ± 0.113
Red	2.505 ± 0.117	3.201 ± 0..032	2.946 ± 0.118	12.968 ± 0.026	11.342 ± 0.068	10.820 ± 0.157
Black	11.371 ± 0.251	3.087 ± 0.047	2.209 ± 0.643	19.412 ± 0.298	12.162 ± 0.144	10.477 ± 0.077

Table 4.2 I C_{50} and FRAB values of wild grape extracts from different colors of wild grape fruits (green, red and black).

Fruit colors	IC_{50} (µg/mL) ± SD			FRAP (mM $FeSO_4$/100g) ± SD		
		Juice powder			Juice powder	
	Juice	Water	Methanol	Juice	Water	Methanol
Green	0.974 ± 0.882	160.280 ± 5.921	ND	304.704 ± 0.555	729.100 ± 1.469	382.590 ± 2.937
Red	39.620 ± 2.556	234.670 ± 5.341	ND	365.000 ± 0.962	525.900 ± 1.256	242.240 ± 2.002
Black	1.380 ± 0.071	52.723 ± 2.842	2.842 ±0.075	612.120 ± 1.923	490.000 ± 2.885	430.850 ± 0.962

ND= no detection

4.3 Antibacterial activity

4.3.1 Antibacterial activity of the fresh juice extracts

The antibacterial activity of the wild grape juice was assayed by agar well diffusion method against 17 bacterial strains (Table 4.3). The juice from green fruit color showed high effective against *S. aureus* ATCC 25293, *S. flexneri* DMST 4423, *E. coli* ATCC 25922, *Enterobacter* sp., *E. coli* 0157: H7 DMST 12733 and *S. aureus* MRSA DMST 20625 **(**DIZ**)** with inhibition zone ranging from 10-13 mm, but the antibacterial activity for *S. typhi* DMST 5784, *S. flexneri* DMST 17569, *E. cloace*, *S. typhi* gr. D, *S. paratyphi* ATCC 14028, *S. typhi* DMST 16122, *S. typhimurium* ATCC 14028, *B. cereus* ATCC 11778, *Ps. aeruginosa*, *K. pneumoniae* and *S. dysenteriae* has hot. All of juices have affect against 3 strains of bacterial including *S. typhimurium* ATCC 14028, *Ps. aeruginosa* and *K. pneumonia*e. The juice of black fruit color found the lowest antibacterial activity. It has the antibacterial activity against only *S. typhi* DMST 16122 (20 mm), *S. flexneri* DMST 4423 (19 mm) and *S. aureus* MRSA DMST 20625 (10 mm). The results suggested that the juice from red color of wild grape fruits showed the most effective antibacterial activity against almost of tested bacterial strains, except *S. typhimurium* ATCC 14028, *Ps. aeruginosa* and *K. pneumoniae*.

Table 4.3 Diameter of inhibition zone of wild grape extracts from extract different fruit colors (green, red and black).

Bacterial	**Diameter of inhibition zone (mm)**		
	Fruit color		
	Green	Red	Black
S. typhi DMST 5784	-	14	-
S. flexneri DMST 17569	-	13	-
E. cloace	-	11	-
S. aureus ATCC 25293	12	11	-
S. typhi gr. D	-	16	-
S. paratyphi ATCC 14028	-	13	-
S. typhi DMST 16122	-	13	20
S. flexneri DMST 4423	13	15	19
E. coli ATCC 25922	10	20	-
S. typhimurium ATCC 14028	-	-	-
Enterobacter sp.	13	15	-
B. cereus ATCC 11778	-	13	-
E. coli 0157: H7 DMST 12733	-	13	-
Ps. aeruginosa	13	11	-
K. pneumoniae	-	-	-
S. aureus MRSA DMST 20625	11	13	10
S. dysenteriae	-	16	-

(-) = no detection

4.3.2 Antibacterial activity of the water extracts

The water extract from wild grape juice powder showed high effective against *S. typhi* DMST 5784 and *E.coli* 0157: H7 DMST 12733 with ranging from 16 mm. The extract showed slightly effective against *B. cereus* ATCC 11778 (DIZ=14 mm), *S. aureus* ATCC 25293, *Ps. aeruginosa* and *S. typhi* gr. D. with DIZ of 13 mm. Moreover, the extract indicated also antibacterial against *S. typhi* DMST 16122, *E. coli* ATCC 25922, *S. aureus* MRSA DMST 20625, *S. flexneri* DMST 4423 and *S. dysenteriae* with DIZ ranging of 11-12 mm. The water extract of green fruit color has not antibacterial ffect against *S. flexneri* DMST 17569, *E. cloace, S. paratyphi* ATCC 14028, *S. typhimurium* ATCC 14028, and *K. pneumonia*e. The water extract of red fruit color showed effective against *S. typhi* DMST 5784 and *E.coli* 0157: H7 DMST 12733 with DIZ 14 mm. In addition, it also showed antibacterial activity against 3 strains including *S. typhi* gr. D., *S. flexneri* DMST 4423 and *S. dysenteriae* with DIZ ranging from 10-11 mm. On the other hand, the water extracts of black fruit color showed antibacterial activity against only *S. typhi* DMST 5784 and *E.coli* 0157: H7 DMST 12733 in low power with DIZ arrange of 10-11 mm. The results indicated that the water extract from wild grape juice powder of green fruit color showed the most effective antibacterial activity against almost of tested bacterial strains, except *S. flexneri* DMST 17569, *E. cloace, S. paratyphi* ATCC 14028, *S. typhimurium* ATCC 14028, and *K. pneumonia*e as shown in Table 4.4.

Table 4.4 Diameter of inhibition zone of water extracts from wild grape juice powder of different fruit colors (green, red and black)

Bacterial	**Diameter of inhibition zone (mm)**		
	Fruit color		
	Green	Red	Black
S. typhi DMST 5784	16	14	11
S. flexneri DMST 17569	-	-	-
E. cloace	-	-	-
S. aureus ATCC 25293	13	-	-
S. typhi gr. D	13	11	-
S. paratyphi ATCC 14028	-	-	-
S. typhi DMST 16122	12	-	-
S. flexneri DMST 4423	11	11	-
E. coli ATCC 25922	12	-	-
S. typhimurium ATCC 14028	-	-	-
Enterobacter sp.	11	11	-
B. cereus ATCC 11778	14	-	-
E. coli 0157: H7 DMST 12733	16	14	11
Ps. aeruginosa	13	-	-
K. pneumoniae	-	-	-
S. aureus MRSA DMST 20625	11	-	-
S. dysenteriae	11	10	-

(-) = no detection

4.3.3 Antibacterial activity of the methanolic extracts

The methanolic extract from wild grape juice powder of green fruit color showed high effective against *S. aureus* ATCC 25293, *S. typhi* DMST 16122 and *E. coli* ATCC 25922 with DIZ ranging from 19-23 mm. It also showed high effective against *S. typhi* DMST 5784, *E.coli* 0157: H7 DMST 12733, *S. aureus* MRSA DMST 20625 and *S. dysenteriae* with DIZ ranging from 14-15 mm. Moreover, the methanolic extract wild grape juice powder of green fruit color indicated antibacterial activity against *S. flexneri* DMST 17569, *E. cloace*, *S. typhi* gr. D, *S. flexneri* DMST 4423, *B. cereus* ATCC 11778 (DIZ = 11-12 mm). The methanolic extract of red fruit color showed the highest activity against *S. aureus* ATCC 25293 (24 mm), followed by *S. typhi* DMST 16122 (20 mm). It also showed high activity against *S. typhi* DMST 5784, *S. flexneri* DMST 4423, *E. coli* ATCC 25922 and *S. dysenteriae* with DIZ of 13-14 mm. It revealed antibacterial activity with low efficacy against *S. flexneri* DMST 17569, *E. cloace*, and *S. typhi* gr. D (DIZ = 10-11 mm). In addition, it showed the lowest effective against *B. cereus* ATCC 11778 with DIZ of 8 mm. The results clearly indicated that the methanolic extract of wild grape juice powder of black fruit color has not effective against almost tested bacterial, except *S. dysenteriae* (DIZ = 12 mm). The results were summarized in Table 4.5.

