ADDIS ABABA UNIVERSITY
ADDIS ABABA INSTITUTE OF TECHNOLOGY
SCHOOL OF CHEMICAL AND BIOENGINEERING

M.Sc. Program in Process Engineering

EXTRACTION AND CHARACTERIZATION OF ANTIOXIDANT FROM ORANGE PEELS

A Thesis Submitted to Addis Ababa Institute of Technology, School of Chemical and Bio Engineering in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Process Engineering.

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ADDIS ABABA, ETHIOPIA

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This is to certify that the thesis is prepared by Assefa Alene, entitled: **Extraction and characterization of antioxidant from orange peels** and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Process Engineering obeys with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ACRONYMS

AA ……Antioxidant activity
AGEs…… Advanced glycation end products
ANOVA……Analysis of variance
ATP……...Adenosine triphosphate
BHA……..Butylated hydroxyl anisole
BHT……...Butylated hydroxyl toluene
CAT…….. Catechin
CNS……...Central nervous system
CV……Coefficient of variation
DPPH…….2, 2-diphenyl-1-picrylhydrazyl
DSC……...Differential scanning calorimeter
EDTA…… Ethylenediaminetetraacetic acid
FCCCD……Face centered central composite design
FDA……..Food and drug administration
GAE…….....Gallic acid equivalent
GRAS……...Generally recognized as safe
HDPE…….....High- density polyethylene
HN……Hydroxyl nonenal
IA……Inhibition activity
LDL……..Low-density lipoprotein
LDPE……....Low- density polyethylene
MOA…….Malondialdehyde
nm…….Nanometer
OS……. …....oxidative stress
PRESS……Predicted residual sum of squares
RNS……Reactive nitrogen species
ROS……Reactive oxygen species
RSA……Radical scavenging activity
RSM……Response surface methodology
Std.Dev…….....Standard deviation

SD……Standard deviation

TBHQ……..Tertiary butylhydroqunine

TFC……..Total flavonoid content

TPC……..Total phenol content

VIF……..Variance inflation factor

WOF…….Warmed- over flavor

ABSTRACT

Antioxidants are substances which are able to prevent or inhibit oxidation processes in human body as well as in food products. Natural antioxidants have gained considerable interest in recent years for their role in preventing the auto oxidation of fats, oils and fat containing food products by replacing synthetic antioxidants. In this study, orange peel was selected as a source of natural antioxidant. The aim of this study was to investigate the antioxidant yield and antioxidant activity of two different varieties of orange peels namely Washington and Valencia using maceration extraction method with ethanol-water mixture binary solvent. Extraction yield was checked at different levels of extraction conditions. The extraction yield obtained ranged from 18-29% for Washington and 14.4-25.9% for Valencia. From the model regression equation developed, the linear terms of temperature, concentration and time had positive effect on response yield. The quadratic terms (pure and interaction quadratic terms) had negative effect on extraction yield except AB interaction for Valencia extract. Concentration had a more weighty linear effect on yield as compared to extraction temperature and time. Response yield was positively affected by linear terms and negatively affected by quadratic terms for Washington extract. From the linear effects, concentration had the highest positive effect on yield; and from quadratic effects, C^2 had the highest negative effect. Antioxidant potential was examined by measuring total phenolic content (TPC), total flavonoid content (TFC), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA) using spectrophotometer. The total phenol content ranged from 29.24 to 135.22 mg GAE/g extract for Washington and 61.94 to 182.58 mg GAE/g extract for Valencia. The total flavonoid content of Washington extract was 22.120 mg CAT/g extract and for Valencia, it was 25.456 mg CAT/g extract. DDPH radical inhibition activity ranged from 47.07 to 92.80 % for Washington and 55.91 to 93.40% for Valencia. Hence, Valencia extract has more potent in free radical inhibiting activity. Antioxidant potential of the extract was also examined by IC_{50} (concentration of extract or sample at which 50% of free radicals were inhibited). It was determined using linear regression. The IC_{50} value for Washington and Valencia, it was 1.1269mg/ml and 0.6731mg/ml respectively. The lower IC_{50} value the more potent antioxidant activity. Hence, Valencia extract has more potential in antioxidant activity than Washington extract.

Keywords: *Citrus sinensis, Orange peel, Total phenol content, Total flavonoid content, Antioxidant activity, DPPH free radical scavenging activity.*

CHAPTER ONE

INTRODUCTION

1.1 Background

Antioxidants simply means against oxidation. They are the substances able to prevent or inhibit oxidation processes in human body as well as in food products. They are added to food products like oil, bread, cookies, biscuits and dairy products to enhance their shelf life by preventing lipid peroxidation and protecting from oxidative damage. Physiological effects of antioxidants have been harnessed for management of public health. Oxidation and reduction for antioxidants can be claimed that the mechanism of therapeutic and pharmacological actions of phytochemicals is in accord with at least one of the oxido- reduction definition of antioxidants. Hence all the pharmacological exhibition of phytochemicals expresses one form of antioxidants or other. The anti-oxidative properties have been employed in preservation of lipid food systems thereby circumventing undesirable changes such as objectionable odor and flavor, rancidity and bleaching of fatty food colors, consequently prolong the shelf-life of the food (Giese, 1996).
Exposure to oxygen and sunlight are the two main factors in the oxidation of food, so food is preserved by keeping in the dark and sealing it in containers or even coating it in wax, as with cucumbers. These antioxidants are especially important class of preservatives because like bacterial or fungal spoilage, oxidation reactions also occur relatively rapidly in frozen or refrigerated food causing their spoilage (Duda-Chodak, 2007).

Natural preservatives, including constituents that are now recognized as antioxidants, have been used in foods since prehistoric times. Smoking meats and fish and treating foods with spices have long been preserving foods, albeit, without an understanding of the underlying preservation mechanisms that are now known to include the antioxidant function. At the beginning of the last century food stabilization was limited to these long- practiced techniques. With the rise of an increasingly capable chemical industry and early investigations into the mechanism of oxidation in foods, the development of antioxidants as intentional food additives began in earnest in the 1920s and became a full-fledged industry in the 1940s and 1950s. Gum guaiac was approved as an antioxidant additive for the stabilization of lard in the 1930s (Decker *et al*., 2010) and lecithin and tocopherols followed in the early 1940s (Decker *et al*., 2010). Great strides were made in the

middle of the 20th century with the rise of the synthetic plastics and synthetic chemicals industries.

While many of the antioxidants used in the plastics industry are toxic or have other properties that render them unsuitable for use in foods, a few of the compounds developed originally for polymers have become staples in the food industry. The development and use of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) was seen as just another aspect of the progress of technology. The middle to latter part of the 20th century, however, saw the rise of a more skeptical attitude toward the use of synthetic chemicals, in all walks of life. This shift spurred efforts to better understand and utilize antioxidant substances found in nature. While significant progress to this end occurred mainly in the 1970s and 1980s, early work on the anti-oxidative effects of spices and endogenous antioxidants in oils can be traced back to the 1920s and 1930s (Decker *et al*., 2010).

Although, oxidative damage to foods can be prevented or delayed using improvements in food processing and preparation, refrigeration and packaging but they are much more expensive in comparison to the cost of adding antioxidants in terms of origin, antioxidants can be synthetic or natural; though synthetic antioxidants are effective in preventing lipid peroxidation but their activity is surrounded by league of limitations that are centrally linked to health risk and poor process carry-through. However, exploitation of natural antioxidants especially from plant sources has greatly increased in recent years. The development on application from natural resources is favored by a number of factors notably: (a) safety, since they are part of food man has been eating for thousand years. (b) effectiveness, since they survive processing operations(c) their use is not guided by regulatory rules (d) their source is renewable (Daramola *et al*., 2009).

The natural antioxidants are a stable part of nutrition as they occur in almost all edible plant products. Polyphenols are the most numerous group of antioxidant components, and they are present in fruits and vegetables, their products, leguminous plants, grains, teas, herbs, spices and wines (Horubała, 1999, Borowska, 2003). Consumption of food containing a lot of polyunsaturated fatty acids raised the significance and usage of substances that protect them against oxidation (Duda-Chodak, 2007). The growing demand for natural antioxidants observed in food and cosmetic industries forces the search for new sources of these compounds. Numerous scientific investigations point at consecutive rich sources of antioxidants, especially among

fruits, but only few of them involve waste parts of fruits, i.e. seeds and peels. The natural antioxidant supplementation is a generally accepted method of prolonging the stability and storage life of food products, in particular the ones including fat. However, the artificial compounds with antioxidant properties, like butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT), have a limited allowance for food due to their potential cancerogenicity (Jayaprakasha *et al*., 2003).

Synthetic antioxidants such as BHA, BHT, PG and TBHQ have potential health hazards. Recently, the interest of consumers in the use of natural antioxidants has increased due to the belief that they will offer more health benefits than synthetic antioxidants. This scenario has led to the search for natural compounds with anti-oxidative properties and indeed, different natural products have been investigated as a source of antioxidants. Special attention has been paid to wastes generated in the food industry, such as peel, wastewaters and seeds. In particular, phenolic compounds isolated from plants are recognized as the most promising group of molecules that help to prevent oxidation and maintain product.

1.2 Statement of Problem

Fats and oils are the chief components of many foods, and their presence is often needed for the development of flavor, texture and color attributes. The quality of lipid containing products is determined based on the oxidative stability of fats and oils. Fats and oils containing unsaturated fatty acids are highly susceptible to oxidation. The oxidative deterioration of fats and oils reduce the shelf life of lipid- containing foods, due to decrease food safety and nutritional quality. Hence, the problems associated with it can be countered by the development of food preservation processes need to keep the oxidative stability of fats and oils and extend the shelf-life of foods, by the application of antioxidants.

Orange Peels have been recognized as an ecological burden for the society. These peels are source of natural antioxidants. A cheap, efficient and environmentally sound utilization of these wastes is needed. Polyphenols are often present in higher concentration in the outer non edible part of the fruits like peel compared to inner edible part (Hegde *et al*., 2015). Use of orange peels as a source of antioxidants may have considerable economic benefit to food processors.

Synthetic antioxidants have been used to retard or minimize oxidative deterioration of foods. It has been discovered that some of synthetic antioxidants especially BHT and BHA are carcinogenic thereby they are being discouraged in international market as food additives. This leads to provoking interest in seeking for safer means of natural antioxidants of plant origin that will serve the same purpose of preventing oil rancidity. The replacement of synthetic antioxidants by natural ones may have benefits due to health implications and functionality such as safety, potential nutritional benefits, therapeutic effects, solubility in both oil and water, of interest for emulsions, in food systems.

The increasing consciousness of consumers with regard to natural foods and food additive safety has obliged the food industries created a need for identifying alternative natural and probably safer sources of food antioxidants and include natural antioxidants in various products to delay oxidative degradation of lipids, improve quality and nutritional value of foods, and replace synthetic antioxidants. Therefore, investigation of natural antioxidants has been a major research interest in utilization of orange peels and other plant materials for possible antioxidant potential.

1.3 Objectives

1.3.1 General Objective

The general objective of this thesis is to extract and characterize antioxidant from orange peels.

1.3.2 Specific Objectives

- To extract and concentrate the natural antioxidant extracts from orange peel varieties using ethanol-water binary mixture as a solvent.
- To study the effects of operating conditions on the extraction yield and identify the optimal levels which give the maximum yield.
- To evaluate the potent antioxidant activity of the extract by determining the total phenolic content, total flavonoid content and percentage free radical scavenging activity of the extract.
- To study the comparison between the two varieties in terms of quantitative extraction yields and potent antioxidant activities (total phenol contents, total flavonoid contents and the free radical scavenging activities) of the extracts.

1.4 Significance of Study

- Utilization of orange peels as source antioxidants will reduce the ecological burden of society (pollution) and have considerable economic benefit to food processors.
- Antioxidants are used as food additives to guard against food deterioration. These are added to food products like oil, bread, cookies, biscuits and dairy products to enhance their shelf life by preventing rancidity, lipid peroxidation and protecting from oxidative damage. The antioxidants extracted from these peels can replace the synthetic antioxidants, used in food products as preservatives, which may harmful and hazardous to human.
- Antioxidants are emerging as prophylactic and therapeutic agents. These are the agents, which scavenge free radicals otherwise reactive oxygen species and prevent the damage caused by them. Free radicals have been associated with pathogenesis of various disorders like cancer, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders and are implicated in aging (Kumar, 2006).

CHAPTER TWO

REVIEW OF LITERATURE

2.1 History of Orange Plant

Citrus *sinensis* sweet orange is a fruit with a small evergreen tree (8–15ft), or shrub in the Rutaceae (citrus family), native to tropical and subtropical areas. It originated from southern China, where it has been cultivated for millennia. Orange is now grown commercially worldwide in tropical, semi-tropical, and some warm temperate regions, and has become the most widely planted fruit tree in the world (Omoba, 2015).

2.2 World Orange production

Orange is the world's most popular fruit. It constitutes about 60% of the total citrus world production (Ibrahium, 2012). Annually more than 55 million tons of oranges are grown globally out of which 80 percentage of the oranges produced are processed in industry for juice production (Hegde *et al*., 2015). Orange fruit extracts are used as functional ingredients in several industrial products and these industrial activities generate humongous quantities of wastes(peels, seeds and pulps) rich in polyphenol that are often disposed into the environment apart from using only as animal feed. Main phenolic constituents of Citrus fruits are flavonone and flavone glycosides, hydroxycinnamates, coumarines, psoralens and polymethoxyalted flavones (Hegde *et al*., 2015).

Table 2.1 World Orange Production, 2007-08 seasons

Country	**Oranges**
	1000 metric tones
Argentina	800
Australia	350
Brazil	15912
China, Peoples of republic of	5450
EU-27	5999
Israel	162
Mexico	4000

Morocco	659
South Africa, Republic of	1280
Turkey	1472
United States	9237
Others	8
Total	45329

Source: Foreign Agricultural Service, United States Department of Agriculture

Table 2.2 Top five orange producing countries, 2011 season

Country	**Orange production**	**% of world production**	**% change from 2010**
	2011		
	metric tones		
Brazil	19,811,064	28.52%	+7.06%
United states	8,078,480	11.63%	-8.03%
China	6,013,829	8.65%	+7.32%
India	4,571,000	6.58%	-23.3%
Mexico	4,079,678	5.87%	+0.69%

Source: FAOSTAT data, 2014 (last accessed by Top 5 of Anything: January 2014)

2.3 Orange Production in Ethiopia

Various kinds of fruits grow in different regions of Ethiopia yielding varying quantities of fruits within the private peasant holdings in the traditional way. There are also a few fruit farms that are run by enterprises in the modern way. The volume of fruit production obtained from the peasant farms is small signaling the absence of development in fruit farming. As the data obtained, less than half a million hectares of land is under permanent crops in Ethiopia. Fruit crops constituted more than 8% of the permanent crop area yielding more than 2 million quintals of fruits. Bananas, mangoes, papayas and orange shared 59.65%, 10.85%, 8.11% and 5.08% of the land under fruit crop and 63.11%, 8.07%, 14.55% and 7.46% of the fruit production, in that order (CSAE, 2003). The statistics show that SNNPR and Oromia Regions shared about 17 thousand (46.07%) and 15 thousand (40.80%) hectares of the country's total area under fruit crops, respectively. More than one million quintals (50.00%) and 826 thousand quintals (40.00%) of the country's total fruit production went for SNNPR and Oromia Regions, in that

order. It is also indicated that more than 700 thousand (57%) and 500 thousand (38%) quintals of the country's total bananas was produced in SNNPR and Oromia regions, respectively and orange 154,624.72 quintals from both. The census data reveals that about half of the permanent crops produced in Ethiopia were used for sale and the remaining for household consumption and other purposes. Thus 51% of the crop was sold and 44% was consumed at home. The utilization by crop type within the permanent crop group ranges between 31% and 72% for household consumption and between 24% and 63% for sale. Permanent crop utilization by region is mainly similar to the country's pattern (CSAE, 2003).

Table 3.3 Orange Production in Ethiopia from 2000 – 2006 (in Meher Season)

Year	Quantity of Orange Produced(1000 tones)
2000	14
2001	15
2002	15
2003	16
2004	16
2005	17
2006	51

Source: Wiersinga, 2006

2.4 Processing Orange

When orange is processed, eaten fresh and used for juice; the fruit is made up of peel, pulp, seeds, and juice. In contrast with other types of fruits, around 34% of the fruit is used for juice production, yielding about 44% of peels as byproducts (Omoba, 2015).

Orange fruits have peculiar fragrance partly due to flavonoids and limonoids present in the peel and these fruits are good sources of vitamin C and flavonoids. The antioxidant/radical scavenging capacity and reducing power ability of different extracts of orange peel were investigated and results showed that ethanolic extract showed the highest values for yield i.e. total phenolic content, total flavonoid content and chelating and antioxidant activities. It was also observed that solvent played a vital role in the extraction of the plant constituents, specifically; methanol and ethanol were highly polar among the solvents used. Orange peels are a rich source of Vitamin C which is considered as a most important water- soluble antioxidant. The major role

of Vitamin C is the prevention of scurvy; this causes the disease which leads to the formation of spots on the skin, spongy gums and bleeding from the mucous membranes. Vitamin C is unstable compounds which are degraded by both aerobic and anaerobic pathways. The loss of Vitamin C might be a critical factor for the shelf life of some products as citrus juice concentrates.

2.4.1 Orange Peels

The orange peels are generally wasted when orange fruits are mainly used by juice processing industries. Since the juice yield of citrus is less half of the fruit weight. A very large amount of oranges byproduct wastes, such as peels which are formed every year. From waste materials, there is always an increased attention in bringing useful products and citrus wastes are no exceptions. Suitable methods have to be adopted to utilize orange peel and pulp for the conversion into value-added products. Environmental pollution can also be reduced. The citrus peels are rich in nutrients and contain many phytochemicals; they also can be efficiently used as drugs or as food supplements. There is an increase in the number of antibiotic resistance pathogens; there is always a search of an alternative drug that is regarded as safe. The orange fruit is highly nutritious and rich in minerals, proteins, carbohydrates, and fat (Arora, 2013).

Orange Peels, pulps and seeds cumulative represent between 50 to 65% of total weight of the fruits and remain as the primary byproduct. If not processed further, it becomes waste produce odor, soil pollution, harborage for insects and can give rise to many studies have reported antioxidant and serious environmental pollution (Ibrahium, 2012).