Table 4.5 Diameter of inhibition zone of methanolic extracts in different colors of wild grape fruits (green, red and black colors).

Bacterial	**Diameter of inhibition zone (mm)**		
	Fruit color		
	Green	Red	Black
S. typhi DMST 5784			
	14	13	-
S. flexneri DMST 17569	12	11	-
E. cloace	12	11	-
S. aureus ATCC 25293	23	24	-
S. typhi gr. D	12	10	-
S. paratyphi ATCC 14028	-	-	-
S. typhi DMST 16122	19	20	-
S. flexneri DMST 4423	11	13	-
E. coli ATCC 25922	20	14	-
S. typhimurium ATCC 14028	-	-	-
Enterobacter sp.	11	8	-
B. cereus ATCC 11778	14	-	-
E. coli 0157: H7 DMST 12733	14	-	
Ps. aeruginosa	-	-	-
K. pneumoniae	-	-	-
S. aureus MRSA DMST 20625	14	-	-
S. dysenteriae	15	14	12

(-) = no detection

4.4 MIC and MBC Investigation

The selected bacterial of *S. typhi* gr. D, *S. typhi* DMST 16122, *S. flexneri* DMST 4423, *E. coli* ATCC 25922 and *S. dysenteriae* were chosen for MIC and MBC assay since the wild grape juice extract showed the highest antibacterial activity. As shown in Table 4.6, the MIC and MBC values were found between 500-250 µg/mL of juice. On the other hand, *S. typhi* DMST 5784 and *E.coli* 0157: H7 DMST 12733 were selected to MIC and MBC assay of water extract from wild grape juice powder. The results indicated that both MBC and MIC values were 500 µg/mL. Furthermore, *S. aureus* ATCC 25293, *S. typhi* DMST 16122, *E. coli* ATCC 25922 and *S. dysenteriae* were selected to MIC and MBC assay of methanolic extract from wild grape juice powder. The results showed the MIC and MBC values were found between 500-250 µg/mL.

Table 4.6 MBC and MIC values of different extracts on selected bacteria.

Bacterial (wild grape fruit color)	**MBC (**µg/mL)	**MIC (**µg/mL
Fresh juice		
S. typhi gr. D (Red)	500	500
S. typhi DMST 16122 (Black)	250	250
S. flexneri DMST 4423 (Red)	250	250
E. coli ATCC 25922 (Red)	250	250
S. dysenteriae (Red)	500	500
Water extract		
S. typhi DMST 5784 (Green)	500	500
E.coli 0157:H7 DMST 12733(Green)	500	500
Methanolic extract		
S. aureus ATCC 25293 (Green/Red)	500	500
S. typhi DMST 16122 (Green/Red)	250	250
E. coli ATCC 25922 (Green)	250	250
S. dysenteriae (Green)	500	500

Discussion

Many kinds of fruits, vegetables, spices and medicinal plants have been reported to be good sources of phytochemicals. Those of the phytochemicals have been found to play protective roles against chronic degenerative diseases (Tsao and Deng, 2004; Lako *et al*., 2007). The phytochemicals include polyphenols, carotenoids and vitamin found to gradually study and interested since they are more effective activity on human health (Liu, 2007). In addition, these phytochemicals are also composed of biological activities such as antioxidant and antimicrobial activities. This work attempted to screen some phytochemicals, especially phenolics and flavonoids in the fresh juice of wild grape (*Ampelocissus martini* Planch.), a local herbal medicinal plant of Thai. Phenolics are secondary metabolism products in plant which were important on the growth of plants (Zheng *et al*., 2011). The quantitative investigation of the phytoconstituents of juice extracts of different colors of wild grape fruits are moderately present of total phenolics (TPC) which were higher contents than those of water and methanolic extracts, especially the extract from black fruit color. The total flavonoids (TFC) in all of extracts were similar profile of TPC since the wild grape juice has the highest of TPC. The contents of phytochemicals were varied companson between colors. The black fruit color showed the highest content both TPC and TFC. In previous report, the activities phytochemical compositions in plant were affected by many factors such as cultivars, maturity, environmental factors, colors as well as the types and quantity of phytochemicals (Lako *et al*., 2007; Iriti and Faoro, 2006). The relationship between phytochemicals and antioxidant activity has been widely reported (Tsao and Deng, 2004; Velioglu *et al*., 1998; Wang *et al*., 2011). Polyphenol and flavonoids have been used for prevention of various diseases caused from free radicals (Alghazeer et al., 2012; Deepa *et al*., 2009). The phenolics act as terminators of free radical from oxidation reaction, while flavonoids are responsible for the radical scavenging effects (Atanassova *et al*., 2011). Generally, the extract with high total phenolic contents had higher antioxidant activity (Yang *et al*., 2009; Burns *et al*., 2000; Irudayaraj *et al*., 2010). The results from this work indicated that juice of green fruit color exhibited of high potential of antioxidant activity according from DPPH, but the lowest by FRAP assay. The highest of value found FRAB in water extract and found moderate content in

red and black fruits colors. This might be affected by the chlorophyll components in the green fruits color (Irudayaraj *et al*., 2010) and the anthocyanin in the black fruits color (Velioglu *et al*., 1998). Interestingly, the extract of black fruit color showed very low of antioxidant activity. It might be suggested that the extract preparation and solvent used should be improved to give the anthocyanin content as well as its activity. The function of reducing power of the phytochemicals presented in the juice was reflected by the antioxidant capacity. Many studies have been shown that many flavonoids and phynolics contribute significantly to the total antioxidant activity of many fruits including grape, vegetable and medicinal plants (Luo *et al*., 2002; Negro *et al*., 2003; Bourgou *et al*., 2008). Wild grape is similar to cultivated grape (Genus *Vitis*) including stem and fruits. The cultivated grape has been reported as a rich source of phytochemicals and shown protective effects on many regenerative diseases (Yang *et al*., 2009; Chuang *et al*., 2011). However, the study about phytochemical composition in wild grape has been rarely available information. The phytochemicals and antioxidant activity of the wild grape juice were found to be the same trend with the extracts from grape. The wild grape fruit extracts presented of high degree of antibacterial activity on Gram negative bacteria over 12 selected strains especially juice and methanolic extracts of green fruit color. This result suggested that the antibacterial activity of the fresh juice due to the present of different kinds of phytochemicals. It is well known that the secondary metabolites called phytochemicals were produced for plant against microbial pathogens. Previous reports suggested that medicinal plants are considered to be potent source of novel compounds having biological activities such as antioxidant and antimicrobial activities (Alghazeer *et al*., 2012; Edziri *et al*., 2010). In many years ago, the scientists have been attempted to develop of drug resistance in human pathogenic microorganisms instead of commercial antimicrobial drugs (Patra et al., 2011). The extracts of wild grape fruits were screened for antibacterial activity against 15 human pathogenic bacteria and 2 strains of normal flora *in vitro*. The obtained results from this work indicated that all extracts of wild grape fruits are active against the selected bacteria with different types of bacteria and efficacies of each wild grape extracts. This might be caused from the structural differences of bacterial cell wall (Scherrer and Gerhardt, 1971). In general, the drug compounds inhibited the growth and metabolism of bacteria to prevent their infection (Dash et al., 2008). With phytochemical

investigation, the extracts showed high amount of total phenolic and flavonoid contents which also presented of high antioxidant activity. These phytochemicals might also be concerned the antibacterial activity by direct correlation. With previous reports, many bioactive produced by plant have been reported to protect plants against bacteria, fungi and pests (Patra *et al*., 2011; Aboa *et al*., 2006). Therefore, it is not surprise that the plant extract should be composed of antibacterial activity. Most of extracts revealed that green fruit color composed of high effective against tested bacteria than other colors. The young growth stage, many matabolited were synthesized to help in plant growth and prevent some harmful factors. The extract of black fruit color has lower of antibacterial activity than red and green fruit colors, respective. This result might be said that black fruit color of wild grape is the last growth stage before falling; therefore, many kinds of active compounds may not synthesized and decreased (Nagendrappa *et al*., 2005). In terms of MIC and MBC values, the wild grape juices possess high antibacterial activity. The result suggested that the potential is mostly reflected by the high concentration of phytochemicals composed in the extracts. Many have been report about the effect researchers of on phenolic compounds bacteria (Khan *et al*., 2009; Sengul *et al*., 2009; Doughari *et al*., 2008). The phenolics injure the lipid – containing plasma membranes of bacteria and cause the cell to leak its cellular content. In addition, they can changes in membrane permeability and cause cell destruction. They can also penetrate into bacteria cells and coagulate cell content (Boulekbache – Makhlouf *et al*., 2013; Tian *et al*., 2009). However, other active compounds such as steroids, alkaloids or tannins may be involved this activity which showed be further evaluated.

CHAPTER 5

CONCLUSIONS

The crude extracts of wild grape (*Ampelocissus martinii* Planch.) fruits are rich in phytochemical contents which directly related to high antioxidant and antimicrobial activities. The data found in this work might be used for further study of the wild grape extract on various applications such as health supplement and pharmaceutical benefits. The results can be concluded as follows:

1. The wild grape juice of black fruit color has the highest of total phenolic content (TPC) (11.37 ± 0.25 mg GAE/g FW) as well as total flavonoid content (TFC) (19.41 ± 0.30 mg CE/g FW).

2. The water and methanolic extracts of wild grape juice powder of red and black fruits colors showed similar content of TPC (in range of ~3.2-1.5 mg GAE/g FW) as well as the TFC (~12-10 mg CE/g FW).

3. The extracts of green and black colors of wild grape fruit showed high potential of antioxidant activity considering from DPPH assay as IC_{50} = 0.97 ± 0.88 µg/mL and 1.38 ± 0.07 µg/mL, respectively.

4. The wild grape juice showed the highest of FRAB value in black fruit color of 612.120 ± 1.923 mM $FeSO_4$/100g FW, while the water extract from green color of wild grape juice powder has the highest FRAB value of 729.100 ± 1.469 mM $FeSO_4$/100g FW.

5. The wild grape juice of red fruit color showed widely antibacterial activity against infective bacteria.

6. The water extract of green color of wild grape juice powder showed the highest of antibacterial activity than other colors, while methanolic extracts from green and red fruit colors showed similarly and widely antibacterial activity.

7. The MIC and MBC values of the selected extracts were in range of 500-250 µg/mL.

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APPENDICES

Appendix A

Preparation of solutions

1. Preparation of reagents for total phenolic content

1.1 Preparation of 10% Folin-Ciocalteu reagent

A 10% Folin-Ciocalteu reagent was prepared by dilute 10 mL of Folin-Ciocalteu reagent in 90 mL of deionized water.

1.2 Preparation of 10% sodium carbonate

A 10% sodium carbonate solution was prepared by dissolving 10.0502 g of Na_2CO_3 in 100 mL of deionized water.

1.3 Preparation of stock standard (2 mg mL^{-1}) gallic acid

Stock standard solution (2 mg mL^{-1}) of gallic acid was prepared by dissolving 0.0510 g of gallic acid and made up to volume with deionized water in 25 mL volumetric flask.

2. Preparation of reagents for total flavonoid content

2.1 Preparation of 5% sodium nitrite solution

A 5% sodium nitrite solution was prepared by dissolving 1.2886 g of $NaNO_2$ in 25 mL of deionized water.

2.2 Preparation of 10% aluminium chloride solution

A 10% aluminium chloride solution was prepared by dissolving 5.0505 g of $AlCl_3$ in 50 mL of 50% methanol.

2.3 Preparation of Stock standard (2 mg mL^{-1}) (±)-catechin

Stock standard solution (2 mg mL^{-1}) of (±)-catechin was prepared by dissolving 0.050 g of (±)-catechin and made up to volume with methanol in 25 mL volumetric flask.

3. Preparation of reagents for Free- radical scavenging activity (DPPH) assay

3.1 Preparation of 0.1 mM DPPH (MW = 394.33)

A 0.1 mM DPPH was prepared by dissolving 0.0232 g of DPPH in 500 mL and made up to volume with methanol in 500 mL volumetric flask.

3.2 Preparation of Stock standard 2 mg mL^{-1} BHA

Stock standard solution (2 mg mL^{-1}) of BHA was prepared by dissolving 0.0521 g of BHA and made up to volume with methanol in 25 mL volumetric flask.

4. Preparation of reagents for Ferric reducing antioxidant power (FRAP) assay

4.1 Preparation of 300 mM sodium acetate buffer, pH 3.6

A 0.025 M sodium acetate buffer (pH 3.6) solution was prepared by dissolving 24.624 g of $CH_3COONa \cdot 3H_2O$ in 500 mL of deionized water. The pH value of 0.3 M of the solution was adjusted using CH_3COOH and made up to volume with deionized water in a 1000 mL volumetric flask.

4.2 Preparation of 10 mM TPTZ (MW= 312.32)

A 10 mM TPTZ solution was prepared by dissolving 0.0789 g of TPTZ in 25 mL and made up to volume with 40 mM HCl in 25 mL volumetric flask.

4.3 Preparation of 20 mM ferric chloride (MW= 162.21) solution

A 20 mM ferric chloride solution was prepared by dissolving 0.1655 g of $FeCl_3$ in 50 mL and made up to volume with deionized water in 50 mL volumetric flask.

4.4 Preparation of 40 mM hydrochloric acid (MW= 36.441; 37%; d = 1.19)

A 40 mM hydrochloric acid was prepared by dilute 3.30 mL of 37% HCl in 1000 mL and made up to volume with deionized water in 1000 mL volumetric flask.

4.5 Preparation of 10 mM Ferrous sulphate solution

Stock standard solution of 10 mM $FeSO_4$ was prepared by dissolving 0.0140 g of $FeSO_4 \cdot 7H_2O$ in 5 mL and made up to volume with methanol in 5 mL volumetric flask.