The peel and seeds results in a considerable amount of by-products which might be a source of environmental pollution since they are prone to microbial spoilage. Citrus by-products could be of use as functional ingredients in the production of functional foods, since they are good sources of dietary fiber and bioactive compounds. Peel is a good source of phenolic compounds which may potentially be used in food formulations or when extracted can be used as natural antioxidants to prevent oxidation of selected food. The citrus peel and seeds are very rich in phenolic compounds, such as phenolic acids and flavonoids; the peel is richer in flavonoids than seeds. Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants, they are oxidized by radicals, resulting in a more stable, less-reactive radical. They can be divided into a variety of classes such as flavones (flavone, apigenin, and luteolin),

flavonols (quercetin, kaempferol, myricetin, and fisetin), flavanones (flavanone, hesperetin, and naringenin), and others. Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activity. Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen-derived radical called peroxynitrite. Epicatechin and rutin are also powerful radical scavengers. The scavenging ability of rutin may be due to its inhibitory activity on the enzyme xanthine oxidase. Rutin is also known for its anti-inflammatory and vasoactive properties, as well as for its capability to diminish capillary permeability and to reduce the risk of arteriosclerosis, whereby reducing coronary heart disease, possibly through the diminishing of platelet aggregation. Rutin was suggested as one of the most potent natural inhibitors of advanced glycation end products (AGEs) accumulation in human body, suggesting that its anti-glycation activity may mainly be due to its radical scavenging activity. Specific flavonoids are known to chelate iron thereby removing a causal factor for the development of free radicals. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties (Omoba, 2015).

Table2.4 Extraction yield of some selected citrus fruits

Fruit peels	Extraction yield (%)
Pomegranate	27.5
Orange	23.9
Lemon	25.8

Source: Singh, 2014

Table 2.5 Total polyphenol content of fruit peels

Types of peel	Total polyphenols(mg/g d.w.) × 100
Banana	415.67±10
Orange	849.3 ± 21.8
Lemon	966.2 ± 16.5
Water melon	335.3 ± 20.8
Apple	1 790.5 ±27.5
Melon	466.5 ±8.8
White grapes	849.3± 21.8

Source: Joshi, 2012

2.5 Food oxidation

Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Food oxidation is a free radical chain reaction that causes major quality losses in the food industry. Lipid oxidation during preparation, processing and storage can lead to the development of rancidity and deterioration of oil and lipid containing food products. The addition of synthetic antioxidants to these foods is one of the most efficient ways to reduce rancidity, minimize the production of toxic oxidation molecules and to extend the food's shelf life. The oxidative reactions proceeding in food are the main cause of its deterioration. They are responsible for the nutritional value losses, as well as aroma, taste and texture degradation. Moreover, the products of biological compounds oxidation, by interaction with important for organism function molecules, can upset cell homeostasis, and act cytotoxically resulting in different diseases like tumors, heart failure, cataract, brain dysfunction (Maniak and Targonski, 1996).

One of the major causes of quality in foods is lipid peroxidation which leads to quality deterioration, rancidity, discoloration and loss of nutrients such as vitamins (Nawar, 1996; Hidalgo *et al*., 1998). The deteriorative process of oxidation occurs naturally in all foods, not just those with high fat content (Giese, 1996). This makes the addition of antioxidants useful in most fat containing foods.

Han- Seung *et al*., (2004) listed in their report several factors such as light, relative humidity, temperature, availability of oxygen and some metals that affect the production lipid oxidation products. For example, lipid oxidation is likely the most common mechanism of oxygen uptake in fried foods such as potato chips. Also lipid oxidation in metal and meat products is one the major causes of spoilage and deterioration of organoleptic properties leading to off-flavor development, colour degradation and nutritive loss (Genot *et al*., 1997). This undesirable occurrence can be inhibited by the use of antioxidants.

2.5.1 Lipid Oxidation in Foods

Lipids are the primary components of many foods, and their presence is often needed for the development of flavor, texture and color attributes. However, lipids are highly unstable and are readily attacked by oxygen, leading to a chain of chemical reactions that generate undesirable flavor and odor compounds. These oxidative reactions can be accelerated by metals (e.g., iron,

copper), light, temperature, enzymes and bacteria. Lipids can be characterized as saturated or unsaturated fatty acids with the term 'saturated' referring to the fact that all carbon atoms are bound to as much hydrogen as possible. Unsaturated fatty acids have one (mono-unsaturated) or more (poly-unsaturated) double bonds between carbon atoms. Foods containing high levels of unsaturated fats such as meat and meat products, dairy, fish and oils are especially susceptible to oxidative reactions as oxygen is able to attack those double bonds, leading to the formation of free-radicals and other oxidation products. Off-flavor resulting from the oxidation of lipids is the most common factor limiting the shelf-life of lipids and lipid containing products. Lipid oxidation, therefore, is a major economic concern as it renders products unacceptable to consumers. The food industry suffers significant losses as a result of decreased product shelf-life caused by "warmed-over flavor" (WOF) development, rancidity and diminished nutritional quality, all of which stem from lipid oxidation (Ballard, 2008).

Oxidation of lipids not only affects flavor and odor development, but also impacts food texture, color and nutritive value. Secondary products of lipid oxidation including malondialdehyde (MDA) and 4-hydroxynonenal (4-HN) have been known to interact with proteins and amino acids. These types of interactions can lead to undesirable color and textural changes. For example, foods can experience a darkening in color as a result of a condensation reaction between oxidation products and proteins. Furthermore, textural changes that occur in oxidized products may be attributed to the oxidative induction of protein crosslinks. Lipid oxidation products are also capable of destroying essential fatty acids and lipid-soluble vitamins. In milk, several key nutrients including riboflavin (Vitamin B2) and ascorbic acid (Vitamin C) are destroyed due to light-induced lipid oxidation (photo-oxidation) (Ballard, 2008).

2.5.2 Oxidation Mechanisms of Fats and Oils

Different chemical mechanisms are responsible for the oxidation of fats and oils during processing, storage, and cooking. Two types of oxygen, atmospheric triplet oxygen and singlet oxygen can react with fats and oils. Triplet oxygen, having a radical character, reacts with radicals and causes autoxidation. The non-radical electrophilic singlet oxygen does not require radicals to react with; it directly reacts with the double bonds of unsaturated fats and oils with high electron densities, which is called type II photosensitized oxidation.

2.5.2.1 Autoxidation

Fats and oils should be in radical forms to react with triplet oxygen in autoxidation. Lipids are normally in non-radical singlet state and heat, metals, or light accelerates their radical formation (Choe and Min, 2005). Hydrogen attached to the carbon between two double bonds is easily removed due to low bond dissociation energy (Choe and Min, 2009). The lipid radical reacts with triplet oxygen very quickly at normal oxygen pressure and forms lipid peroxy radical. The lipid peroxy radical abstracts hydrogen from other lipid molecules to form lipid hydro-peroxide and another lipid radical. The radicals automatically catalyze the reaction and the autoxidation is called free radical chain reaction. When radicals react with each other, non-radical species are produced to stop the reaction.

2.5.2.2 Photosensitized oxidation

Light accelerates lipid oxidation, especially in the presence of photosensitizers such as chlorophylls. Chlorophylls in singlet state become excited upon absorption of light energy in pico second (Choe and Min, 2006). Excited singlet state chlorophylls become excited triplet state via intersystem crossing. Excited triplet state chlorophylls react with triplet oxygen and produce singlet oxygen by energy transfer, returning to their ground singlet state. Singlet oxygen is able to diffuse over larger distances, about 270 nm (Choe and Min, 2009), to react with electron-rich compounds. Since singlet oxygen is electrophilic due to a completely vacant 2 pπ orbital, it directly reacts with high-electron-density double bonds via 6-membered ring without lipid radical formation. The resulting hydroperoxides by singlet oxygen are both conjugated and nonconjugated. Production of nonconjugated hydroperoxides does not occur in autoxidation. The reaction rate of lipid with singlet oxygen is much higher than that with triplet oxygen (Frankel, 1985).

2.5.2.3 Thermal oxidation

Heating of oil produces various chemical changes including oxidation. The chemical mechanism of thermal oxidation is basically the same as the autoxidation mechanism. The rate of thermal oxidation is faster than the autoxidation (Choe and Min, 2007), and the unstable primary oxidation products, hydroperoxides, are decomposed rapidly into secondary oxidation products such as aldehydes and ketones.

Thermal oxidation of oil produces many volatiles and nonvolatiles (Choe and Min, 2009). Volatiles such as aldehydes, ketones, short-chain hydrocarbons, lactones, alcohols, and esters are produced from decomposition of hydroperoxides by the same mechanisms as the autoxidation. Many nonvolatile polar compounds and triacylglycerol dimers and polymers are produced in thermally oxidized oil by radical reactions.

2.5.2.4 Enzymatic oxidation

Lipid oxidation is catalyzed by lipoxygenase in a nonradical mechanism (Niki, 2004). Lipoxygenase is an iron-bound enzyme with Fe in its active center. Lipoxygenase oxidizes unsaturated fatty acids having a 1-cis, 4-cis-pentadiene system resulting in oil deterioration, and oils containing linoleic, linolenic, and arachidonic acids are favored substrates.

Lipoxygenase with iron in the ferric state (LOX-Fe^{3+}) forms a stereospecific complex with the unsaturated fatty acid having a 1,4-pentadienyl system (RH), and it abstracts hydrogens from interrupted methylenes in the fatty acids. It binds to pentadienyl radical which is rearranged into a conjugated diene system, followed by the reaction with oxygen to produce lipid peroxy radicals (ROO^-). The iron in the enzyme is reduced to the ferrous state (LOX-Fe^{2+}). Lipid peroxy radicals are reduced to ROO^- by lipoxygenase with iron in a ferric state again, and the attachment of a proton, which is produced by the oxidation of hydrogen abstracted from fats and oils by lipoxygenase, results in release of hydroperoxides (Belitz and Grosch, 1999).

2.5.3 Formation of free radicals

Normally, bonds would not split in a way that leaves a molecule with odd, unpaired electrons. However, when weak bond split, free radicals are formed. Free-radicals are very unstable and react quickly with other compound trying to gain stability.

Generally, free radicals attack the nearest stable molecules abstracting its electron to attain stability. When the attacked molecule loses its electron, it becomes a free-radical itself, these formations of free-radicals continue on and on and finally result in the disruption of the substance especially in fatty foods. Environmental factors such as pollution, radiation, cigarette smoking and herbicides can also spawn free-radicals in the body but, if antioxidants are not available to check the free-radical production it becomes excessive and cause damage to body

and any substance in which oxidation occurs. Of particular interest is the free radical damage in the body system, fatty foods and other substance like polymer and antioxidants mechanism of action in inhibiting these damages (Hamid *et al*., 2010).

2.5.4 Free radicals damage and diseases

Free radicals contribute too many different diseases. Chemically, a substance is oxidized when electrons are removed and reduced when electrons are added. All chemical reactions involve the transfer of electrons. The body generates energy by gradually oxidizing its food in a controlled manner and storing it in the form of chemical potential energy called ATP (Adenosine triphosphate).

Free radicals are generated largely during the production of ATP in the mitochondria. During this process, radicals coming out from the mitochondria from reactive oxygen species such as superoxide anion (O_2^-) and hydroxyl radicals ($HO^{\cdot}$) and other reactive oxygen species such as singlet oxygen (O_2^1), destroy the body system especially the site where the free radicals is been generated. The ultraviolet light that penetrate the skin and the air pollutant that is high in smog which we inhale generates free radicals too. Food, like lipid in the presence of (Fe^{3+} , Fe^{2+}) lead to the production of hydrogen peroxide from which further hydroxyl radicals are generated in a reaction that appear to depend on the presence of iron ions (Hamid *et al*., 2010).

- The acceleration of hydroperoxide decomposition to form peroxyl radicals and alkoxyl radical

  $$Fe^{3+} + ROOH \rightarrow Fe^{2+} + ROO^{\cdot} + H^{+}$$
  $$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^{\cdot} + OH^{-}$$

- Formations of alkyl free radicals by direct reaction with fats and oils

  $$Fe^{3+} + RH \rightarrow Fe^{2+} + R^{\cdot} + H^{+}$$

- Activation of molecular oxygen for singlet oxygen formation

  $$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O^{2-}$$

2.6 Antioxidants

Antioxidants are substances capable of slowing or preventing the oxidation of other molecules and may protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free

radicals might otherwise cause. Free radical damage may lead to cancer. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols. Some of antioxidants include beta-carotene, lycopene, vitamins C, E, A, and other substances (Sies, 1997).

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells. As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline. For many years chemists have known that free radicals cause oxidation which can be controlled or prevented by a range of antioxidants substances (Bjelakovic *et al*., 2007). It is vital that lubrication oils should remain stable and liquid should not dry up like paints. For this reason, such oil usually has small quantities of antioxidants such as phenol or amine derivatives, added to them. Although plastics are often formed by free radical action, they can also be broken down by the same process, so they too, require protection by antioxidants like phenols or naphthol. Low density polythene is also of protected by carbon black which absorbs the ultraviolet light which causes radical production (Sies, 1997).

2.6.1 Biological Activities of Antioxidant

Antioxidants are the chemical substances that reduce or prevent oxidation and have the ability to counteract the damaging effects of free radicals in tissues, and thus are believed to protect against cancer, heart disease, cardiovascular diseases and several other diseases. They scavenge radicals by inhibiting initiation and breaking of chain reaction, suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide, and quenching superoxide and singlet oxygen (Moure, 2000).

The protection that fruits and vegetables provide against several diseases has been attributed to the various antioxidants. In living systems, dietary antioxidants (α-tocopherol, α-carotene, ascorbic acid) protect against damage. Several studies have shown that phenolic compounds reduce in vitro oxidation of low density lipo- protein; particularly those phenolics with multiple hydroxyl groups which are generally the most efficient for preventing lipid and low density lipoproteins (LDL) oxidation(Moure, 2000).

2.6.2 Types of Antioxidants

2.6.2.1 Natural Antioxidant

Natural antioxidants are compounds from plant or animal sources that retard oxidative rancidity of oils, fats, and fat-soluble components, thus protecting them while delaying the development of unpleasant flavors and odors resulting from oxidation.

Antioxidants are present naturally in most raw food sources. Processing can remove or degrade some of these antioxidants. Therefore, a supplementation with suitable antioxidant compounds is needed to maintain acceptable quality of the products. Especially oils, fats, and products with a high fat content are susceptible to oxidation and require the addition of antioxidants. The most widely used antioxidants are synthetic ones, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG). Doubts about the safety of synthetic antioxidants arose first in the 1960s and led to an increased interest and a broad research on natural antioxidants. Natural antioxidants are primarily phenolic compounds that may occur in all parts of a plant. They are multifunctional and can act as free radical terminators, metal chelators, and singlet oxygen quenchers. The common plant phenolic antioxidants are tocopherols, flavonoids, and related compounds like coumarins, cinnamic acid derivatives and chalcones, phenolic diterpenes, and phenolic acids. Another widespread antioxidant in nature is ascorbic acid, but today its extraction from natural sources is not significant as all ascorbic acid used for food is chemically synthesized.

To be considered as an antioxidant for practical use, a compound extracted from a natural source must meet several criteria, including (a) absence of any toxic or physiological effect, (b) no impartation of any strong odor, flavor, or color to the product, and (c) considerable antioxidant

activity at small concentrations in the product. In fact, antioxidants present in or added to foods are functional at very small concentrations, usually up to 0.02%.

Usually, in literature surveys on antioxidants, compounds that have no antioxidant activity themselves but enhance the activity of antioxidants, called synergists, are included.

Fruits are rich with antioxidants that help in lowering incidence of degenerative diseases such as cancer, arthritis, arteriosclerosis, heart disease, inflammation, brain dysfunction and acceleration of the ageing process (Feskanich *et al*., 2000). It has been reported that plant polysaccharides or their derivatives have strong antioxidant activities and can be explored as novel potential antioxidants (Jiang *et al*., 2005).

2.6.2.1.1 Phenolic Compounds

Plants are a major source of phenolic compounds, which are synthesized as secondary metabolites during normal development in response to stress conditions, such as wounding and UV radiation among others (Stahl and Sies, 2003; Close *et al*., 2005). Plants may contain simple phenolics, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignins and lignans (Naczk and Shahidi, 2006). Distribution of phenolics in plants at the tissue, cellular and subcellular levels is not uniform. Insoluble phenolics are found in cell walls, while soluble phenolics are present within the plant cell vacuoles (Randhir and Shetty, 2005). Cell wall phenolics may be linked to various cell components such as sugars (Chaoui and El Ferjani, 2005) Phenolic compounds are considered to be the most important antioxidants of plant materials. They constitute one of the major groups of compounds acting as a primary antioxidant or free radical terminators. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. In addition, they possess ideal structural properties for free radical scavenging properties (Kanatt *et al*., 2007). The low correlations confirm that phenolic compounds are not the only contributor to the antioxidant activities. The type and quantity of phenolic compounds and the presence of non-phenolic antioxidants may also contribute to the antioxidant activity of the extracts. Non-phenolic antioxidants such as vitamin C, vitamin E and β- carotene might also be accountable in enhancing the antioxidant activity (Dzulkifli, 2013). Determination of total phenolics is one of important parameters to estimate the amount of antioxidants. The phenolic compounds found in fruits and vegetables have received substantial interest because of their potential antioxidant benefits. Phenolic compounds undergo

a complex redox reaction with the phosphortungstic and phosphomolybdic acids present in the Folin– Ciocalteu reagent. It was reported that intense heat from boiling water or extracting solvent is able to release cell wall phenolics or bound phenolics due to the breakdown of cellular constituents causing more polyphenols to be extracted (Toor and Savage, 2006).

2.6.2.1.2 Flavonoid Compounds

The broad spectrum of biological activities within the group and the multiplicity of actions displayed by certain individual members make the flavonoids group, one of the most intriguing classes of biologically active compounds and thus these are often termed 'bioflavonoids' (Singla et al., 2001).

Flavonoids, the most potent anti-oxidative compounds of plant phenolics occur in vegetable, fruits, berries, tea leaves and herbs (Skrede and Wrolstad, 2002). Chemically, flavonoids and isoflavonoids are one-electron donors. They are derivatives of conjugated ring structures and hydroxyl groups that have the potential function as antioxidants in cell culture in vitro, or in cell free systems (Sun *et al*., 2011).

Flavonoids are very important constituents of plants because of the scavenging ability conferred by their hydroxyl groups. The flavonoids may contribute directly to anti-oxidative action. Flavonoid compounds from plants are known to be good natural antioxidants. Flavonoid compounds at certain concentrations markedly slowed down the rate of conjugated diene formation (Sun *et al*., 2011). Flavonoids occur in particularly all parts of plants including fruits, vegetables, nuts, seeds, leaves flowers and bark (Middleton, 1984). Phytochemicals are very important because some symptoms originally thought to be due to vitamin C deficiency such as bruising due to capillary fragility were found in early studies to be relieved by crude vitamin C extract but not by purified vitamin C. Bioflavonoids were found to be essential components in correcting this bruising tendency and improving the permeability and integrity of the capillary lining. These bioflavonoids include hesperidin, citrin, rutin, flavones, flavonoids, catechin and quercetin (Singla *et al*., 2001).

2.6.2.2 Synthetic Antioxidants

Synthetic antioxidants have been widely used to extend the shelf-life of various food materials. The current preference for synthetic antioxidants can be attributed to their proven effectiveness in a variety of food systems and their relative low cost when compared to natural antioxidants. The most commonly used synthetic antioxidants in the food industry include BHT, BHA and TBHQ. The use of PG has been limited by its tendency to cause undesirable color changes. BHT and BHA are hydrophobic phenolic antioxidants that inhibit free-radical initiated chain reactions. Protection against lipid oxidation may occur as a result of the formation of a BHT radical, which is thought to have a lower reduction potential than that of lipid peroxyl radicals. BHA is commonly used in combination with BHT or PG which creates a synergistic effect. TBHQ is less volatile than BHA and BHT but is stable at higher temperatures. Due to the stability of TBHQ at elevated temperatures, it has proven to be more effective in polyunsaturated vegetable oils (Ballard, 2008).

Although synthetic antioxidants are extremely effective at slowing oxidation, there have been recent consumer concerns over potential adverse health effects associated with these compounds. Studies have reported that BHT and BHA cause a wide range of health problems including, enlarged liver, increased liver microsomal enzyme activity and conversion of some ingested materials into toxic and carcinogenic substances, especially if they are present in excessive amounts. Many processors wish to avoid adding synthetic antioxidants to foods to eliminate these health concerns and to be able to state on the label that the product is “all natural” (Ballard, 2008).

2.6.3 Source of Natural Antioxidants

In recent years, much research has been focused on identifying sources of natural antioxidants that can be used to replace their synthetic counterparts. Strong natural antioxidant compounds were found in fruits, vegetables, trees and leaves. High activity antioxidants were found in olive oil and in fruit juices (Moure, 2000).

The main sources for the extraction of natural antioxidants are of plant origin. Some animal sources, such as shrimp, have also been reported (Pasquel and Babbitt, 1991). The most common

plant sources are cereals, citrus fruits, cocoa and coffee beans, herbs and spices, oilseeds, olives, onion and garlic, tea, as well as some miscellaneous products.

2.6.3.1 Citrus Fruits

Citrus fruits and especially oranges contain antioxidant components (Tzia and Liadakis, 2003). These antioxidants are mainly concentrated in the outer part of the peel, called flavedo. Extracts of orange and grapefruit flavedo had significant activity against *d*-limonene oxidation, but extracts of flavedo from lemon and lime had very little activity. On the contrary, extracts of lemon peel offer effective protection from peroxidative damage in living systems. Antioxidant properties of extracts from orange flavedo were attributed to tocopherols. Also citrus essential oils and terpenes showed antioxidant activity in several food products (Dewdney and Meara, 1977). However, flavonoids isolated from various citrus extracts seem to be the most important antioxidant components present in citrus fruits. More than 60 individual flavonoids have been identified in citrus fruits (Horowitz and Gentili, 1977). Among them, flavanones are the most abundant, and especially hesperetin and naringenin are presented in the pulp of all citrus fruits (Tzia and Liadakis, 2003). Highly methoxylated flavones occur in much lower concentration but exhibit the greatest antioxidant activity. Two isoflavones are referred as the main antioxidant components of osage orange peel, while a flavanone, eriocitrin and its aglycon, eriodictyol, are referred as the main antioxidant components of lemon peel.

2.6.3.2 Cereals

Several cereals have been reported to possess antioxidant activity. Oat has been the most widely investigated because it has greater activity than the others and a bland flavor. Oat flour has been incorporated in dairy products, potato crisps, sausages, mayonnaise, etc., and improved their stability. Malt and barley flour or meal acted as antioxidants on fats too (Dewdney and Meara, 1977).

Extracts of groats and hulls from oat were more active than synthetic phenolic antioxidants in the protection of oils during frying and of emulsions (Duve and White, 1991). The antioxidant activity of oat hulls and groats is attributed to several phenolic compounds, mainly ferulic, *p*-coumaric, caffeic, vanillic, *p*-hydroxybenzoic acids and vanillin. The extract of defatted oat kernels also showed antioxidant activity similar to BHT and PG that was attributed to caffeic and

ferulic acid and their derivatives. Oat hulls contain more phenolic acids than oat flour; therefore, oat hulls are more efficacious for antioxidant extraction (Tzia and Liadakis, 2003).

Byproducts of other cereals could be used as raw materials for antioxidant extraction, too. Buckwheat hulls extracts showed a good antioxidant activity, which was attributed to the presence of dihydroxy phenolic components, flavonols, and flavone glucosides (Tzia and Liadakis, 2003).

2.6.3.3 Cocoa and Coffee Bean

Cocoa bean husk powder and especially water and alcohol extracts were reported as potential antioxidants (Mueller, 1954). Fractions with antioxidant activity were isolated from the extracts, but the active substances were not identified. The brown pigment was suggested as the antioxidant factor, though it was not active to all substrates. Roasted coffee powder showed antioxidant properties in oils. However, the antioxidant activity of the coffee powder was lower than that of its constituents: caffeic acid and quinic acid. Another potent antioxidant component in coffee is chlorogenic acid (Dewdney and Meara, 1977).

2.6.3.4 Herbs and Spices

Herbs and spices are the most broadly examined raw materials for the occurrence and extraction of antioxidants. Rosemary has exhibited the greatest antioxidant activity of all spices and herbs. The extracts of rosemary were more effective than BHA and BHT in the protection of fat and of products with a high fat content. Bleached, odorless, and tasteless antioxidants have been produced from rosemary and commercially have exploited since the early 1980s (Chang *et al*., 1977). The most active components of rosemary, identified in the earlier studies, were carnosol, rosemaridiphenol, and rosemariquinone. Nowadays several commercial rosemary extracts are available and their most active compounds are reported to be carnosol, carnosic acid, and rosemarinic acid (Richheimer *et al*., 1996).

Sage belongs to the same family as rosemary the Labiatae family and possesses almost equivalent antioxidant activity. Many investigations on rosemary antioxidant efficiency also include data on sage efficiency, and the same procedures for the extraction and purification of the antioxidant fraction from both spices have been suggested. The main antioxidant constituents

in sage were also found to be carnosol, carnosic acid, and rosemarinic acid, followed by other related phenolics. In addition to the main sage species, i.e., *Salvia officinalis*, several other varieties have also shown remarkable antioxidant efficiency (Protogeras *et al*., 1998).

Other plants of the Labiatae family are potent sources of natural antioxidants. Oregano is one of the most promising. Extracts of oregano retarded lipid oxidation, while the essential oil of the spice also contained antioxidant components. Thyme seems to be another promising spice: extracts of the plant showed appreciable antioxidant activity and its essential oil was effective. Dittany and marjoram extracts exhibited antioxidant activity too. Several other herbs, spices, and medicinal plants are rich in antioxidant components (Tzia and Liadakis, 2003).

2.6.3.5 Oilseeds

Oilseeds contain several antioxidant compounds that protect them against rancidity. Therefore, whole seeds or the byproducts obtained after oil extraction could be potential sources of antioxidants. Methanolic extracts of cottonseed exhibited antioxidant activity attributed mainly to flavonoids (Whittern *et al*, 1984). Flavonoids are also claimed to be the major antioxidants of soybean methanolic extracts. Most of these flavonoids remain in the waste during soybean curd processing and could be recovered. Canola meal is another potent source of antioxidants with a total phenolic content remarkably higher than both cottonseed and soybean meals. Other oilseeds studied with positive results include sunflower seed and sesame seed (Fukuda *et al*., 1985).

2.6.3.6 Olives

Olives and olive oil are rich in antioxidant compounds, especially tocopherols. In addition to tocopherols, several other phenolic compounds, i.e., hydroxytyrosol, caffeic, protocatechuic, ferulic, syringic, and vanillic acids, were identified in the extracts of virgin olive oil and were found effective in prolonging the shelf life of the refined olive oil (Satue *et al*., 1995). Rape, a major byproduct of olive oil production, was used for the extraction of antioxidants. The extracts contained various phenolic acids, catechol, and tyrosol, and inhibited oxidative deterioration of refined vegetable oils. Also, olive leaves contain phenolic antioxidant compounds, and some of them were identified (Notte and Romito, 1971).

2.6.3.7 Onion and Garlic

Onion contains appreciable amounts of the well-known flavonoid quercetin and exhibits a remarkable antioxidant activity. Onion skins are also rich in quercetin and quercetin derivatives, and their methanolic extracts were effective antioxidants. Garlic was found effective for some substrates, i.e., linoleic acid or minced pork, but ineffective in lard (Tzia and Liadakis, 2003).

2.6.3.8 Tea

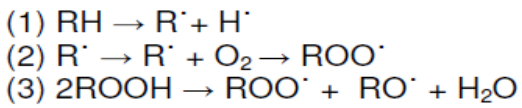
Tea leaves are a well-known source of natural antioxidants, especially catechins. Fresh or mildly processed commercial tea has been found to contain large quantities of these polyphenols, up to 30% of its dry mass (Price and Spitzer, 1994). Green tea extracts were 21–24% more effective at radical quenching than black tea extracts in both water and lipid-soluble media (Gardner *et al*., 1998). Aqueous extracts of green tea retarded the oxidation of oils and fats more than commercial rosemary extract, and were also effective in emulsions and fish meat. Commercial tea extracts are available with various catechin contents (Tzia and Liadakis, 2003).

2.6.4 The chemistry of antioxidants

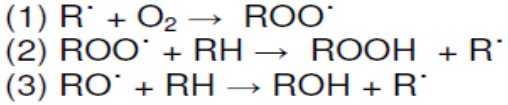
It involves the mechanism of action of antioxidant. Two principle mechanisms of action have been proposed for antioxidants. The first is a chain-breaking mechanism by which the primary antioxidants donate electrons to the free radicals present in the system, example lipid radicals. The second mechanism involves removal of ROS (reactive oxygen species) and RNS (reactive nitrogen species) initiator by quenching chain initiator catalyst.

Chain reactions of free radicals

- Initiation stage



- Propagation stage



- Termination stage

(1) $R^{\cdot} + R^{\cdot} \rightarrow R - R$
(2) $R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$
(3) $ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + O_2$
(4) Antioxidants + O_2 → oxidized antioxidants (Hamid *et al*., 2010)

Further, in free radical chain reactions, when fats are in contact with oxygen, it forms unsaturated fatty acids which give rise to free radicals. Also hydroperoxide which exist in trace quantities prior to oxidation reaction, break down to yield radicals, which abstract a hydrogen atom from another molecule and become a hydroperoxide producing further radicals. The antioxidants added to it, will neutralize the free radicals by donating one of their own electrons ending the reactions. These occur generally in the body.

2.6.5 Mechanisms of Antioxidants in the Oxidation of Foods

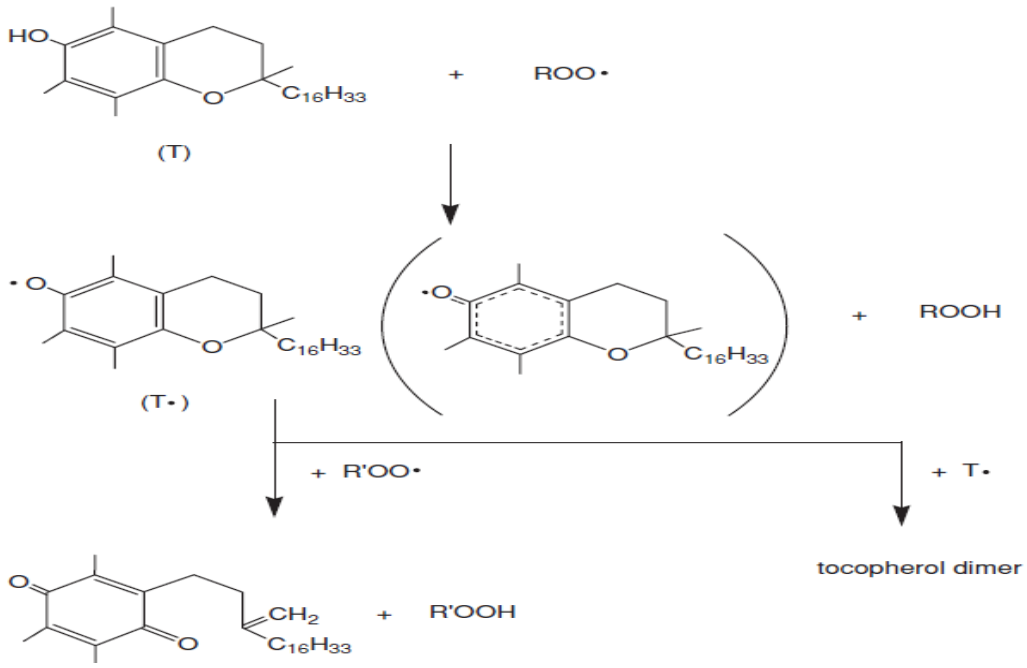
Antioxidants slow down the oxidation rates of foods by a combination of scavenging free radicals, chelating metals, quenching singlet oxygen and photosensitizers, and inactivating lipoxygenase.

2.6.5.1 Free radical scavenging

Antioxidants scavenge free radicals of foods by donating hydrogen to them, and they produce relatively stable antioxidant radicals with low standard reduction potential, less than 500 mV (Choe and Min, 2005). Rates of hydrogen abstraction from antioxidants are higher than that of lipids. The higher stability of antioxidant radicals than that of food radicals is due to resonance delocalization throughout the phenolic ring structure. The effectiveness of antioxidants to scavenge free radicals of foods depends on the bond dissociation energy between oxygen and phenolic hydrogen, pH related to the acid dissociation constant, and reduction potential and delocalization of the antioxidant radicals. Hydrogen transfer from antioxidants to the peroxy or alkyl radicals of foods is more thermodynamically favorable when the bond dissociation energy for O–H in the antioxidants is low. Bond dissociation energy for O–H of phenolic antioxidants decreases in the order of $\delta > \gamma > \beta >$ α-tocopherol (Choe and Min, 2009). Bond dissociation energy for O–H of phenolic antioxidants is affected by surrounding solvents; it is higher in polar solvents such as acetonitrile and tert-butyl alcohol than nonpolar benzene. Thus, polar solvents decrease the radical scavenging activity of the antioxidants due to the intermolecular hydrogen bonding between oxygen or nitrogen in a polar solvent and OH group in phenolic antioxidants.

The bond dissociation energy for O–H of the phenolic antioxidants also predicts the stabilization of antioxidant radicals. The lower the bond dissociation energy for the O–H group of the antioxidants, the more stable the antioxidant radical. The antioxidants with low bond dissociation energy are thus more efficient hydrogen donors and better antioxidants. The O–H bond strength of phenolic antioxidants is affected by substitution of hydrogen in a benzene ring. The antioxidant activity of the phenolic antioxidants is dependent on the balance between the electron donating effect of the substituents and the steric crowding around the phenolic OH groups which is related to the position of the substituents. Any substituent destabilizing the ground-state phenolic antioxidants, and/or stabilizing the phenoxy radical form of the antioxidants, reduces the O–H bond strength. Substituents such as an alkyl or a 2^{nd} hydroxyl group improve stabilization of the antioxidant radicals and increase radical scavenging activity.

α-Tocopherol reacts with alkyl peroxy radicals more rapidly than alkyl radicals since the difference in reduction potential between tocopherol radicals and alkyl peroxy radicals (500 mV) is higher than that between tocopherol radicals and alkyl radicals (100 mV) (Choe and Min, 2009). Tocopherol donates hydrogen at the 6-hydroxy group on a chromanol ring to alkyl peroxy radical, and alkyl hydroperoxide and tocopherol radical are formed. Tocopherol radical is relatively stable due to a resonance structure. Tocopherol radical can react with lipid peroxy radical to produce tocopherol semiquinone having no vitamin E activity, or react with each other for the formation of tocopherol dimer. Tocopherols slowly and irreversibly react with superoxide anion radicals in organic solvents and produce tocopherol radical, but the reaction is insignificant in aqueous solution (Arudi *et al*., 1983).



tocopherol semiquinone

Figure 2.1 Reaction of α-tocopherol with lipid peroxy radical

Flavonoids should have special structural features for scavenging free radicals: the ortho-dihydroxy or catechol group in the B-ring, the conjugation of the B-ring to the 4-oxo group. Quercetin, rutin, and luteolin satisfy the requirements and are known as some of the most efficient radical scavengers among the nonvitamin plant phenols. Catechin, an efficient radical scavenger, does not have a 2, 3-double bond and 4-carbonyl group, but it has many hydroxy groups to donate hydrogen. Catechol-structured flavonoids scavenge lipid peroxy radicals by donating hydrogen and become more stable phenoxy radicals (Choe and Min, 2009). Phenoxy radicals undergo disproportionation and produce phenolic quinone and a dihydroxy phenolic compound (Shahidi and Wanasundara, 1992).