Appendix B

Calibration curves of standard in DPPH assay

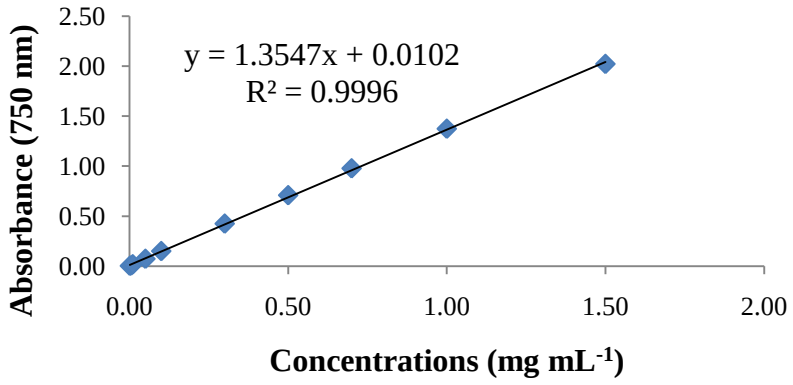


Figure AB1 Calibration curve of standard gallic acid.

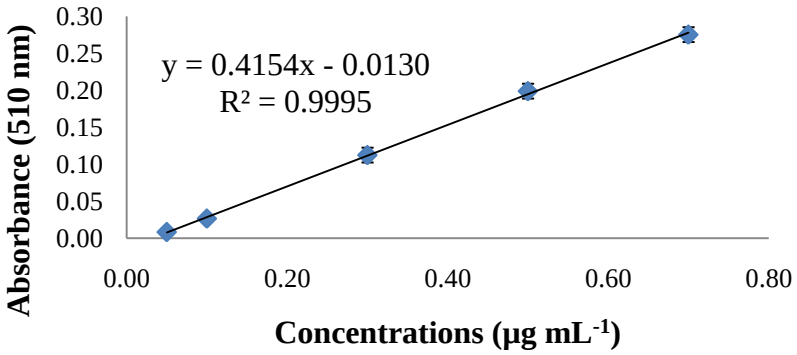


Figure AB2 Calibration curve of standard (±)-catechin.

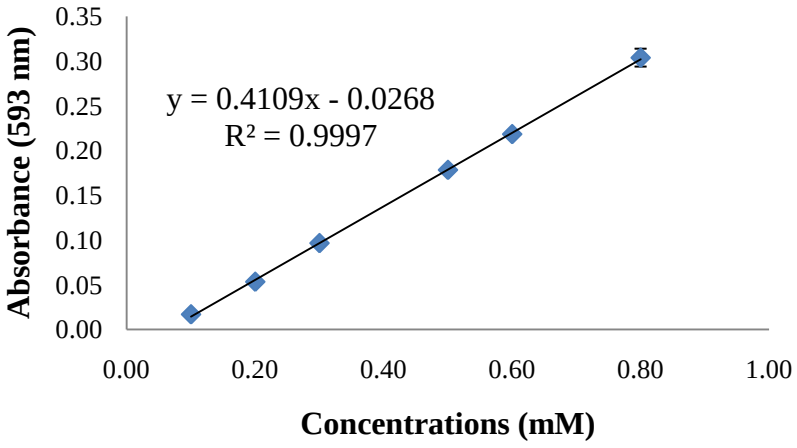


Figure AB3 Calibration curve of standard ferrous sulphate.

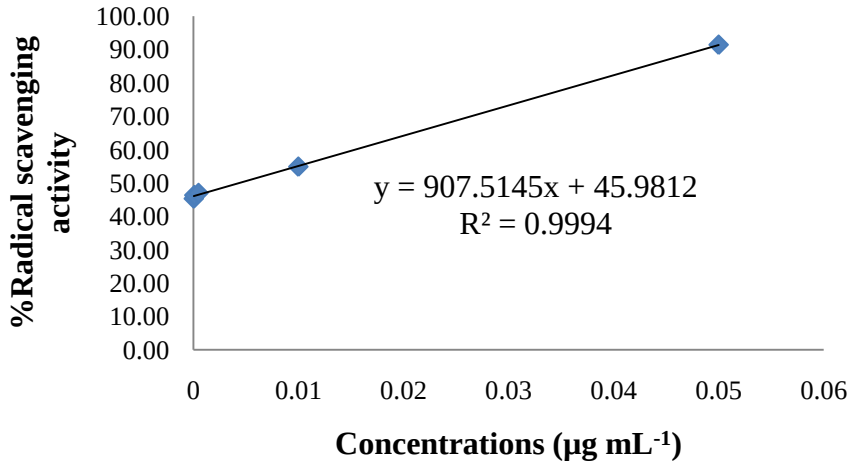
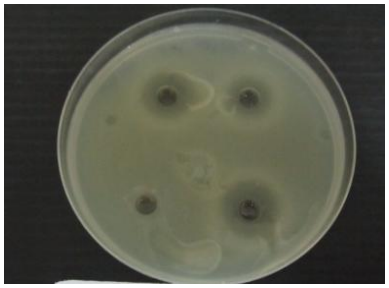


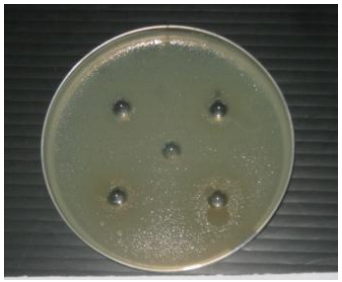
Figure AB4 Calibration curve of standard BHA.

Appendix C
Antibacterial activity of wild grape fruits extracts

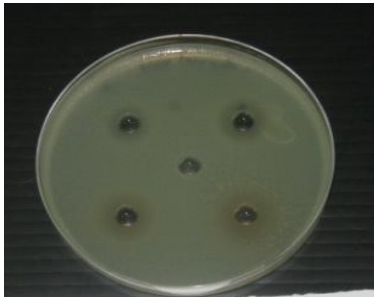


(a)

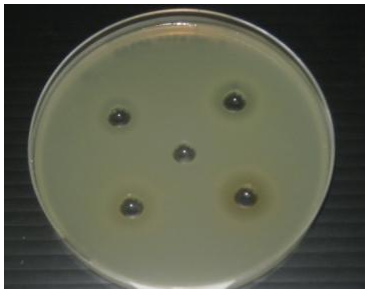
(b)



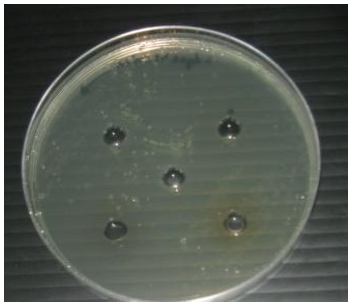
(c)



(d)



(e)



(f)

Figure AC1 Diameter inhibition zone of wild grape fruit extracts on some bacteria;(a)*S. typhi*gr.D(b)*S. typhi* DMST 16122 (c) *S. flexneri* DMST 4423(d)*S. flexneri* DMST 4423(e)*E. coli* ATCC 25922(f)*S.* dysenteriae.