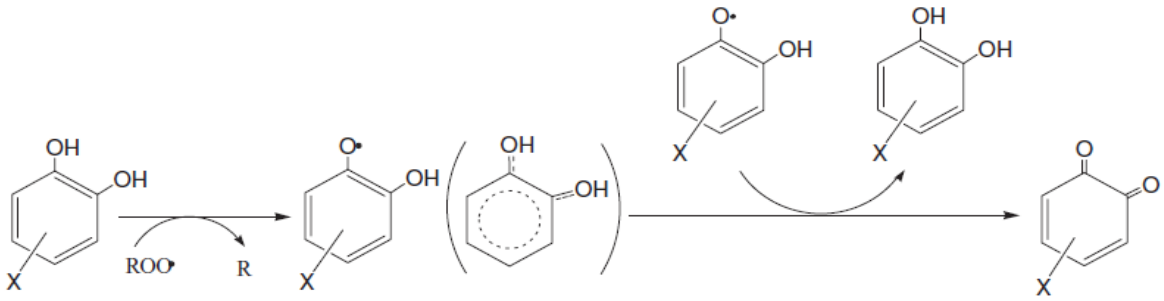


Figure 2.2 Reactions of catechol structured flavonoid with lipid peroxy radicals

Carotenoids can give electrons and then donate hydrogen. Two electrons rather than one are transferred per carotenoid with 2 reduction potentials, E_1 and E_2. Ease of electron donation of carotenoids depends on the nature of substituents on the carotenoids. Reduction potential for sequential transferring 2 electrons is different in canthaxanthin and astaxanthin, generally $E_1 < E_2$, while lycopene, β-carotene, and zeaxanthin have similar E_1 and E_2 values (Jeevarajan and Kispert, 1996). Electron donation of carotenoids containing terminal electron acceptor group is difficult and the 2nd electron donation occurs at quite a different potential to the 1st oxidation step. As the electron-accepting strength of the end groups decreases, ΔE ($E_1 - E_2$) decreases or cation radical can be reduced to carotenoid radical with a reduction potential E_3 which is generally much lower than E_1(Jeevarajan and Kispert, 1996).

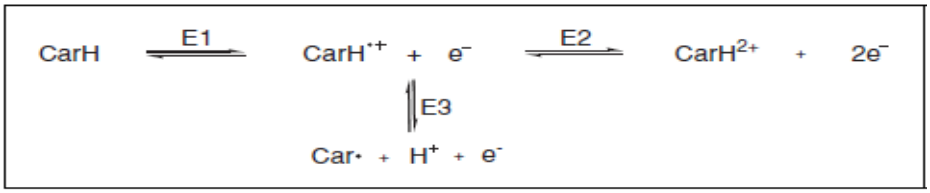


Figure 2.3 Hydrogen release from carotenoids (CarH) via electron donation

β-Carotene may donate hydrogen to lipid peroxy radical with some limitations and produce carotene radical. Carotene radical is a fairly stable species due to delocalization of unpaired electrons in its conjugated polyene, and has enough lifetime for a reaction with lipid peroxy radicals at low oxygen concentration and forms non-radical carotene peroxides. Carotene radical can also undergo oxygen addition, and subsequent reaction with another carotene molecule, and produce carotene epoxides and carbonyl compounds of carotene (Car) and produce carotene peroxy radical (ROO–Car•), especially at oxygen pressure higher than 150 mm Hg(Choe and Min, 2009).

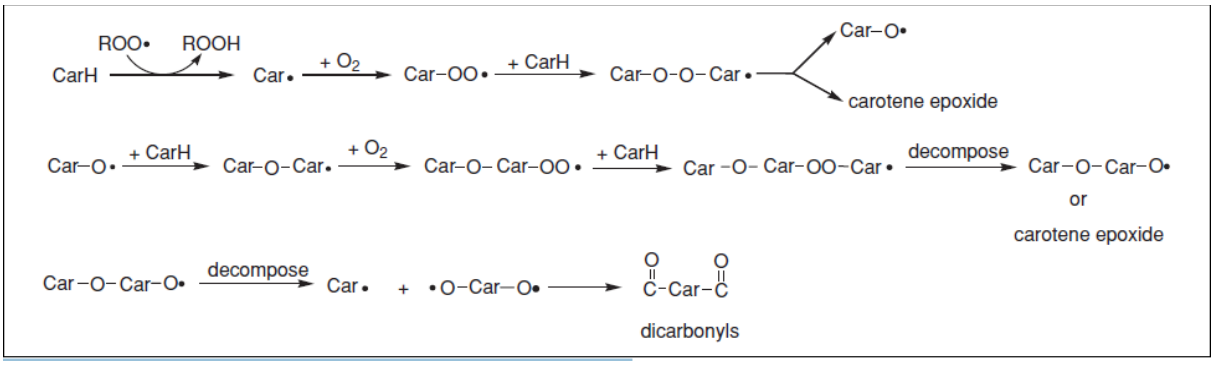


Figure 2.4 Reaction of β-carotene and lipid peroxy radicals

β-Carotene peroxy radical reacts with triplet oxygen to form peroxy radical of carotene peroxide (ROO– Car–OO•), which then abstracts hydrogen from another lipid molecule and produces lipid radicals (R′•. The resulting lipid radicals propagate the chain reaction of lipid oxidation, thus *β*-carotene acts as a prooxidant:

$$Car + ROO\bullet \rightarrow ROO - Car\bullet$$

$$ROO - Car\bullet + {}^{3}O_2 \rightarrow ROO - Car - OO\bullet$$

$$ROO - Car - OO\bullet + R'H \rightarrow ROO - Car - OOH + R'\bullet$$

Ascorbic acid and glutathione scavenge free radicals by donating hydrogen to food radicals, producing more stable ascorbic acid and glutathione radicals than food radicals. Ascorbic acid radicals become dehydroascorbic acid by loss of proton. Amino acids containing sulfhydryl or hydroxy groups such as cystein, tyrosine, phenylalanine, and proline also inactivate free radicals. Inactivation of food radicals by proteinaceous compounds might be a result of competition between proteinaceous compounds and lipid for high-energy food radicals, rather than an actual chain breaker.

2.6.5.2 Metal chelating

Metals reduce the activation energy of the oxidation, especially in the initiation step, to accelerate oil oxidation. Metals catalyze food radical formation by abstracting hydrogen. They also produce hydroxy radicals by catalyzing decomposition of hydrogen peroxide or hydro peroxides. Ferric ions decrease the oxidative stability of olive oil by decomposing phenolic antioxidants such as caffeic acid. Crude oil contains transition metals such as iron or copper, often existing in chelated form rather than in a free form. Oil refining decreases metal contents. Edible oils manufactured without refining, such as extra virgin olive oil and roasted sesame oil, contain relatively high amounts of transition metals (Decker, 2002).

Metal chelators decrease oxidation by preventing metal redox cycling, forming insoluble metal complexes, or providing steric hindrance between metals and food components or their oxidation intermediates. EDTA and citric acid are the most common metal chelators in foods. Most chelators are water-soluble, but citric acid can be dissolved in oils with some limitation to chelate metals in the oil system (Choe and Min, 2009). Phospholipids also act as metal chelators.

Flavonoids can also bind the metal ions and the activity is closely related with the structural features: 3′, 4′-dihydroxy group in the B ring, the 4-carbonyl and 3-hydroxy group in the C ring, or the 4-carbonyl group in the C ring together with the 5-hydroxy group in the A ring. Lignans, polyphenols, ascorbic acid, and amino acids such as carnosine and histidine can also chelate metals (Decker, 2002).

2.6.5.3 Singlet oxygen quenching

Singlet oxygen having high energy of 93.6 kJ above the ground state triplet oxygen reacts with lipids at a higher rate than triplet oxygen (Choe and Min, 2009). Tocopherols, carotenoids, curcumin, phenolics, urate, and ascorbate can quench singlet oxygen. Singlet oxygen quenching includes both physical and chemical quenching. Physical quenching leads to deactivation of singlet oxygen to the ground state triplet oxygen by energy transfer or charge transfer. There is neither oxygen consumption nor product formation. Singlet oxygen quenching by energy transfer occurs when the energy level of a quencher (Q) is very near or below that of singlet oxygen:

$$^1O_2 + Q \rightarrow {}^3O_2 + {}^3Q$$

$$^3Q \rightarrow {}^1Q\,(\text{no radiation})$$

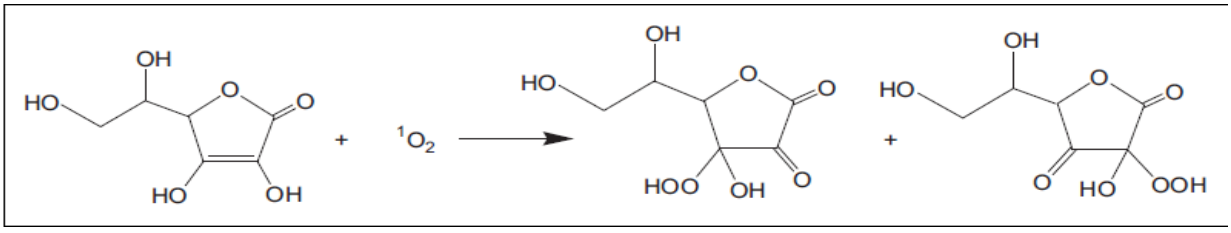


Figure 2.5 Formation of ascorbic acid hydro peroxides by singlet oxygen

Carotenoids with 9 or more conjugated double bonds are good singlet oxygen quenchers by energy transfer. The singlet oxygen quenching activity of carotenoids depends on the number of conjugated double bonds in the structure and the substituents in the *β*-ionone ring. *β*-Carotene and lycopene which have 11 conjugated double bonds are more effective singlet oxygen quenchers than lutein which has 10 conjugated double bonds (Choe and Min, 2009). The presence of oxo and conjugated keto groups, or cyclopentane ring in the structure increases the singlet oxygen quenching ability; however, *β*-ionone ring substituted with hydroxy, epoxy, or methoxy groups is less effective (Viljanen *et al*., 2002). When a quencher has high reduction potential and low triplet energy, it quenches singlet oxygen by a charge transfer mechanism.

These types of quenchers are amines, phenols (including tocopherols), sulfides, iodide, and azides, which all have many electrons. The quencher donates electron to singlet oxygen to form a singlet state charge transfer complex and then changes the complex to the triplet state by intersystem crossing. Finally, the triplet state charge transfer complex is dissociated into triplet oxygen and a quencher:

$^1O_2 + Q \rightarrow [O_2{-}{-}{-}{-}Q^+]^1 \rightarrow [O_2{-}{-}{-}{-}Q^+]^3 \rightarrow {}^3O_2 + Q$

Chemical quenching of singlet oxygen is a reaction involving the oxidation of a quencher rather than a quenching, thus producing breakdown or oxidation products of a quencher. *β*-Carotene, tocopherols, ascorbic acid, amino acids (such as histidine, tryptophan, cysteine, and methionine), peptides, and phenolic are oxidized by singlet oxygen, and they are all chemical quenchers of singlet oxygen. Reaction of ascorbic acid with singlet oxygen produces an unstable hydroperoxide of ascorbic acid. Tocopherol reacts irreversibly with singlet oxygen and produces tocopherol hydroperoxydienone, tocopherylquinone, and quinone epoxide.

2.6.5.4 Photosensitizer inactivation

Foods contain sensitizers such as chlorophylls and riboflavin (Choe and Min, 2009), which are activated by light. Photo activated sensitizers transfer the energy to triplet atmospheric oxygen to form singlet oxygen, or transfer an electron to the triplet oxygen to form a superoxide anion radical, and these reactive oxygen species react with food components to produce free radicals. Carotenoids having fewer than 9 conjugated double bonds prefer the inactivation of photosensitizers instead of singlet oxygen quenching; singlet oxygen quenching is preferable by carotenoids with 9 or more conjugated double bonds (Viljanen *et al*, 2002). Energy of the photosensitizer is transferred to the singlet state of carotenoids to become a triplet state of carotenoids, which is changed to the singlet state by transferring the energy to the surrounding or emitting phosphorescence (Stahl and Sies, 1992). The edge-to-edge distance for a direct quenching of triplet state of chlorophyll by carotenoids must be less than the van der Waals distance (0.36 nm), which enables some overlap between electron orbitals of these 2 pigments (Edge and Truscott, 1999).

2.6.5.5 Inactivating lipoxygenase

Lipoxygenase is a catalytic enzyme in the oxidation of lipids and is inactivated by tempering, which is heat treatment with moisture. Steaming of ground soybeans at 100 ^{0}C for 2 min or 116 ^{0}C under 44.5Nfor 1 min decreases the lipoxygenase activity by 80% to 100%, with a decrease in peroxide values, which improves the sensory quality of crude soybean oil (Choe and Min, 2009).

2.6.6 Structure of antioxidants

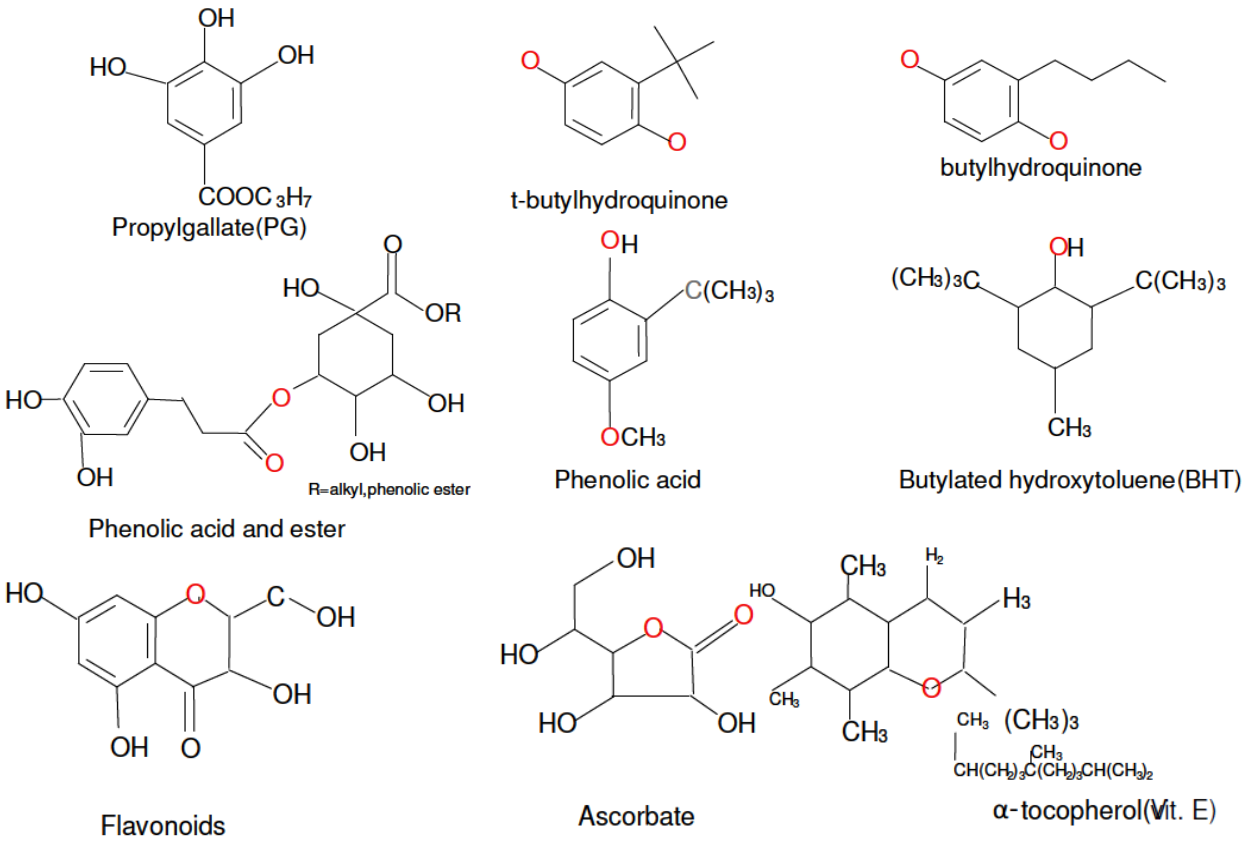


Figure 2.6 Structures of antioxidants

2.7 Extraction Method

2.7.1 Solvent Extraction Method

The most common techniques that were employed to obtain high yield of antioxidant activity is direct by using solvent. The solvent used for the extraction is a major importance for the recovery of the antioxidant component, the coextraction of undesirable substances and the process yield. Selective extraction methods should be practiced since active compounds in plants that exhibit biological activities are usually present in low concentration.

The type of the solvent used to extract antioxidants from orange peel can affect single electron transfer and hydrogen atom transfer, which are key aspects in the measurements of antioxidant capacity. The polarity of the solvent and that of the different antioxidant compounds affects the efficiency of the extraction and the activity of the obtained extracts. Water, methanol, ethanol, acetone, chloroform, hexane, aqueous solutions of the aforementioned solvents and ethyl acetate are commonly used as extraction solvents (González-Montelongo *et al*., 2010). However acetone is the most commonly used as extraction solvent due to its extensive validation by FDA. Several studies focused on the efficiency of different organic solvent used in extraction process. Organic solvent with high polarity are more effective in quantitative recovery than nonpolar solvent (Atiah, 2010).

The most frequently used technique for the isolation of plant antioxidants is solvent extraction that is, maceration and percolation. Conversely, the extract yields and resulting antioxidant activities of the plant materials are strongly reliant on the type of extracting solvent, due to the presence of different antioxidant compounds of diverse chemical characteristics and polarities that may or may not be soluble in a particular solvent. Usually polar solvents are used for the recovery of polyphenols from a plant matrix.

2.7.1.1 Maceration

In this process, the powdered plant material is placed in contact with the menstruum, usually alcohol but sometimes water, in a stoppered container stand at room temperature for a longer period of time with frequent shaking until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration or decantation after standing (Handa *et al*., 2008).

This simple widely used procedure involves leaving the pulverized plant to soak in a suitable solvent in a closed container. Simple maceration is performed at room temperature by mixing the ground plant material with the solvent (solid to solvent ratio; 1:5 or 1:10) and leaving the mixture for several days with occasional shaking or stirring. The extract is then repeated from the plant particles by straining. The process is repeated for once or twice with fresh solvent. Finally the last residue of extract is pressed out of the plant particles using a mechanical press or a centrifuge. Kinetic maceration differs from simple one by continuous stirring. The method is

suitable for both initial and bulk extraction. The main disadvantage of maceration is that the process can be quite time-consuming, taking from a few hours up to several weeks.

2.7.1.2 Percolation

Percolation differs slightly from maceration. The powdered plant material is dampened with the menstruum, left for four hours then packed into a percolator. Sufficient menstruum is added to cover the powdered plant material and left for twenty-four hours. The liquid is then allowed to very slowly drain from the bottom of the percolator (about twenty drops per minute). More menstruum is added and the process continued until the volume in the collecting flask reaches about three-quarters of the required volume. The marc is pressed, this liquid added to the flask, more menstruum added to make the specified volume then the whole liquid is clarified (Handa *et al*., 2008).