MIC

(a)

(b)

(c)

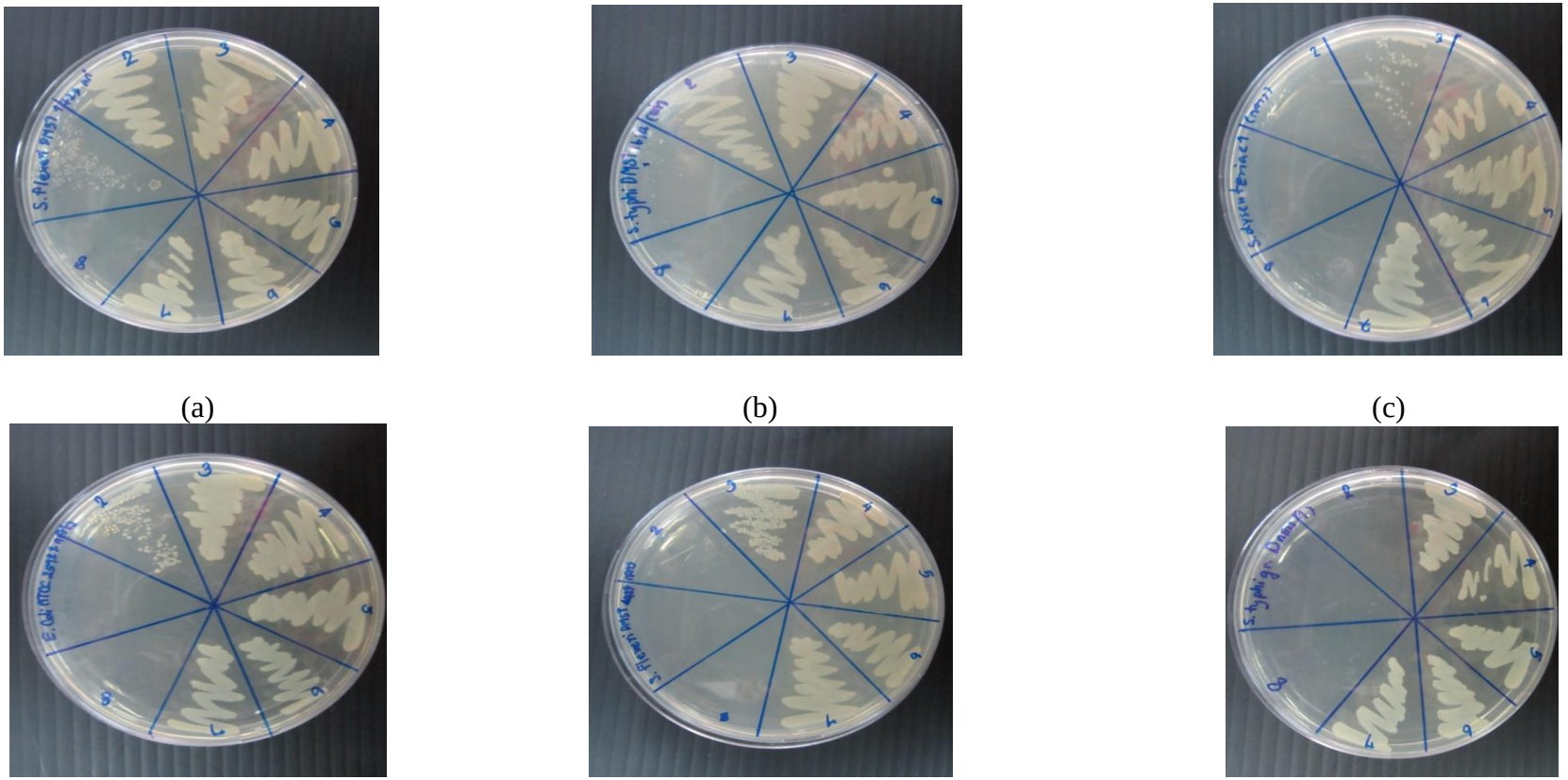
(d)

(e)

(f)

Figure AC2 showed the MIC investigation of selected bacteria; (a)*S. typhi*gr.D, (b)*S. typhi* DMST 16122, (c) *S. flexneri* DMST 4423,(d)*S. flexneri* DMST 4423,(e)*E. coli* ATCC 25922,(f)*S.* dysenteriae.

MBC



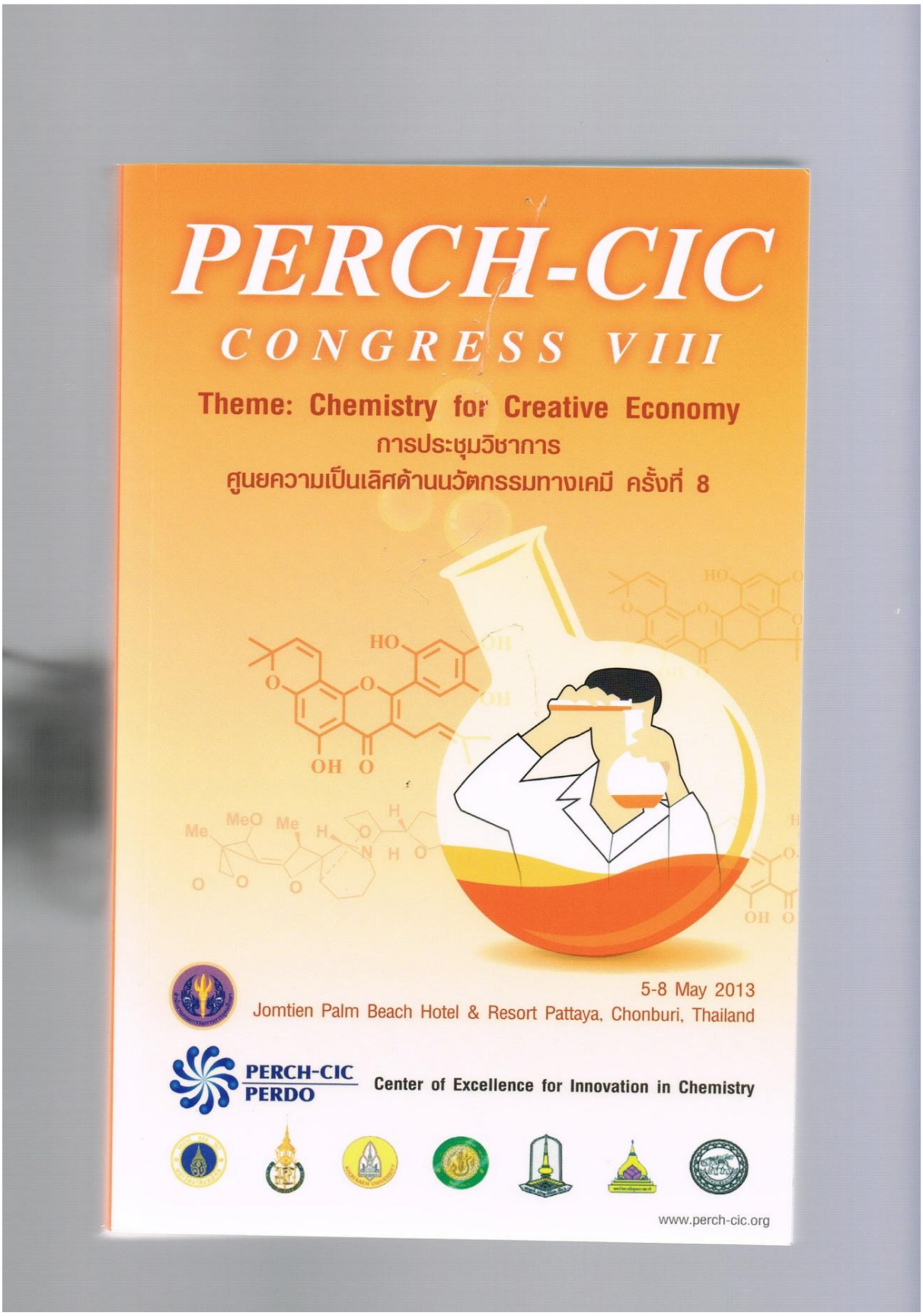
(a) (b) (c)

(d) (e) (f)

Figure AC3 showed the MBC investigation of selected bacteria; (a)*S. typhi*gr.D, (b)*S. typhi* DMST 16122, (c) *S. flexneri* DMST 4423,(d)*S. flexneri* DMST 4423,(e)*E. coli* ATCC 25922, (f)*S.* dysenteriae

Appendix D

Research output



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Phytochemicals and Biological Activities in Crude Extract of Wild Grape (*Ampelocissus martinii* Planch.) Fruits

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Introduction and Objective

Free radical is a molecule that affects the body's biochemical balance and causing damage to living systems. With previous reports, all of this damage can be protected by some phytochemicals. Plant is an important source, especially phenolic compounds. This work interested in wild grape (*Ampelocissus martinii* Planch.), since it is very similar both stem and fruits to the cultivated grape. This fruit has been reported as the high source of phytochemicals. Therefore, the aim of this work was to investigate some phytochemicals and their activities in crude extract of wild grape fruits.

Methods

The crude extracts of wild grape fruits (green, red and black colors) were prepared by fingers forcing, then filtrated and kept in 4˚C temperature until use. The antioxidant activities were determined for free- radical scavenging activity using a stable 2,2'-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) and ferric reducing antioxidant power (FRAP) assay. Total phenolic content (TPC), and total flavonoid content (TFC) were analyzed by Folin-Ciocalteu, colorimetric aluminum chloride, methods, respectively.

Results

The result found that the crude extract of black color show the highest FRAP value (612.120 mM FeSO4/100gFW), then red (365 mM FeSO4/100gFW) and green (304.47 mM FeSO4/100gFW). The IC_{50} for DPPH radical-scavenging activity of the green, red and black color extracts were about 0.974, 39.62 and 1.38 $\mu g/mL^{-1}$, respectively. Analysis of the extracts from green, red and black color of wild grape fruits showed the TPC of 3.632, 2.505 and 11.371 mg GAE/g FW, while TFC were 16.832, 12.968 and 19.412 mgCE/g FW, respectively.

Conclusion

The crude extracts of black color show the highest 0f FRAP value as well as TPC and TFC. However, the crude extract of green showed the highest of antioxidant activity indicated by the lowest of IC_{50} value.

Keywords: Antioxidant, Free radicals, IC_{50}, Phytochemicals

Selected References: 1. Wu, X.J. and Hansen, C. *Journal of Food Science*, **2008**, 73, 1-8.

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3

PHYTOCHEMICAL AND BIOLOGICAL ACTIVITIES IN FRESH JUICE EXTRACTS OF WILD GRAPE (*AMPELOCISSUS MARTINI* PLANCH.) FRUITS

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ABSTRACT

The fresh juice extracts of wild grape (*Ampelocissus martini* Planch.) fruits in different colors; green, red and black were used for investigation of their phytochemicals and antioxidant activity. This fruit is much similar when compared to the cultivated grape, an important source of phytochemicals, and never been reported about their biological activities. The fresh juice extracts were evaluated for total phenolic content (TPC), and total flavonoid content (TFC) by using Folin-Ciocalteu assay and colorimetric aluminum chloride assay, respectively. The antioxidant activities were carried out using DPPH and FRAP assays. In addition, the antibacterial activity of the extracts was also performed by agar well diffusion method. The fresh juice extract of wild grape fruits showed high contents of TPC and TFC, especially the juice extract from black color (11.37±0.25 mg GAE/g FW and 19.41±0.30 mM $FeSO_4$/g FW for TPC and TFC, respectively). However, the fresh juice extracts from green color exhibited the highest antioxidant activities. In contrast, the antibacterial activity of the juice extract from red color of wild grape fruits were wide against 14 strains and at least 6 strains of pathogenic bacteria were also susceptible against the juice extract of green color. In conclusion, this work confirmed the strong antioxidant and antibacterial activities of wild grape fruits extracts. The result suggested that this fruit might be used as a powerful source of phytochemicals and biological activities.

Keywords: *Ampelocissus martini* Planch., Antioxidant, Antibacterial, Phytochemical

INTRODUCTION

In recent years, studies on phytochemical extracted from plants have been gradually increased.[1-4] Since many phytochemicals were believed to protect the body from free radicals[5] which are released when cells or the body are in oxidative stress and result in damage of living systems. Sometime, this damage is related with diseases and disorders such as cancer, multiple types of cardiovascular diseases, immune diseases, and aging.[6] It is well known that the product of oxidation stress can be treated by substances called antioxidant. Antioxidants are substances that delay or prevent oxidative stress even present at low quantity.[7] Plants have been reported as an important source of many substances that could be used in prevention of human diseases. From the past, there are strong evidences that medicinal plants are being used for treatment of diseases and revitalizing body system worldwide, especially in ancient civilizations including Thailand. Until now, medicinal plants are an important part of traditional medicine.[8] Phytochemicals such as phenolics and flavonoids are classified as secondary metabolites.[9] They usually comprised of various biological activities[10] and are being used as pharmaceutical drugs.[11-14] Over past decades, synthetic substances were increasingly denied; therefore, plant metabolites have been explored and applied for medical application.[15]

Wild grape (*Ampelocissus martini* Planch.) is a native tropical plant found generally in the north and northeast of Thailand. The fruit of wild grape and stage of fruit development is very similar to cultivated grape. Therefore, the wild grape fruit may be contained of some secondary metabolites as well as their biologicals. As far as our literature is concerned, the information on phytochemical and biological activities of wild grape fruit is rarely available. Thai people (especially in the northeast region) have been consuming it as food and used as medicine for a long history. Therefore, the aim of this work was to screen some phytochemicals and evaluate the biological activities of the wild grape fruit extracts.

MATERIALS AND METHODS

Plant Material

Fresh fruits of wild grape (*Ampelocissus martini* Planch.) were collected from Kheeleg village, Tambon Huayhinlad, Suwannaphumi district, Roi-Et province, Thailand in August 2012. The fruits were washed twice with water and grouped (green, red and black colors). All fruits were kept at 4 °C and used as required.

Preparation of Fresh Juice

The wild grape fruits of each group were weighed and extracted by hand fingers forcing to obtain the fresh juice at room temperature. The yield of the extract was about 30% (w/v) in terms of starting material. The fresh juice extract was filtrated using Whatman No. 1 paper filter, and then stored in refrigerator at 4 °C (less than 35 h) for further studies of phytochemicals and biological activities.