A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstruum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

2.7.1.2.1 Cold Percolation

The extraction of plant material is carried out in a percolator which is a tall cylindrical vessel with a conical bottom and a built-in false bottom with a filter cloth. The percolator is connected to a condenser and a receiver for stripping solvent from the marc.

The powdered material is fed into the percolator along with a suitable solvent (ethyl alcohol or another non-polar solvent). The material is left in contact with the solvent until equilibrium of the active principle is achieved. The solvent extract, known as miscella, is taken out from the bottom discharge valve of the percolator. Fresh solvent is added into the percolator and the

miscella is drained out after acquiring equilibrium. Overall, the plant material is washed four to five times until it gets exhausted. All washes from the percolator are pooled and concentrated.

The solvent in the marc is stripped out by passing steam from the bottom of the percolator. The solvent and steam vapors rise and are condensed in a tubular condenser. The condensate, which is a mixture of alcohol and water, is collected in a receiver and then subjected to fractional distillation to get 95% pure ethyl alcohol which is again used as a fresh solvent.

This type of percolation is not efficient as it takes a long time to reach equilibrium due to the slow mass transfer rate. The mass transfer rate can be enhanced if some sort of movement is created between the particles and the solvent. This can be achieved either by providing inside agitation with a mechanical stirrer or by repeated circulation of the extract back to the percolator. The first method is cumbersome and power intensive whereas the latter has been successful. A circulation pump that continuously circulates the miscella back to the top of the percolator gives a better mass transfer rate and reduces the equilibrium time considerably. Still, this type of percolation is energy-consuming as large amounts of miscella from multiple washes must be concentrated to remove the solvent.

To overcome this problem, a battery of percolators can be connected in series. If three washes are required for completion of the extraction, four percolators are connected in series with their respective miscella storage tanks.

At a particular time, one percolator is out of circuit, for charging and discharging the material and also for stripping solvent from the marc, whereas the other three percolators are in operation. Material is fed into all the percolators and the solvent is fed into the first percolator. When the equilibrium in the first percolator is reached, the extract from the first percolator is sent to the second percolator. The first percolator is again filled with fresh solvent. The extract of second percolator is transferred to the third, the extract of first is transferred to second, and fresh solvent is added to the first. The extract of the third percolator is transferred to the fourth percolator. After attaining equilibrium, the extract from the fourth percolator is drained off. The extract of the third percolator goes to fourth, the extract of second goes to third, and the extract of first goes to second percolator. The material of the first percolator, which has received three washes, is completely exhausted. This percolator is taken out of the system for stripping the solvent and discharging the extracted marc. This is again filled with fresh plant material and the sequence is

repeated with other percolators. In this way, solvent of each percolator comes in contact three times with solid material and gets fully enriched with active principle. The enriched extract is sent for solvent recovery and concentration. Thus, instead of concentrating three volumes of solvent, only one volume has to be concentrated; this saves energy and the process is efficient.

2.7.1.2.2 Hot Percolation

Increasing the temperature of the solvent increases the solubility of the active principle, which increases the concentration gradient and therefore enhances the mass transfer of active principle from solid material to the solvent, provided the active principle is not heat sensitive. This is achieved by incorporating a heat exchanger between the circulation pump and the feed inlet of the percolator. The extract is continuously pumped into a tubular heat exchanger which is heated by steam. The temperature of the extract in the percolator is controlled by a steam solenoid valve through a temperature indicator controller. This sort of arrangement can be incorporated in single percolators or in a battery of percolators as needed.

2.7.1.3 Hot Continuous Extraction (Soxhlet)

Soxhlet apparatus is a type of instrument for extraction of medicinal ingredients from plant material, which consists of an extractor, a distillation still, a tubular condenser for the distillation still, a tubular condenser for the recovery of solvent from the marc, a receiver for collecting the condensate from the condenser, and a solvent storage tank. The plant material is fed into the extractor, and solvent is added until it reaches the siphon point of the extractor. Then, the extract is siphoned out into the distillation still, which is heated with steam. The solvent vapors go to the distillation condenser, get condensed and return to the extractor. The level of the solvent in the extractor again rises to the siphon point and the extract is siphoned out into the distillation still. In this way, fresh solvent comes in contact with the plant material a number of times, until the plant material is completely extracted. The final extract in the distillation still, which is rich in active principle, is concentrated and the solvent is recovered (Handa *et al*., 2008).

In this method, the finely ground plant material is placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber of the Soxhlet apparatus. The extracting solvent in flask is heated, and its vapors condense in condenser. The condensed extractant drips into the thimble containing the crude powder, and extracts it by contact. When the level of liquid in

chamber rises to the top of siphon tube, the liquid contents of chamber siphon into flask. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This affects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.

2.7.3 Selection of Extraction Method

Maceration type of extraction process takes place by direct contact of pant material with extraction solvent. Even though the extraction took long time from 1 day to 3 day or more up to 7 days (Handa *et al*., 2008), the antioxidant activity of extract didn't degradable and denatured since extraction has been taking place at room temperature. The antioxidant extracted by maceration is better in antioxidant activity, total phenol and flavonoid content than that of by soxhlet. But the disadvantage of maceration extraction method is time consuming extraction up to 7 days (Ibrahim *et al*., 2011).

The advantage of soxhlet method, compared to maceration, is that large amounts of extract can be extracted with a much smaller quantity of solvent. This saves tremendous economy in terms of time, energy and consequently financial inputs but the extraction process takes place at the boiling of solvent and at this temperature, the antioxidant activity of extracts degrades.

According to the investigation Ibrahim *et al*., (2011), soxhlet extraction method gave the high yield and the maceration extraction gave low yield but maceration gave significantly higher polyphenols (phenols and flavonoids) than that of soxhlet extraction method. The characteristic of antioxidant activity of the extract was determined by the amount of total phenol and flavonoid contents not yield. Not only this but also, maceration type extraction method is simple, convenient and economic. For this reason, maceration extraction method was selected for this study.

2.8 Extraction Processing Conditions

The aim of this study is to extract of antioxidant compounds from orange peel waste and to evaluate its ability to limit food oxidation as a potential alternative to commercial antioxidants.

The extraction conditions are extraction temperature, type of solvent and processing time studied to optimize the yields of phenolics and flavonoids, antioxidant activity and the main phenolic compounds identified.

2.8.1 Type of Extraction Solvent

Solvent extraction is more frequently used for isolation of antioxidants and both extraction yield and anti- oxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potential of compounds with different polarity. Polar solvents are among the most employed solvents for removing polyphenols from water. Ethyl acetate and diethyl ether have been used for extraction of low molecular weight phenols from oak wood and the polyphenols extracted with ethyl acetate from natural materials were reported to have strong antioxidant activity. Ethanol and water are the most widely employed solvents for hygienic and abundance reasons, respectively. Since the activity depends on the poly- phenol compounds and the antioxidant assay, comparative studies for selecting the optimal solvent providing maximum antioxidant activity are required for each substrate. Less polar solvents such as ethyl acetate provided slightly more active extracts than mixtures with ethanol or methanol, or methanol alone for tamarind seed coats although ethanol and methanol extracts also presented high lipid peroxidation-inhibiting activity, comparable to a-tocopherol. Selective extraction of more a polar compound was reported to enhance the antioxidant activity of lentil husk extracts (Moure, 2000).

Table 2.6 Effect of solvent type on % yield of orange peel extracts

Organic solvents	Yield(mg/10ml)	%Yield
Methanol	69.27	28.32
Ethanol	65.82	27.96
Dichloromethane	34.79	13.29
Acetone	49.20	18.21
Hexane	21.76	8.27
Ethyl acetate	58.27	24.92

Source: Ibrahium, 2012

Table 2.7 Effect of different solvents on TPC &TFC of produced OPE

Extract	TPC(mg/g)	TFC(µg/g)	DPPH scavenging activity %
Methanol	165.38	28.36	73.42
Ethanol	169.56	29.75	78.14
Dichloromethane	98.64	17.39	62.06
Acetone	145.79	21.87	65.38
Hexane	63.20	13.89	58.78
Ethyl acetate	85.27	18.36	68.99

Source: Ibrahium, 2012

2.8.2 Extraction Time

Extraction time is one of the factors that affect the extraction of antioxidant from different plant materials and the extraction yield is dependent on the extraction time. Different research studies showed researchers conducted extraction at different extraction time. According to Singh (2014), extraction of antioxidant from orange peel was conducted for 24 h And according to Singh (2015) study, extraction was carried out for 48 h In the other research studies, extraction of antioxidant from orange peel was also performed for 72 h (Park *et al*., 2014).

2.8.3 The Extraction PH

The maximum solubility of polyphenols from fruit peels at pH4 in the organic phase increased antioxidant activity of aqueous fractions after treatment at acidic conditions, probably due to altered phenol composition. The pH has also been considered for the aqueous extraction of antioxidants from oat fiber, the highest yield being attained at pH6 and the highest antioxidant activity at pH10. At alkaline pH, the fractions with high protein and fatty acid contents are solubilized; and due to contradictory data on the higher antioxidant activity of carbohydrate or proteins, the antioxidant activity was probably carried by the protein-rich fraction (Moure, 2000).

2.8.4 Extraction Temperature

The temperature, during extraction, affects the compound stability due to chemical and enzymatic degradation, losses by volatilization or thermal decomposition. Research studies investigated the effects of extraction temperatures (25 – 60 ºC) on total phenolic content (TPC),

total flavonoid content (TFC), antioxidant activity (AA) and yield of extract. The used of 40 ºC to 60 ºC of extraction temperature showed a gradual reduction in TPC, thus decreased in AA for DPPH (Moure, 2000).

2.8.5 Sample drying temperature

The antioxidant activity of samples dried with air at 100 ºC was reduced by 28% and, at 140 ºC by half, with respect to drying at 60 ºC that did not significantly affect either the extractable polyphenols or condensed tannins, with respect to freeze-drying. Drying at 100 ºC caused a reduction of 18.6% and at 140 ºC of 32.6% in the TEPs, which in this material are a complex group of different substances. The maximum yield of phenol attained when the drying temperature was below 50 ºC; increasing the temperature above 60 ºC significantly lowered the phenols content being the most affected by temperature (Moure, 2000).

2.8.6 Concentration of solvent

The first experiment was conducted at different percentages of alcoholic concentration of solvents (0%, 20%, 40%, 60%, 80%, and 100%) for extraction (Woo *et al*., 2013). And according to Sharma et al (2014), the extraction of antioxidant from orange and pomegranate was carried out at 80% v/v of ethanol.

2.8.7 Solid to solvent ratio

The ratio of solvent/solid was in range of 5:1 to 50:1(Wang *et al*., 2011). Fresh orange fruit were peeled and cut in to small pieces with a knife cutter, dried in the oven and ground in to powder and then soaked in solvent at the rate of 10g: 100ml, 20g: 100ml and 30g: 100ml of each solvent for extraction (Jembere, 2002). The solid to solvent was also investigated at rate of 10g: 100ml, 10g: 150ml and 10g: 200ml (Shimelis, 2015).

2.8.8 Particle size of sample

The samples of particle sizes were investigated in range of 10 - 40 mesh (Wang *et al*., 2011). According to Samavardhana *et al*., (2014), extraction of phenolic compounds from plant materials was performed in the range of particle sizes (ground, <20, 20-40 and >40 mesh). Separation of supernatant from solid waste residue was difficult when of extraction was performed at the particle >40 mesh. Hence the efficiency of extraction was lowered. The

efficiency of extraction was also decreased at the particle size <20 mesh due to decrease in surface area of the particles and hence the rate of diffusion phenolic compounds towards the extraction solvent.

2.8.9 Selection of Extraction Factors

Extraction of antioxidant was affected by various factors. Among those factors, only three factors highly affect the extraction were selected for the present study. According to the study Samuagam *et al*., (2013) effects of ethanol concentration, extraction time, and extraction temperature on total phenolic content (TPC) and free radical-scavenging capacity of tropical Fruits' Peel were investigated. The levels of each factors also selected depending on the benchmark of past research studies. Research study Samuagam *et al*., (2013) investigated the effects of extraction temperatures (25 – 60 ºC) on total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (AA) and yield of extract. Selection of 40 to 60 ºC of extraction temperature showed a gradual reduction in TPC, thus decreased in AA for DPPH (Moure, 2000). According to Singh, (2014), extraction of antioxidant from orange peel was conducted for 24 h. And according to Singh (2015) study, extraction was carried out for 48 h. In the other research study, extraction of antioxidant from orange peel was also performed for 72 h (Park *et al*., 2014). Similarly, the optimal ethanol concentration was selected based on the values of DPPH and TPC assays with ethanol concentrations ranging from 0 to 100% and best values of DPPH and TPC assays were reported at 60 and 80% (Samuagam *et al*., 2013).

The remaining factors were selected as the controlled variables. According to Ibrahium, (2012), the total amounts of phenol and flavonoid contents obtained by Ethanol were highest among other polar solvents such as Methanol, Ethyl acetate, Dichloromethane, Acetone and Hexane. Hence, ethanol was selected among polar solvents and became control variable. The highest yield was attained at pH6 and the highest antioxidant activity at pH10 (Moure, 2000). To compromise yield and antioxidant activity, pH was controlled at pH8. Separation of supernatant from solid waste residue was difficult when of extraction was performed at the particle >40 mesh. Hence the efficiency of extraction was lowered. The efficiency of extraction was also decreased at the particle size <20 mesh due to decrease in surface area of the particles and hence the rate of diffusion phenolic compounds towards the extraction solvent decreased. Hence, the particle size was also another controlled variable at 40 mesh size. The solid to solvent was also

another controlled factor for the present study and was controlled at rate of 10g: 100ml (Shimelis, 2015).

2.9 Application of antioxidant

Antioxidants have a wide range of applications in different sectors. From many applications of antioxidants, only the applications of antioxidants in food sectors, medicinal sectors and polymer sectors were included in this study.

2.9.1 Application of antioxidants in food industries

Lipids in foods of vegetable origin are usually more unsaturated than lipids of foods of animal origin, therefore, the initiation rate of oxidation reactions is higher and natural antioxidants, originally present in foods are more rapidly consumed than in lard or tallow and other animal fats. The stabilization of products of vegetable origin against autoxidation is thus less efficient than the stabilization of animal products. Protection factors of comparable antioxidants are several times higher in lard than in edible oils. Edible oils become rancid on storage, the type of rancid off-flavor depending on their fatty acid composition and the presence of minor components. Edible oil producers try to prolong the shelf life of edible oils by different techniques, including the addition of antioxidants. The presence of natural antioxidants should always be taken into account, when appropriate levels of added antioxidants are considered (Shimelis, 2015).

2.9.2 Natural Antioxidants in Polymers

The migratory nature of BHT into Foods and food simulants has caused some concern regarding its continuous use as an antioxidant in food-packaging materials; therefore, α- tocopherol has been increasingly tested as a natural alternative (Hamid *et al*., 2010). Natural antioxidants such, as α- tocopherol, are "generally recognized as safe" (GRAS) by FDA and its degradation products containing mostly tocopherol quinone compounds are harmless. α-Tocopherol would be expected to have a slower migration rate from packaging into a food due to the larger molecular weight of α-tocopherol (Hamid *et al*., 2010). The thermal stability of α-tocopherol appears far superior to BHT by thermo gravimetric analysis showing that α-tocopherol does not begin to volatilize until almost 300 ºC. α-Tocopherol was observed to be susceptible to thermo-oxidative

degradation beginning at 165 ºC as measured by non-isothermal DSC measurements(Hamid *et al*., 2010).

After incorporated α-tocopherol and BHT into a heat seal layer coextruded with an HDPE layer at their initial concentrations, the relative percentage of antioxidant remaining in the packaging film some time latter was significantly higher in α-tocopherol than BHT. During the storage of food products with α-tocopherol and BHT incorporated LDPE film, BHT was depleted much more rapidly than α- tocopherol. The amount α-tocopherol retained in the LDPE film with the presence of food packaged products within the film was significantly less than the film stored without the product. Oatmeal packaged in LDPE pouches resulted in a loss of approximately 400 mg/g of α-tocopherol during four weeks of storage, but the film stored without the product had retained 900 mg/g of α-tocopherol during the same period (Hamid *et al*., 2010). α- Tocopherol is considered to be a more stable antioxidant than BHT when used in an LDPE film in contact with sunflower oil or 95% ethanol due to the much slower transfer from the polymer film to the fatty food simulants (Wessling *et al*., 1998).

2.9.3 Application of antioxidants in medicinal sectors

Antioxidants are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease.

2.9.3.1 Anti-cancer agent

The application of inorganic chemistry to medicine is a rapidly developing field, Novel therapeutics and diagnostic metal complexes are now having an impact on medical practice. Advances in bio-coordination chemistry are crucial for improving the design of compounds to reduce toxic side effects and understand their mechanisms of action. A lot of metal –based drugs are widely used in the treatment of cancer (Hamid *et al*., 2010).

Lycopene shows both antioxidant activity and array of biological effects including cardio protective, anti- inflammatory, anti-mutagenic and anti-carcinogenic activities. The cancer activities of lycopene have been demonstrated both in vitro and in vivo tumor models (Xianquan *et al*., 2005).

The role of selenium in the prevention of cancer has been recently established by laboratory experiments, clinical trials and epidemiological data. Consequently, selenium supplementation has moved from the realm of correcting nutritional deficiencies to one of pharmacological intervention, especially in the clinical domain of cancer chemoprevention and in the control of heart failure (Hamid *et al*., 2010).

2.9.3.2 Anti-aging agent

Lipoic acid protects against diseases of aging, this offer powerful antioxidant protection against three common afflictions (two of them potentially disastrous) association with the aging stroke, heart attack and cataracts. It does it by suppressing the action of free radicals in the cells of the brain, heart and eyes. Lipid acid has an unusual relationship with four other important antioxidants: glutathione, coenzymeQ10, vitamin C and vitamin E. Memory loss is not considered to be a disease at least not until it is a component of a full-fledged dementia, such as Alzheimer's disease-but it is certainly another hallmark of aging. Unlike lipoic acid other antioxidants are either primarily water-soluble or fat-soluble, but not both. This means that they have different (often overlapping) domain are free radical scavengers. What is good is that lipoic acid not only acts as a primary antioxidant in brain cells but serves to boost glutathione levels through the antioxidant network interactions. Diabetes, a terrible yet largely preventable disease, is practically epidemic in the western world, especially the United States, because of our tendency to obesity due to poor diet and lack of exercise. Since lipoic acid is the most versatile and powerful antioxidant in the entire antioxidants defense network. Gene therapy promises to be one of the most exciting and fruitful avenues of medical practice in the twenty-first century and it offer powerful antioxidant protection against common afflictions.