Chemicals

The DPPH (2,2-diphenyl-1-Picrylhydrazyl) was purchased from Sigma-Aldrich (Singapore). Aluminium

chloride ($AlCl_3$) was purchased from Merck (England). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and Folin-Ciocalteu's reagent were purchased from Carlo Erba Reagents. 2,4,6-Tri (2-pyridyl)-s-triazine ($C_{18}H_{12}N_6$) was purchased from Acros organics. (±)-catechin hydrate ($C_{15}H_{14}O_6$), ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) and L-ascorbic acid ($C_6H_8O_6$) were purchased from Univar. All other chemicals and reagents were of analytical grade.

Evaluation of Total Phenolic Content

The amount of total phenolic content (TPC) in the crude extract of wild grape fruits was determined using the Folin-Ciocalteu reagent according to the method of Bonoli *et al.*[16] by using gallic acid as a standard. For the modified procedure, fifty microliters of crude extract was mixed with 3 mL of 10% Folin-Ciocalteu reagent (diluted 10 fold with distilled water). The mixture solution was stand at room temperature for 15 min. After that 1.5 mL of 10% (w/v) sodium carbonate solution was added to the mixture and then left at room temperature for 15 min. The absorbance of all samples was measured at 750 nm using an UV-Vis spectrophotometer (UV-1610, Shimadzu). The experiment was carried out in triplicate and averages of values were calculated. The TPC was analyzed against gallic acid calibration curve standard and expressed as milligrams of gallic acid equivalents (mg GAE) per grams of fresh weight (g of FW).

Evaluation of Total Flavonoid Content

The total flavonoid content (TFC) of crude extract was evaluated according to the modified method of Yang et al.[17] Two hundred and fifty microliters of crude extract was mixed with 1.25 mL of deionized water, 75 µL of 5% sodium nitrite ($NaNO_2$) solution and allowed to stand for 5 min at room temperature. One hundred and fifty microliters of 10% aluminium chloride ($AlCl_3$) were added to the mixture solution and left to react for 6 min at room temperature. Five hundred microliters of 1M sodium hydroxide (NaOH) and 775 µL of distilled water were added to the mixture. The absorbance of all samples was immediately measured at 510 nm. TPC was calculated using the standard curve of (±)-catechin, and expressed as milligrams of catechin equivalents (mg CE) per gram of fresh weight (g of FW).

Free-Radical Scavenging Activity

Free radical scavenging activity of crude extract was determined by using a stable 2,2´-diphenyl-1-picrylhydrazyl (DPPH) following a modified method of Chan et al.[18] A total of 1.0 mL of crude extract was added to 2.0 mL of 0.1 mM DPPH solution. The mixture solution was incubated at room temperature in a dark room for 30 min. Absorbance of all samples was measured at 517 nm using an UV-Vis spectrophotometer. The percentage of radical scavenging activity was calculated using the following equation;

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

Where, $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the crude extract. BHA dissolved in methanol was also analyzed as control. DPPH radical scavenging activity was expressed as IC_{50} value, which represented the amount of antioxidant in the crude extract necessary to reduce the initial DPPH concentration by 50%. The experiment was performed in triplicates.

Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing power of the crude extract was detected using a ferric reducing antioxidant power (FRAP) assay described by Benzie and Strain[19] with some modifications. Briefly, the fresh solution of FRAP reagent contained 2.5 mL of 10 mL 2,4,6-Tri (2- pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl with 2.5 mL of mM $FeCl_3$ and 25 mL of 0.3M acetate buffer pH 3.6 was freshly prepared. The 20 µL of crude extract was mixed with 180 µL of FRAP reagent and allowed to stand at 37 ^{0}C for 4 min. The absorbance of the mixture solution was measured at 593 nm using UV-Vis spectrophotometer. The ethanolic solution of known Fe (II) concentration in the range of 50-500 µM ($FeSO_4$) was used as calibration curve. The ferric reducing ability of the crude extracts was expressed as mM of $FeSO_4$ equivalent concentration (EC) per 100 gram of fresh weight (FW). BHT and quercitin was used as positive controls. The experiment was performed in triplicates.

Antibacterial Activity

The different 17 types of pathogenic bacteria was chosen as substrate for determination of antibacterial activity of the juice extract of wild grape (*Ampelocissus martinii* Planch.). All bacteria including *S. typhi* (DMST 5784), *S. flexneri* (DMST 17569), *E. cloacae*, *S. aureus* (ATCC 25293), *S. typhi* (gr. D), *S. paratyphi* (ATCC 14028), *S. typhi* (DMST 16122), *S. flexneri* (DMST 4423), *E. coli* (ATCC 25922), *S. typhimurium* (ATCC 14028), Enterobacter sp, *B. cereus* (ATCC 11778), *E. coli* (0157:H7 DMST 12733), *Ps. aeruginosa*, *S. aureus* (MRSA DMST 20625), *K. pneumonia* and *S. dysenteriae* were cultured in Mueller-Hinton broth at 37°C for 48 h. The cultured bacteria were diluted with 0.84% normal saline by adjusting turbidity of bacterial suspension as equal to McFarland No. 0.5 for obtaining bacterial density of about 1.5×10^8 cell/mL.

Antibacterial Activity of Aqueous Extracts

The inhibition activity on bacteria of aqueous extract was tested using Agar well diffusion method. The 1 mL of cultured bacteria at equal turbidity of McFarland No.0.5 was swabed and placed into the surface of Mueller-Hinton Agar. The agar media was punctured into 3 holes per culture plates of 0.5 cm diameter. Twenty five micro-liters of the juice extracts were poured into 2 holes of agar and another hole was used as control (without the juice extract). The culture plates were incubated at 37 °C for 24 h. Finally, the diameters of inhibition zones (DIZ) were measured in millimeter (mm) and were recorded as the mean of triplicate experiments. Moreover, the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the fresh juice extracts were carried out using agar two folds serial dilution assay.

Table 1: DPPH radical scavenging and reducing capacity (FRAP) of the fresh juice extract of wild grape

Colors	DPPH radical scavenging IC_{50} (µg/mL) ± SD	Ferric reducing antioxidant power (FRAP) (mM $FeSO_4$/100g FW) ± SD
Green	0.974±0.882	304.740±0.555
Red	39.620±2.556	365.000±0.962
Black	1.380±0.071	612.120±1.923

Table 2: Antibacterial activity of the fresh juice extract of wild grape against pathogenic bacteria

Bacterial strains	Diameter of zone of inhibition (mm)		
	Green	Red	Black
S. typhi DMST 5784	-	14	-
S. flexneri DMST 17569	-	13	-
E. cloacae	-	11	-
S. aureus ATCC 25293	12	11	-
S. typhi gr. D	-	16	-
S. paratyphi ATCC 14028	-	13	-
S. typhi DMST 16122	-	13	20
S. flexneri DMST 4423	13	15	19
E. coli ATCC 25922	10	20	-
S.typhimurium ATCC 14028	-	-	-
Enterobacter sp.	13	15	-
B. cereus ATCC 11778	-	13	-
E.coli 0157: H7 DMST 12733	13	11	-
Ps. aenainosa	-	-	-
S. aureus MRSA DMST 20625	11	13	10
K. pneumoniae	-	-	-
S. dysenteriae	-	16	-

*(-) mean no activity

Table 3: MIC and MBC values of the fresh juice extract of wild grape against selected pathogenic bacteria

Bacterial strains	Juice colors	MIC (µg/mL)	MBC (µg/mL)
S. typhi gr. D	Red	500	500
S. typhi DMST 16122	Black	250	250
S. flexneri DMST 4423	Red	250	250
S. flexneri DMST 4423	Black	250	250
S. dysenteriae	Red	250	250
E. coli ATCC 25922	Red	500	500

Statistical Analysis

Data were expressed as means ± standard deviations (SD) of triplicate experiments.

RESULTS

Total Phenolic and Flavonoid Contents

The fresh juice extracts of wild grape (*Ampelocissus martini* Planch.) fruits in different colors (green, red and black) were analyzed for their phytochemical compositions. The results indicated that wild grape fruit is rich in total phenolic (TPC) and flavonoid (TFC) contents. The highest TPC was found in black color (11.37±0.25 mg GAE/g FW), followed by green (3.63±0.03 mg GAE/g FW), and then red color (2.51±0.12 mg GAE/g FW) with gallic acid as standard. The fresh juice extract of wild grape was good source of TFC which was indicated as mg cathecin equivalent per g of FW. The TFC of all extracts were higher than 10 mg CE/g FW. The highest TFC value was found in the juice of black color (19.41±0.30 mg CE/g FW), followed by green (16.83±0.04 mg CE/g FW), and red color (12.97±0.03 mg CE/g FW), respectively.

Antioxidant Activity

The antioxidant activity of the juice extract was analyzed using DPPH scavenging assay and ferric reducing antioxidant power (FRAP) assay. The DPPH assay results are shown in Table 1. The IC_{50} was expressed as the concentration of antioxidant comprise in the extracts, able to decrease the DPPH amount by 50%. Almost extracts showed free radical scavenging capacity, especially green and black colors. Their capacities were indicated IC_{50} of 0.97±0.88 µg/mL and 1.38±0.07 µg/mL, respectively while red color was 39.62±2.56 µg/mL. Moreover, the reducing ability (FRAP values) of the juice extracts of wild grape fruits was about 304.740±0.555, 365.000±0.962 and 612.120±1.923 mM $FeSO_4$/100g FW for green, red and black colors, respectively.

Antibacterial Activity

Antibacterial activity of the juice extract of wild grape (*Ampelocissus martini* Planch.) fruits was assayed by agar well diffusion method against 17 bacterial strains (Table 2). The juice extract from green color was highly effective against *S. aureus* ATCC 25293, *S. flexneri* DMST 4423,

E. coli ATCC 25922, Enterobacter sp., *E.coli* 0157:H7 DMST 12733 and *S. aureus* MRSA DMST 20625 with inhibition zone ranging from 10-13 mm while it has not shown antibacterial activity for *S. typhi* DMST 578, *S. flexneri* DMST 17569, *E. cloacae*, *S. typhi* gr. D, *S. paratyphi* ATCC 14028, *S. typhi* DMST 16122, *S.typhimurium* ATCC 14028, *B. cereus* ATCC 11778, *Ps. Aenainosa*, *K. pneumonia* and *S. dysenteriae*. All the juice extracts were not effective against 3 strains of pathogenic bacteria; *S.typhimurium* ATCC 14028, *Ps. aeruginosa* and *K. pneumoniae*. The juice extracts of black color had the lowest antibacterial activity. It was found to have antibacterial activity only against *S. typhi* DMST 16122 (20 mm), *S. flexneri* DMST 4423 (19 mm) and *S. aureus* MRSA DMST 20625 (10 mm). The results indicated that the juice extract from red color of wild grape fruits showed the highest effective antibacterial activity against all tested bacterial strains, except *S. typhimurium* ATCC 14028, *Ps. aeruginosa* and *K. pneumoniae*. The more effective antibacterial activity against *S. typhi* gr. D, *S. typhi* DMST 16122, *S. flexneri* DMST 4423, *E. coli* ATCC 25922 and *S. dysenteriae* were chosen for MIC and MBC assay. As shown in Table 3, the MIC and MBC values were found in range of 500-250 µg/mL of selected juice extracts.

DISCUSSION

Many kinds of fruits, vegetables, spices and medicinal plants have been reported to be good sources of phytochemicals. These phytochemicals have been found to play protective roles against chronic degenerative diseases.[20,21] The phytochemicals including polyphenols, carotenoids and vitamin were found to be important for study and interested since they were found more effective in activity on human health.[22] In addition, these phytochemicals are also composed of biological activities such as antioxidant and antimicrobial activities. This work was attempted to screen some phytochemicals, especially phenolics and flavonoids in the fresh juice of wild grape (*Ampelocissus martini* Planch.), a local herbal medicinal plant of Thailand. Phenolics are secondary metabolite products in plant which are important on the growth of plants.[23] The quantitative investigation of the phytoconstituent of juice extracted from different colors of wild grape fruits are moderately existed with total phenolics content (TPC) and total flavonoids (TFC). The contents of phytochemicals were varied depending on the colors of fruit. The black color showed the highest value in both phenolics and flavonoids in comparison to other. With reference to previous reports, the activities as well as the phytochemical compositions in plants were affected by many factors such as cultivars, maturity, environmental factors, colors as well as the types and quantity of phytochemicals.[21,24] Many reports about the relationship between phytochemicals and antioxidant activity were found.[20,25,26] Polyphenol and flavonoid are used for prevention of various diseases caused from free radicals.[9,27] The phenolics act as terminators of free radical from oxidation reaction, while flavonoids are responsible for the radical scavenging effects.[8] Generally, the extract with high total phenolic contents had higher antioxidant activity.[17,28,29] The results in this work indicated that green and black colors of wild grape fruits composed of antioxidant activity in higher potential both DPPH and FRAP assays. This may affected from the chlorophyll components of green color[30] and the anthocyanin in the black color.[25] The function of reducing power of the phytochemicals presented in the juice was reflected by the antioxidant capacity. The antioxidant capacity increased with increasing concentration in all samples. Many studies have been shown that flavonoids and phynolics contribute significantly to the total antioxidant activity of many fruits including grape, vegetable and medicinal plants.[31-33] Wild grape is similar to cultivated grape [Genus *Vitis*] including stem and fruit. The cultivated grape has been reported as a rich source of phytochemicals and shown to have protective effects on many regenerative diseases.[17,34] However, study on phytochemical composition in wild grape has been rarely available information. The phytochemicals and antioxidant activity of the wild grape juice found to be the same as with the extracts from grape. The wild grape juice possessed high degree of antibacterial activity, especially on Gram negative bacteria over 14 selected strains. This suggested that the antibacterial activity of the fresh juice may be due to the presence of different kinds of phytochemicals. It is well known that the secondary metabolites are called phytochemicals and were produced in plant against microbial pathogens. Previous reports suggested that medicinal plants are considered to be potent source of novel compounds with having biological activities such as antioxidant and antimicrobial activities.[9,35] In terms of MIC and MBC values, the wild grape juices possess high antibacterial activity. This suggests that the potential is mostly reflected by the concentration of phytochemicals in the extracts. However, other active compounds such as steroids, alkaloids or tannins may be involved this activity. The evaluation and characterization as well as biological investigation of other compounds are in process.

CONCLUSION

The present work showed that the wild grape juice extracts are composed of high content of phytochemicals, especially phenolics and flavonoids. They are also having high potential of biological activities such as antioxidant and antibacterial activities. Since the wild grape (*Ampelocissus martini* Planch.) is a local herb, it may be exploited in preparation of crude drugs for human health care. It can also be considered for using as natural antioxidant and antibacterial drugs with more potent efficiency in biomedical or pharmaceutical applications.

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