2.9.3.3 Significance of antioxidants in red cells

Erythrocytes contain abnormal haemoglobin with high affinity for red cell. Since HbS disease (sickle cell anemia) is known to have high affinity for red cell membrane and sickle cells are particularly susceptible to membrane lipid peroxidation, the behaviour of erythrocyte antioxidant system has been evaluated heterozygous for sickle cell anaemia (Gutter, 1991).

2.9.3.4 Antioxidants therapy in acute central nervous system injury

Free radicals are highly reactive molecules generated predominantly during cellular respiration and normal metabolism imbalance between cellular production of free radicals and ability of cells to defend against them is referred to as oxidative stress (OS) (Gutter, 1991). OS has been implicated as a potential contributor to acute central nervous system (CNS) injury by ischemic or hemorrhagic stroke or trauma. The production of reactive oxygen species (ROS) may increase, sometimes drastically leading to tissues damage via several different cellular molecular pathways. Radicals can cause damage to cardinal cellular components such as lipids, proteins and nucleic acid (DNA leading to subsequent cell death by modes of necrosis or apoptosis.) The damage can become more widespread due to weakened cellular antioxidant defense systems. Moreover, acute brain injury increases the level of excitoxic amino acids (such as glutamate), which also produce ROS, thereby promoting parenchymatous destruction. Therefore, treatment with antioxidants may theoretically act as tissue damage and improve both the survival and neurological outcome, several such agents of widely varying chemical structures have been investigated as therapeutic agents for acute CNS injury, although, a few of the antioxidants showed some efficacy in animal models or in small clinical studies. Better understanding of the pathological mechanisms of acute CNS injury would characterize the exact primary targets for drug intervention improved antioxidant design should take into consideration the relevant and specific harmful free radical. A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially superoxide and hydroxyl radicals) and high-energy oxidants such as peroxynitrite as mediators of inflammation, shock and ischemia/reperfusion injury (Hamid *et al*., 2010).

2.10 Factors Affecting Antioxidant Activity

The quality of natural extracts and their ant oxidative performances depends not only on the quality of the original plant, the geographic origin, climatic condition, harvesting date and storage but also environmental and technological factors affect the activities of antioxidants from residual sources(Moure, 2000).

CHAPTER THREE

MATERIALS AND METHODS

Chemicals and reagents were bought from Cherkos Market Center, Addis Ababa. Some chemicals and reagents were available in the Center of Food and Nutrition, College of Natural Science, Addis Ababa University. The necessary apparatus are found in the School of Chemical and Bio-Engineering Laboratory, Addis Ababa Institute of Technology, Addis Ababa University and Center of Food and Science Nutrition, College of Natural Science, Addis Ababa University. The experiment was done in the school of chemical and Bio-Engineering laboratories, Addis Ababa Institute of Technology, Addis Ababa University. Experimental result analyses were analyzed in the Center of Food Science and Nutrition, Collage of Natural Science, Addis Ababa University.

3.1 Materials

3.1.1 Raw Materials

Two fresh orange varieties were collected from Upper Awash Fruit and Vegetable Industry Enterprise in December 18/12/ 2015. The two varieties were Citrus Sinensis var. Washington and Citrus Sinesis var. Valencia. The study on these two varieties of orange peel was advantageous on selecting and concluding the better in terms of extraction yield, total phenol content, total flavonoid content and radical scavenging activity.

3.1.2 Chemicals

Chemicals like Ethanol, Methanol, Folin Ciocalteu Reagent, Gallic acid, catechin, sodium carbonate, ascorbic acid, aluminum chloride ($AlCl_3$), DDPH, Sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), were used during the investigation of the laboratory works. Ethanol and methanol were bought from Charkos Market Center, but all other listed chemicals were available and used in the Center of Food and Nutrition, College of natural science, Addis Ababa University.

3.1.3 Apparatus

Apparatus like Knife Cutter, Oven dryer, Electrical grinder, conical flask, Orbital shaker, Whatman filter paper, Rotary vacuum evaporator, UV-Spectrophotometry were used during laboratory work. Knife cutter, electrical grinder and Whatman filter paper were bought from the Market, oven dryer, conical flask and orbital shaker were used in the School of Chemical and Bio Engineering, Addis Ababa Institute of Technology, Addis Ababa University and rotary vacuum evaporator and UV-Spectrophotometer were used in the Center of Food and Nutrition, College of Natural Science, Addis Ababa University.

Table 3.1 Chemicals and their function

Chemicals	Function
Ethanol-water mixture	As extraction solvent
Folin- Ciocalteu reagent	Reagent used for formation of blue color complex
Gallic acid	Positive control or standard for phenol determination
Catechin	Standard or positive control for total flavonoid determination
Sodium carbonate	For prevention of precipitation and turbidity of reagent
Ascorbic acid	Standard for radical scavenging activity
Aluminum chloride	Reagent for formation of yellowish complex
DPPH	Reagent for formation of deep purple color complex

Table 3.2 Apparatus and their function

Apparatus	Function
Oven dryer	To dry wet peels
Knife	To reduce the size of peels in to pieces
Electric grinder	To obtain powder
Conical flask	To Soak the powder peels in to the solvent
Shaker	To distribute the solvent and facilitate extraction
Filter paper	To isolate the supernatant from residue
Rotary evaporator	To remove solvent from extract
Spectrophotometer(UV)	To determine absorbance of solution

3.2 Methods

3.2.1 Experimental Setup

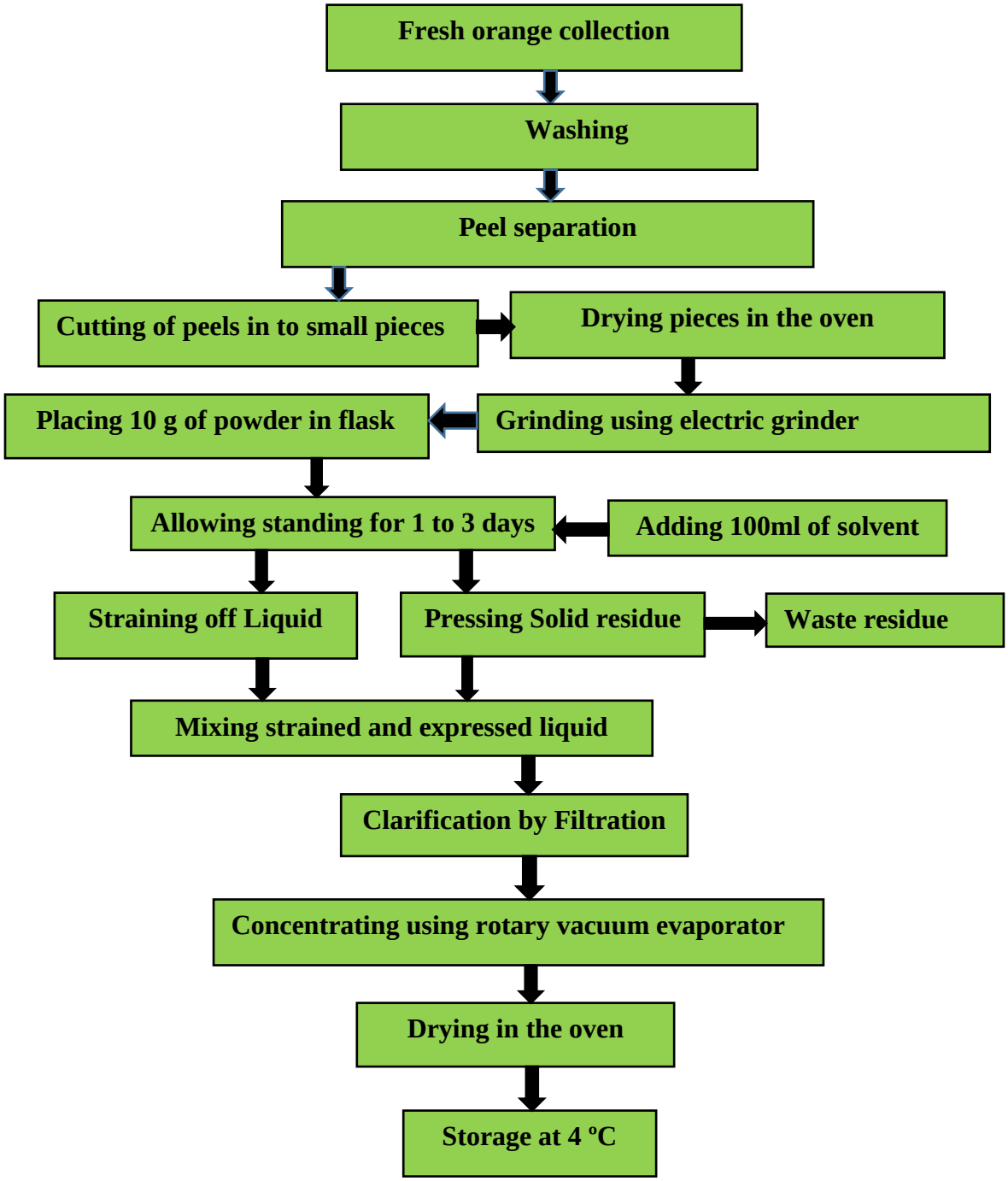


Figure 3.1 Equipment setup for extraction antioxidant from orange peel

3.2.2 Preparation of Orange Peels

The two fresh orange varieties (Citrus Sinensis Var. Washington and Citrus Sinensis Var. Valencia) were collected from Upper Awash Fruit and Vegetable Processing Industry Enterprise. To remove impurities, debris and dust particles, fresh oranges were washed using water. Then the peel was separated from the juice, pulp and seeds. Once the peel was separated, it was cut in to small piece using a knife. To remove the moisture content, the peel was dried on oven for a period of 6-7 days, at an ambient temperature of 30 ºC (Arora, 2013). The dried samples were ground properly using an electrical grinder, to obtain the powder. The powder of the peels was stored separately in air tight bottles (Arora, 2013).

3.2.2.1 Moisture content of orange peel

Moisture content is the amount of liquid moisture mass found in the total mass of the fresh wet orange peels. Moisture content of peels was determined by using weight difference method (initial and final weight difference method). The known weight of wet fresh orange peels was dried in oven dryer for a period of 6-7 days, at an ambient temperature of 30 ºC. The dried sample was weighed. The difference between the mass of wet and dry sample is the moisture content of sample peels.

Moisture content (%) = $\left(\frac{loss\ in\ weight}{weight\ of\ sample}\right) * 100$ …….. (1)

3.2.2.2 Ash content of orange peel

Ash is the in organic residue remaining after the water and organic matter have been removed by ignition or complete oxidation of organic matter in the presence of oxidizing agents, which provides a measure of the total amount of minerals within orange peels.

About 5g of dry orange peel was taken, cleaned porcelain dishes and weighed accurately. Hot air oven method was applied to remove the moisture. Then the sample was burnt on a gas burner. This was done to avoid the loss of sample in the muffle furnace under higher temperature. Then sample was transferred into the muffle furnace and burnt for 4 to 6 hours at a temperature of 550 ºC and ignited until light gray ash resulted (or to constant weight). The sample was then cooled in a desiccator and weighed. The ash content expressed as:

Ash content (%) = $\left(\frac{weight\ of residue}{weight\ of\ sample}\right) * 100$ ……..... (2)

3.2.3 Extraction of Antioxidant from Orange Peels

The dried powder of peels was extracted by maceration method using different concentrations of ethanol as a solvent separately. 10 g of the dried powder was soaked in 100 ml of ethanol-water binary mixture solvent in conical flask, plugged with cotton wool and then kept in shaker at 120 rpm for given time , at given temperature. After extraction time was completed, the extract was filtered through Whatman filter paper No.41 for removal of peel particles and concentrated under rotary vacuum evaporator at 40 ºC. The dry extract was stored at 4 ºC (Singh, 2014).

3.2.3.1 Characterization of Extract

3.2.3.1.1 Determination of Extract Yield

The extraction yield is a measure of the solvent's efficiency to extract specific components from the original material and it was defined as the amount of extract recovered in mass compared with the initial amount of whole plant. It is presented in percentage (%) and was determined for each techniques tested. The dry extract obtained after filtration was weighed to obtain the extraction yield.

Extraction yield(%) = $\frac{Dried\ weight\ of\ extract}{Total\ dried\ weight\ of\ peel\ powder} * 100$ ……..…. (3)

3.2.3.1.2 Determination of Specific Density of Extract

The specific gravity of solid extract can be determined by the method of ratio of the weight of 1 ml of solid extract to the weight of 1 ml of water or the ratio of density of 1 ml of extract to density of 1 ml of water at room temperature.

Procedure

Stock solution (50 mg/ml) of extract was prepared by dissolving 100 mg of dry extract in 2 ml of solvent (ethanol). Then 1 ml of stock solution was taken and weighed and the mass was known (0.83mg). The weight of ethanol in the stock solution (0.78 g) was determined by subtracting the weight of pure extract (0.05mg) found in the 1ml of stock solution from the total weight (0.83mg) of 1 ml stock solution. Once the mass of ethanol in the stock solution was known, 1 ml

of pure ethanol was weighed and the mass of pure 1ml of ethanol was known (0.82g) and it was used to determine the volume of 0.78 g of ethanol (0.95ml). Once the volume of ethanol in 1ml stock solution was known, it was deduced that the rest in 1ml of stock solution was the volume occupied by the extract (0.05ml). The density of extract was determined by dividing the mass of extract to the volume of the extract (1.0 g/ml). Then 1ml of water was taken and its mass was weighed (1.00g). The density of water was determined (1 g/ml). Finally, the specific volume of extract was evaluated as the ratio of mass of extract to the mass of water at the same volume (density of extract to density of water).

$$\text{Specific density} = \frac{\text{densty of extract}}{\text{density of water}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

3.2.3.1.3 Determination of pH value of extract

The pH value of extract was directly measured by pH meter at room temperature. After extracting, the supernatant was separated from the solid residue and the supernatant was concentrated and dried, stock solution (50 mg/ml) was prepared by dissolving 500mg of dry extract in to 10ml of solvent. Once the stock solution was prepared, pH meter was adjusted using pH buffer solution. Then the pH value of extracts was measured by pH meter at room temperature.

3.2.3.1.4 Determination of Color of Extract

The color value or color intensity was measured from the UV- spectrophotometer absorbance. The color value of extract solution was typically determined by measuring the absorbance in the visible range at 517nm absorbance wavelength at room temperature. It is an indicated as a value converted to the absorbance in a 10 %(w/v) solution. The color value of extract solutions was determined by the following correlations:

$$\text{Color value} = \frac{10 * A}{W} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

Where, “A” is absorbance and “W” is weight of extract in gram

3.2.3.1.5 Determination of Total Phenolic Content

The total phenol content was determined according to Folin- Ciocalteu’s reagent method of modified Singh (2014). 1ml of Gallic acid or extract solution and 1 ml Folin-Ciocalteu’s reagent

was mixed and the mixture was incubated at room temperature for 3 min and blue color complex was formed due to redox reaction and lead to increase in absorbance of the extracts. Then 1 ml of sodium carbonate solution was added. The solution was adjusted to 10 ml with distil water or (7ml distil water was added), mixed well and further incubated for 90 min at room temperature and the absorbance was measured at 725 nm using a Visible Spectrophotometer (UV-7804C). Hence, the more rapidly the absorbance increases, the more potent the antioxidant activity of the extract. Gallic acid was used as a positive control.

A total phenol value was expressed in terms of Gallic acid equivalent (mg of Gallic acid/g of extracted compound) (Singh, 2014). The total phenol content was calculated using the following relationship:

$$T = \frac{C*V}{W} \qquad \text{..... (6)}$$

Where C= Gallic acid equivalent concentration obtained from the calibration curve (mg/ml)

V= volume of stock solution of extract (ml)

W = dry weight of extract found in the stock solution (g)

T = total phenol content (mg of GAE/g dry extract)

If dilution of the solution was used for correct concentration to obtain the required absorbance, Dilution factor was not forgotten.

$$T = \frac{C*DF*V}{W} \qquad \text{..... (7)}$$

Where DF = dilution factor

Procedures

Reagent preparation

$NaCO_3$ reagent solution was prepared by weighing 13g of $NaCO_3$& dissolving in 5 ml methanol using 250 ml flask and diluted with distil water; incubate at room temperature for 24 hours. Folin-Ciocalteu's reagent solution was prepared by taking 1ml of Folin-Ciocalteu's into 9ml of distil water.

Preparation of blank solution

1ml of Folin-Ciocalteu's reagent solution was added to the test tube containing 1ml of methanol, mixed well and incubated for 3 min. After 3 min, 1ml of saturated Na_2CO_3 solution was added

and adjusted the solution to 10 ml with distil water or (added 7ml distil water), mixed well and incubated in the dark for 90 min at room temperature.

Preparation of standard solution

Gallic acid was used as a positive control or standard for determination of total phenolic content of the extract. A stock solution (50 mg/ml) of Gallic acid was prepared by weighing 2.5g of Gallic acid and dissolving in 5ml methanol and diluted to 50 ml Volumetric Measuring flask using distil water. Aliquots of 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240µL of standard Gallic acid were withdrawn from the stock solution and mixed with 980, 960, 940, 920, 900, 880, 860, 840, 820, 800, 780 and 760µL of methanol solvent to get Gallic acid concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 mg/ml respectively in separate test tubes. Then 1 ml of Folin-Ciocalteu's reagent was added, mixed well and incubated for 3 min. After 3 min, 1ml of saturated Na_2CO_3 solution was added and adjusted the solution to 10 ml with distil water (added 7ml distil water), mixed well and incubated in the dark for 90 min at room temperature. After 90 min incubation, the absorbance was measured against the blank (the same mixture without the Gallic acid) at 725nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in triplicate.

Preparation of sample solution

Sample stock solution of Washington extract (50 mg/ml) was prepared by dissolving 1.15g of extract in to 23 ml of ethanol and stock solution of Valencia extract (50 mg/ml) was prepared by dissolving 0.7 g of extract in to 14 ml of ethanol. From each stock solution, 20, 40, 80, 120, 160, 200 and 240µL of sample solution were withdrawn and mixed with 980, 960, 920, 880, 840, 800 and 760µL of methanol to get sample concentrations of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/ml respectively in separate test tubes. Then 1 ml of Folin-Ciocalteu's reagent was added, mixed well and incubated for 3 min. After 3 min, 1ml of saturated Na_2CO_3 solution was added and adjusted the solution to 10 ml with distil water (added 7ml distil water), mixed well and incubated in the dark for 90 min at room temperature. After 90 min incubation, the absorbance was measured against the blank (the same mixture without the extract) at 725nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in triplicate. The total phenol content was calculated and expressed as milligrams of Gallic acid equivalents (mg of GAL/g dry extract) using the Gallic acid calibration curve and the curve used to determine the corresponding Gallic acid concentration of the samples.

3.2.3.1.6. Determination of Total Flavonoid Content

The flavonoid content was determined according to aluminum chloride colorimetric method by modification of Singh (2014). Briefly, a dose of 0.25 mL of the extract or catechin standard solution was mixed with 0.75mL of methanol in a test tube, followed by adding 75μL of a 5% $NaNO_2$ solution. After 6 min, 150μL of a 10% $AlCl_3.6H_2O$ (freshly prepared) solution was added and allowed to stand for another 5 min before adding 0.5 mL of 1 M NaOH. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the sample) at 510 nm using a UV-Visible Spectrophotometer (UV-7804C). The results were calculated and expressed as milligrams of catechin equivalents (mg of CAE/g dry extract) using the calibration curve of catechin. Linearity range of the calibration curve was 10 to 1000 μg/mL (r = 0.99). The non-flavonoid polyphenols was taken as the difference between the total phenol and total flavonoid content (Omoba, 2015).

The total flavonoid content was expressed as milligrams of catechin equivalents (mg of CAE/g dry extract) using the calibration curve of catechin. The total flavonoid content was calculated by the following relationship:

$$T = \frac{C*V}{W} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (8)$$

Where C = Catechin equivalent concentration obtained from the calibration curve (mg/ml)

V= volume of stock solution of extract (ml)

W = dry weight of extract found in the stock solution (g)

T = total flavonoid content expressed as (mg of CAE/g dry extract)

Procedures

Preparation Reagents

5% $NaNO_2$ solution was prepared by dissolving 5 g of $NaNO_2$ in to 100 ml of distill water and 10% $AlCl_3.6H_2O$ was prepared by placing 10 mg $AlCl_3$ in to 100 ml of distill water. Similarly, 1 M NaOH was prepared by adding 0.4 g of NaOH in to 10 ml of distill water.

Preparation of blank solution

To the test tube containing 1ml of methanol, 75μL of a 5% $NaNO_2$ solution was added. After 6 min, 150µl of a 10% $AlCl_3.6H_2O$ was added followed by addition 1 M NaOH after another 5 min stand and the mixture was brought to 2.5 mL with distilled water and mixed well.

Preparation of standard solution

A stock solution (0.5 mg/ml) of catechin was prepared by dissolving 0.05 g of catechin in to 100 ml of methanol. Aliquots were withdrawn from the stock solution to get catechin concentrations 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 mg/ml by mixing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of catechin stock solution with 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and 0 ml of methanol respectively in the different test tubes. 75μL of a 5% $NaNO_2$ solution was added in each test tube. After 6 min, 150µl of a 10% $AlCl_3.6H_2O$ was added to each test tube. Again after another 5 min, 0.5 mL of 1 M NaOH was added and the mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the catechin) at 510 nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in triplicate. The standard catechin calibration curve was developed by plotting linear regression curve of absorbance versus catechin concentration (mg/ml).

Preparation of sample solution

Sample stock solution of Washington extract (3.9535 mg/ml) was prepared by dissolving 0.17g of extract in to 43 ml of ethanol and stock solution of Valencia extract (3.4884 mg/ml) was prepared by dissolving 0.15 g of extract in to 43 ml of ethanol. From each stock solution, 0.25 ml was withdrawn from the stock solution and mixed with 0.75 ml of methanol to get a sample concentration of 0.9884 mg/ml for Washington extract and 0.8721 mg /ml for Valencia extract in the distinct test tube. 75μL of a 5% $NaNO_2$ solution was added in each test tube. After 6 min, 150µl of a 10% $AlCl_3.6H_2O$ was added to each test tube. Again after another 5 min, 0.5 mL of 1 M NaOH was added and the mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the catechin) at 510 nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in triplicate.

3.2.3.1.7 Determination of DPPH Radical Scavenging Activity

Radical Scavenging Activity was determined according a modified version of the DPPH method by Singh (2014). Stock solution of the extract was prepared. 0.004% DPPH solution was prepared by dissolving 0.01 g DPPH dissolve in 250 ml methanol in volumetric flask. Then 1 mL of extract or standard ascorbic acid from stock solution was added into 4 mL of DPPH solution. The mixture was shaken vigorously and was left to stand in the dark for 30 minute. The absorbance of the resulting solution was measured using a UV-Visible Spectrophotometer (UV-7804C) at 517 nm by monitoring the decrease in absorbance. Ascorbic acid was used as standard. The scavenging activity of the extract was calculated using the formula: Scavenging activity % = 100 × (1– AS/AC), Where AS is absorbance of the solution, when extracts has been added at a particular level and AC is the absorbance of the DPPH solution, without extract or simply control (Singh, 2015).

Procedures

Preparation of reagent and blank solution

0.004% DPPH was prepared by dissolving 0.01 g of DPPH in to 250ml of methanol in volumetric flask. To the test tube containing 1ml of methanol, 4 ml of 0.004 % DPPH was added and mixed well.

Preparation of standard solution

Ascorbic acid was used as a positive control or standard for determination of radical scavenging activity. A stock solution (50mg/ml) of ascorbic acid was prepared by dissolving 2.5 g of ascorbic acid in to 50 ml of methanol. Aliquots were withdrawn from the stock solution to get ascorbic acid concentrations 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/ml by mixing 20, 40, 80, 120, 160, 200 and 240µL of standard ascorbic acid with 980, 960, 920, 880, 840, 800 and 760µL of methanol solvent respectively in dissimilar test tubes. Then 4 ml of 0.004% DPPH was added to each sample concentrations in the test tubes and mixed well then incubated the samples for 30 minute in the dark at room temperature. After 30 min incubation, the absorbance was measured against the blank (the same mixture without the ascorbic acid) at 517 nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in duplicate.

Preparation of sample solution

Sample stock solution of extract (50 mg/ml) was prepared by dissolving 1.15g of extract in to 23 ml of ethanol. From the stock solution, aliquots were withdrawn to get different concentrations. 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/ml of sample solution were prepared by mixing 20, 40, 80, 120, 160, 200 and 240 µL of aliquots from the stock solution with 980, 960, 920, 880, 840, 800 and 760µL of ethanol in different test tubes respectively. Then 4 ml of 0.004% DPPH was added to each sample concentrations in the test tubes and mixed well then incubated the samples for 30 min in the dark at room temperature. After 30 min incubation, the absorbance was measured against the blank (the same mixture without the ascorbic acid) at 517 nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in duplicate.

Free radical scavenging activity or inhibition activity of free radical DPPH percentage (IA %) was calculated using the following correlation:

$$IA\% = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} * 100$$

$$IA\% = \frac{AC - AS}{AC} * 100 \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(9)}$$

3.2.4 Experimental Design and Data Analysis

In this study, Design expert 6.0.8 software experimental design method, Response Surface Methodology (RSM) design was appropriate for second order model to determine the effect of the three operating variables of the extraction of antioxidant from orange peel waste. These operating variables were extraction time, extraction temperature and ethanol concentration. The response variable was the extraction yield. A second order model is generally used to approximate the response once it is realized that the experiment is close to the optimum response region where a first order model is no longer adequate. A second order model is usually sufficient for the optimum region.

Central composite designs in which the axial points represent the middle levels for all but one of the factors are referred to Face Centered Central Composite Designs **(FCCCD).** For these designs, α = 1 and all factors are run at three levels, which are -1, 0 and 1 in terms of the coded values. Face centered is desirable for this study because the experimenter have prior information

about the location of the optimum. Therefore; designs provide equal precision in all directions is not preferred in this case. The response variable was the extraction yield. Three levels, three factors and six times replication at center point was used, and total of 20 experiments were done. The independent variables were extraction temperature, extraction time and ethanol concentration and these variables were selected to optimize the conditions for extraction of antioxidant. The data required to optimize the process conditions for antioxidant extraction was collected by conducting the twenty experiments; and was analyzed by the Design Expert software 6.0.8 and suitable model equation was gained. And in order to minimize errors the experimental work was randomized.

The significant effect of treatment was judged with the help of 'F' (variance ratio). Calculated F value was compared with the table value of F at 5% level of significance. If calculated value exceeded the table value the effect will be considered to the significant. The significance of the study was tested at 5% level. The IC_{50} values, total phenol contents and total flavonoid contents were calculated from linear regression analysis.

Table 3.3 Selected factors and corresponding symbols and labels

Factors	Symbol	Labels		
		-1	0	1
Temperature (ºC)	A	25	42.5	60
Ethanol concentration(%v/v)	B	40	60	80
Time (h)	C	24	48	72

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results and discussion on physical properties orange peels

4.1.1 Moisture content of peels.

Moisture content was determined using a correlation in the equation (1). As illustrated in the table 4.1, the moisture content of both orange peel varieties was conducted in triplicates and the average moisture content was taken as Mean ± SD. From the result shown, 75.43% (w/w) of Valencia orange peel was weighed by liquid moisture content and similarly, 66.23% (w/w) of Washington orange peel was covered by liquid moisture mass. The moisture contained in Valencia peel was higher than the moisture contained in Washington orange peel. Conversely, 24.57% (w/w) of fresh Valencia orange peel was solid dry mass and 33.77% (w/w) of fresh Washington orange peel was constituted by solid dry mass. Hence, Washington orange peel had higher solid dry mass than Valencia orange peel.

Table 4.1 Moisture contents of orange peels

Peel	Fresh peel weight(g)	Dry peel weight(g)	Loss in weight(g)	Moisture content (%)	Mean ± SD (%)
Valencia Peel	1.0	0.246	0.754	75.4	75.43 ± 0.023
	1.0	0.244	0.756	75.6	
	1.0	0.248	0.752	75.3	
Washington Peel	1.0	0.336	0.664	66.4	66.23 ± 0.023
	1.0	0.338	0.662	66.2	
	1.0	0.340	0.660	66.1	

4.1.2 Ash contents of orange peel

The ash content was calculated the using correlation written in the equation (2). Ash content of the two orange peel varieties was shown in the table 4.2. Ash content was performed in triplicates. Mean with standard deviation was taken as average percentage ash content. 0.8% (w/w) of Valencia peel was constituted by ash and 0.8567% (w/w) of Washington peel was covered by ash. Hence, Washington orange peel had slightly higher inorganic ash content than

Valencia. In general interpretation from the result, the composition of ash content in both orange peel varieties was very low as compared to the total dry solid mass contained in the orange peels; deduce that orange peel varieties are better in phytochemical and other nutritive compounds.

Table 4.2 Ash contents of orange peels

Peel	Sample weight(g)	Residue weight(g)	Ash content (%)	Mean ± SD (%)
Valencia Peel	5.0	0.0415	0.83	0.8000 ± 0.0265
	5.0	0.039	0.78	
	5.0	0.0395	0.79	
Washington Peel	5.0	0.0435	0.87	0.8567 ±0.0153
	5.0	0.042	0.84	
	5.0	0.043	0.86	

4.2 Results and Discussion on Extraction Yield

Extraction of antioxidant extracts from orange peels was depending on the various factors that affect the extraction yield significantly. The factors selected in this study were extraction temperature, extraction time and concentration of ethanol solvent. All these factors were individually and interactively affect the extraction yield of phytochemical polyphenol compounds. The levels of extraction temperature were 25.00, 42.50 and 60.00 ºC, levels of extraction time were 24, 48 and 72 h, and levels of ethanol concentration were 40, 60 and 80%. For these three levels three factors, total of 20 experiments were conducted for each variety of orange peels and their corresponding extraction yields were illustrated in the table13 and 17.

4.2.1 Extraction Yield from Washington Peel

20 experimental runs were performed and their corresponding extraction yields were demonstrated in the table 4.3. The extraction yields obtained were ranged 18-29%. The minimum extraction yield was obtained on the experiment run number (13) at low levels of extraction parameters. On other hand, the maximum extraction yield was obtained on the three different experimental run numbers (1, 7 and 17). Operating extraction at center level of both temperature and concentration and high level of extraction time (experimental run number 1) gave highest yield. The highest yield also obtained by operating extraction at center level of both

concentration and time and high level of extraction temperature (experimental run number 7). Similarly, operating extraction at center level of both extraction temperature and time and high level of concentration (experimental run number 17) gave the maximum yield as shown in the table 4.3. The maximum yield obtained was compared with past investigations. Ethanol extract yield of 23.9% was obtained from orange peel (Singh, 2014). The maximum ethanol extract yield of present study was significantly higher than the result reported by (Singh, 2014). According to the study Ibrahium (2012), the maximum yields obtained from Baladi orange peel using methanol and ethanol as a solvent were 28.32% and 27.96% respectively. From the comparison, the maximum yield of the present study was nearly similar with methanol extract but slightly higher from ethanol extract obtained by Ibrahium (2012).

Increasing the temperature from low level to center level, diffusion of both ethanol and water soluble phytochemical compounds (polyphenols and flavonoids etc.) towards ethanol-water binary mixture was facilitated. The extraction yield was proportionally increasing. Increasing the temperature from center level to high level, there was still increasing in extraction yield but the rate of increasing in extraction yield was decreasing as increasing in extraction temperature towards to high level due to the degradation of previously extracted polyphenols as temperature increases. Further increased in extraction temperature above high level, extraction yield was decline automatically due to degradation rate of extracted phytochemical compounds was higher than the extraction rate of new phytochemical compounds at higher temperature as extraction time progress.

Increasing the ethanol concentration from low to center level, the rate of diffusion of ethanol soluble phytochemical compounds towards the solvent was increasing and increasing concentration from center to high level, the rate diffusion of polyphenols and flavonoids towards the solvent was higher due to the amount of ethanol soluble phytochemical compounds were increasing. Further increased ethanol concentration above high level affected the extraction yield in two ways. First, as concentration of ethanol increased, the concentration of water in the solvent decreased, the amount water soluble phytochemical compounds diffused towards solvent decreased. Secondly, as ethanol concentration increased higher and higher, the degradation of soluble polyphenols increased and the extraction yield started to decline. The advantage of

selecting ethanol- water mixture of binary solvent was to combine both water and ethanol soluble phytochemicals and were diffused towards the solvent.

Extraction yield was also depending on extraction temperature. Increasing the extraction time from low to center level, the amount of phytochemicals towards the solvent was increasing. But it was depending on the levels of both extraction temperature and concentration of ethanol. Operating extraction at low and center level of extraction temperature and increasing the extraction time from low to high level, the extraction yield was increasing. Operating the extraction at high level of extraction temperature or concentration and increasing the extraction time from center to high level and further, the total phytochemical compounds diffused were decreased due to degradation of these soluble compounds. Hence the extraction yield was declined.

Table 4.3 Extraction runs and corresponding response yield for Washington orange peel

Std	Run	Block	Factor1 A: Temperature (^{0}c)	Factor2 B:Concentration (% v/v)	Factor3 C: Time (hr)	Response1 Yield (%)
14	1	Block 1	42.50	60.00	72.00	29
11	2	Block 1	42.50	40.00	48.00	25.9
19	3	Block 1	42.50	60.00	48.00	28.4
4	4	Block 1	60.00	80.00	24.00	26.1
7	5	Block 1	25.00	80.00	72.00	26.2
5	6	Block 1	25.00	40.00	72.00	23.9
10	7	Block 1	60.00	60.00	48.00	29
18	8	Block 1	42.50	60.00	48.00	28.1
16	9	Block 1	42.50	60.00	48.00	28.6
8	10	Block 1	60.00	80.00	72.00	28
9	11	Block 1	25.00	60.00	48.00	25
13	12	Block 1	42.50	60.00	24.00	23.9
1	13	Block 1	25.00	40.00	24.00	18
2	14	Block 1	60.00	40.00	24.00	24.1
20	15	Block 1	42.50	60.00	48.00	28.2
6	16	Block 1	60.00	40.00	72.00	28.1

12	17	Block 1	42.50	80.00	48.00	29
3	18	Block 1	25.00	80.00	24.00	22
15	19	Block 1	42.50	60.00	48.00	28.1
17	20	Block 1	42.50	60.00	48.00	28.2

4.2.1.1 Results and Discussion on Experimental Design Analysis

The experimental design selected for analysis of variance was surface methodology (RSM). Under response surface methodology, central composite design (CCD) was selected, specifically, face centered central composite design (FCCCD) in which the alpha value is face centered (α = 1). The response of the analysis was extraction yield. The response yield was ranged from 18 to 29%. The ratio of maximum to minimum yield was 1.61111. A ratio greater than 10 usually indicates a transformation is required. For a ratio less than 3, the power transformations have little effect. The aim of model fit summary was maximizing the "adjusted-R square" and the "predicted-R square". The model assumed for analysis of variance was response surface quadratic model and ANOVA was performed using Partial sum of squares methods. Model significance was checked for both model and model factors, linear model factors temperature (A), concentration (B) and time (C) and, quadratic model factors; pure quadratic terms (A^2, B^2, C^2) and interaction quadratic terms (AB, AC, BC) depending on the F and P values.

Table 4.4 Analysis of variance for Washington extract

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Model significance
Model	**155.72**	**9**	**17.30**	**149.94**	**< 0.0001**	**significant**
A	40.80	1	40.80	353.59	< 0.0001	
B	12.77	1	12.77	110.65	< 0.0001	
C	44.52	1	44.52	385.80	< 0.0001	
A^2	3.96	1	3.96	34.32	0.0002	
B^2	1.55	1	1.55	13.40	0.0044	
C^2	8.42	1	8.42	72.98	< 0.0001	
AB	2.42	1	2.42	20.97	0.0010	
AC	2.21	1	2.21	19.11	0.0014	

BC	1.81	1	1.81	15.64	0.0027	
Residual	**1.15**	**10**	**0.12**			
Lack of Fit	0.96	5	0.19	4.97	0.0516	not significant
Pure error	0.19	5	0.039			
Cor total	**156.88**	**19**				

From the table 4.4, the model F-value of 149.94 implies the model is significant. There was only a 0.01% chance that a "model F-value" this large could be occur due to noise. Value of "Prob > F" less than 0.0500 indicate the model terms are significant. In this case, A, B, C, A^2, B^2, C^2, AB, AC, BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The "lack of Fit F-value" of 4.97 implies there is a 5.16% chance that a "lack of Fit F-value" this large could occur due to noise. Lack of Fit is not significant is good model.

Std. Dev.	0.34	R-Squared	0.9926
Mean	26.39	Adj R-Squared	0.9860
C.V.	1.29	Pred R-Squared	0.9492
PRESS	7.97	Adeq Precision	47.167

The "Pred R-Squared" of 0.9492 is in reasonable agreement with the "Adj R-Squared" of 0.9860. The "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case, the ratio 47.167 indicates an adequate signal. This model can be used to navigate the design space.

4.2.1.1.1 Development of Model equation

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables. The application of RSM offers an empirical relationship between the response function and the independent variables. The mathematical relationships between the response and the independent variables temperature (A), concentration (B) and time (C) in terms of coded and actual factors can be determined by Design Expert software. The model equation that correlates the response (Y) to the extraction process variables in terms of coded factors after excluding the insignificant terms was given in equation.

Table 4.5 Model equation coefficients for Washington peel extract

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	28.24	1	0.12	27.98	28.50	
A-Temperature	2.02	1	0.11	1.78	2.26	1.00
B-Concentration	1.13	1	0.11	0.89	1.37	1.00
C-Time	2.11	1	0.11	1.87	2.35	1.00
A^2	-1.20	1	0.20	-1.66	-0.74	1.82
B^2	-0.75	1	0.20	-1.21	-0.29	1.82
C^2	-1.75	1	0.20	-2.21	-1.29	1.82
AB	-0.55	1	0.12	-0.82	-0.28	1.00
AC	-0.53	1	0.12	-0.79	-0.26	1.00
BC	-0.48	1	0.12	-0.74	-0.21	1.00

Final equation in terms coded factors:

$$\begin{aligned} \text{Yield} = & \\ & +28.24 \\ & +2.02 * A \\ & +1.13 * B \\ & +2.11 * C \\ & -1.20 * A^2 \\ & -0.75 * B^2 \\ & -1.75 * C^2 \\ & -0.55 * A * B \\ & -0.53 * A * C \\ & -0.48 * B * C \end{aligned}$$

The importance of the model developed equation was evaluated from their coefficients of correlation. As shown in the final equation in terms of coded factors, the response yield was affected by both linear terms (A, B, C) and quadratic terms, pure quadratic terms (A^2, B^2, and C^2)

and interaction quadratic terms (AB, AC, BC). All coefficients of linear terms were positive and the response yield was positively affected by linear terms but the coefficients of interaction terms were negative and the response yield was negatively affected by quadratic terms. From the linear effects, concentration had highest effect on response yield. Similarly, pure quadratic term of concentration (C^2) had highest effect on response yield from negative quadratic effects.

Final equation in terms of actual factors:

Yield =

-14.51041

+0.60278 * Temperature

+0.39579 * Concentration

+0.49208 * Time

-3.91837E-003 * Temperature^2

-1.87500E-003 * Concentration^2

-3.03819E-003 * Time^2

-1.57143E-003 * Temperature * Concentration

- 1.25000E-003 * Temperature * Time

- 9.89583E-004 * Concentration * Time

4.2.1.1.2 Model Adequacy Checking

Model adequacy can be easily investigated by the examination of residuals. Examination of the residuals should be an automatic part of any analysis of variance. If the model is adequate, the residuals should be structureless; that is, they should contain no obvious patterns. Through a study of residuals, many types of model inadequacies and violations of the underlying assumptions can be discovered.

An extremely useful procedure is to construct a normal probability plot of the residuals. A normal probability plot of the raw data is used to check the assumption of normality when using the t-test. In the analysis of variance, it is usually more effective (and straightforward) to do this with the residuals. If the underlying error distribution is normal, the plot will resemble a straight

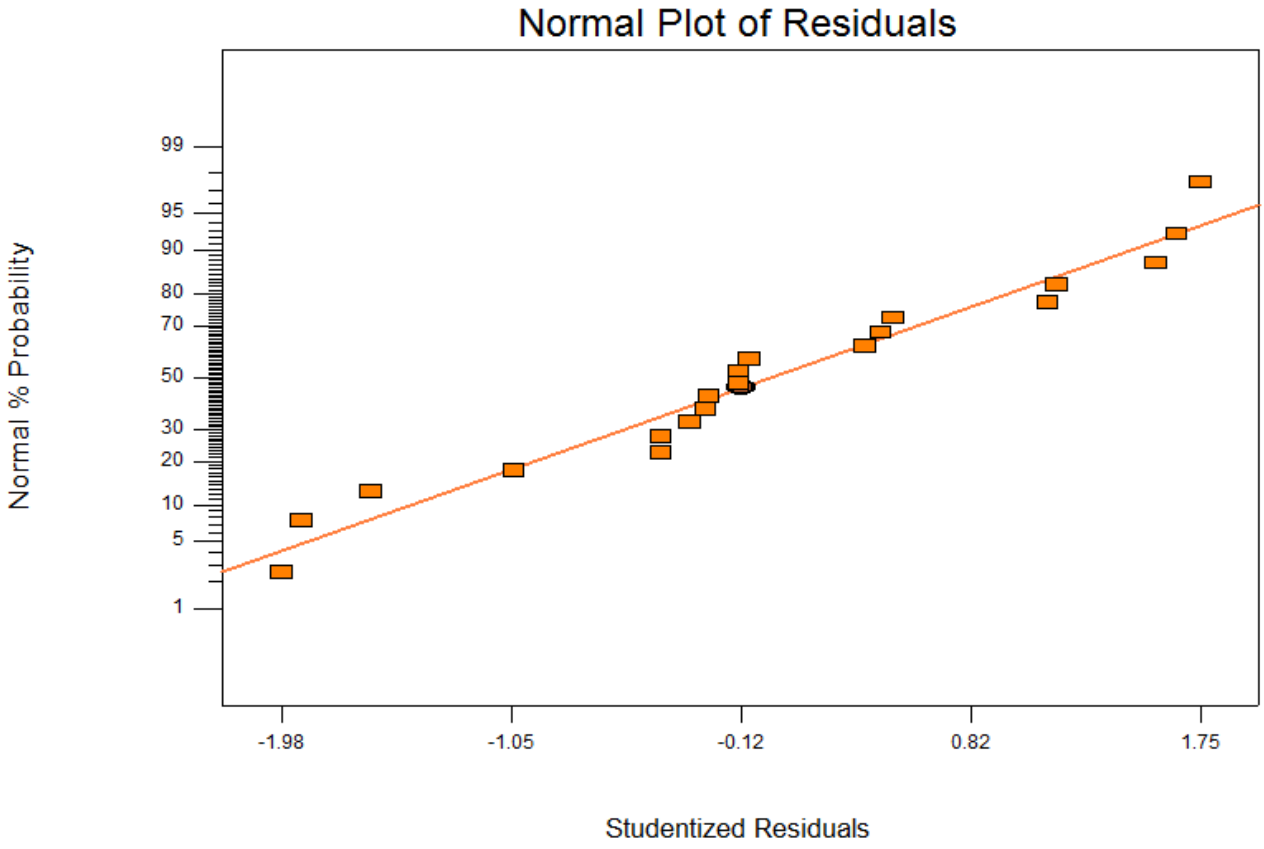
line. In visualizing the straight line, place more emphasis on the central values of the plot than on the extremes.

As illustrated in the figure 4.1 (a), the general impression from examining the display was that the error distribution was slightly skewed at both extreme tails (the right and the left tail) than the normal distribution. The degree of skewed distribution of left tail was slightly higher than the right; with the right tail longer than the left tail. The tendency of the normal probability plot to bend down slightly on the left and bend up slightly on the right side implies that both the left and right tails of the error distribution were somewhat thinner than would be anticipated in a normal distribution but the error distribution in the right tail was slightly thicker than the left tail, that is, the negative residuals were not quite as large as expected. The plot was not grossly non-normal in terms of normal distribution.

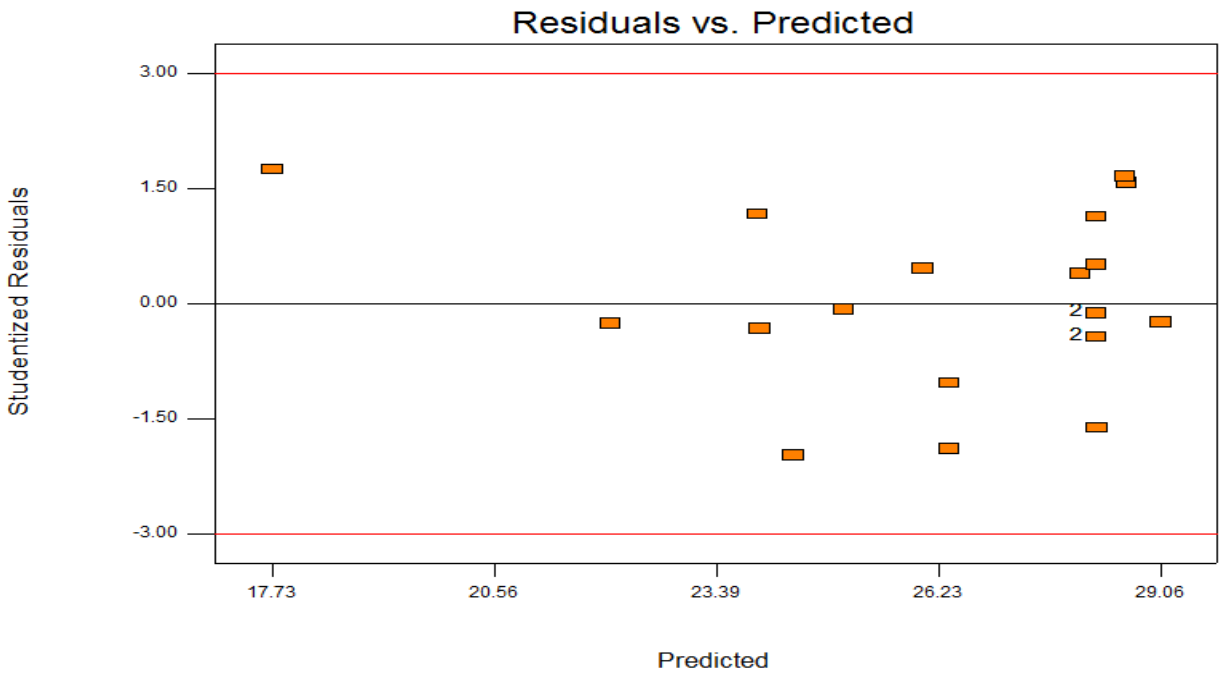
A very common defect that often shows up on normal probability plots is one residual that is very much larger than any of the others. Such a residual is often called an outlier**.** The presence of one or more outliers can seriously distort the analysis of variance, so when a potential outlier is located, careful investigation is mandatory. Frequently, the cause of the outlier is a mistake in calculations or a data coding or copying error. If this is not the cause, the experimental circumstances surrounding this run must be carefully studied. If the outlying response is a particularly desirable value, the outlier may be more informative than the rest of the data. It should be careful not to reject or discard an outlying observation unless we have reasonably non-statistical grounds for doing so. At worst, you may end up with two analyses; one with the outlier and one without. A rough check for outliers may be made by examining the standardized residuals**.** If the errors are N (0, $\sigma 2$*),* the standardized residuals should be approximately normal with mean zero and unit variance. Thus, about 68 percent of the standardized residuals should fall within the limits ± 1, about 95 percent of them should fall within ±2, and fundamentally all of them should fall within ± 3. A residual bigger than 3 or 4 standard deviations from zero is a potential outlier (Montgomery, 2001).

As illustrated in the table 16 and figure 4.1 (c), 55 percent of the standardized residuals were fall within ± 1 standard deviation from zero, 90 percent of the standardized residuals were fallen with in ± 2 standard deviation from zero and 100 percent of standard residuals were fallen in the range

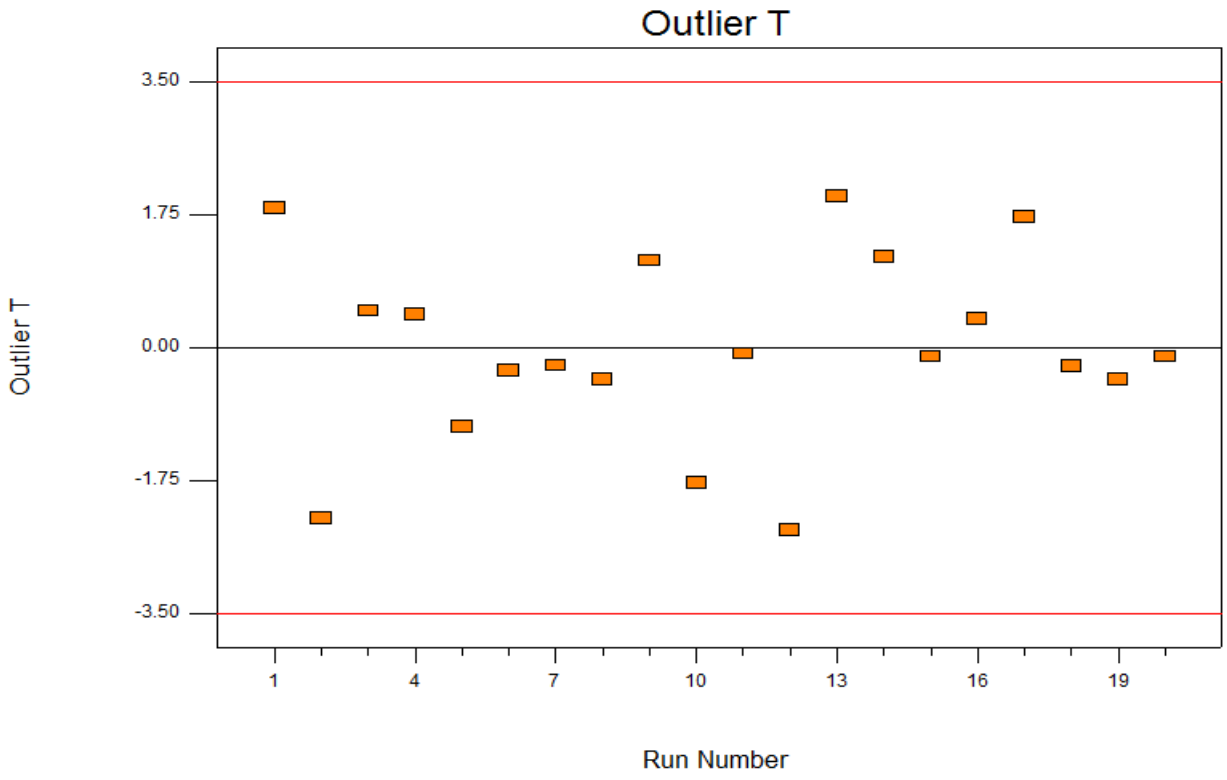
of ± 3. As shown in the figure 4.1 (c), absolute value of a residual bigger than 3.5 standard deviations from zero was a potential outlier. But all of the standard residuals were fallen in the interval of ±3 standard deviation from zero and constitute 100 percent of standard residuals, indicates 100 percent of the residuals were less than ±3 and all were closely lying in the normal % probability plot. The adequacy of the model depends on the percent of standard residuals lying normal % probability curve. Based on the information, the plot was normal and the assumed regression model was a 100 percent adequate in terms of outlier.



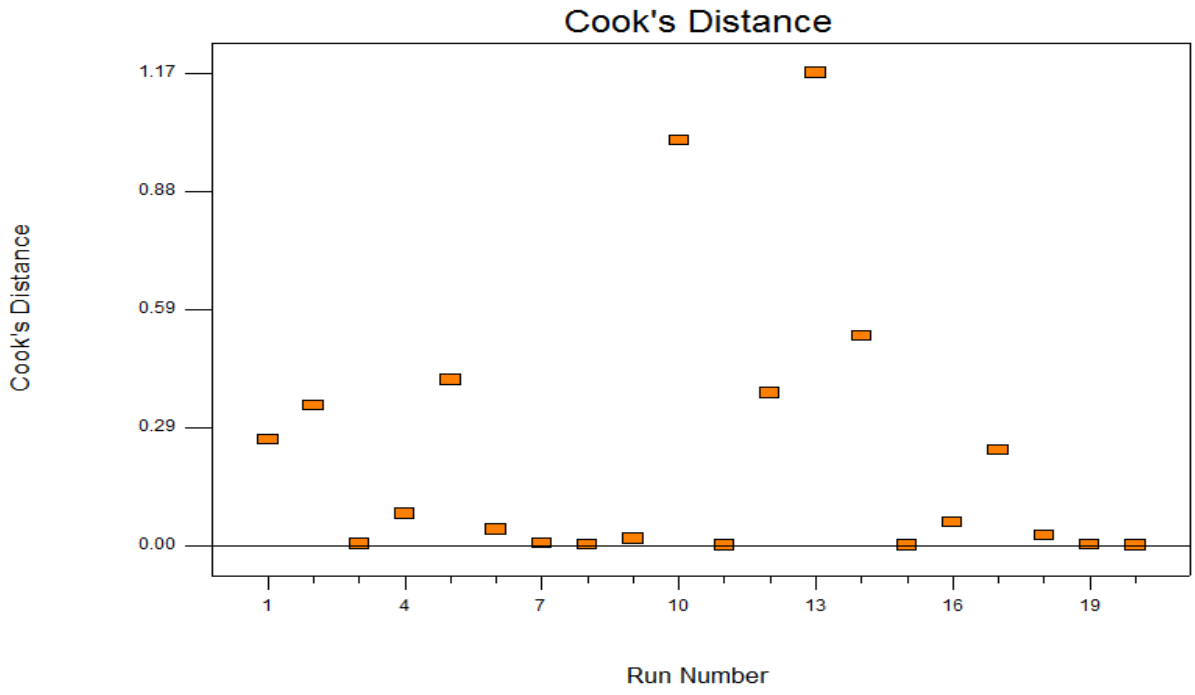
(a) Normal plot of residuals



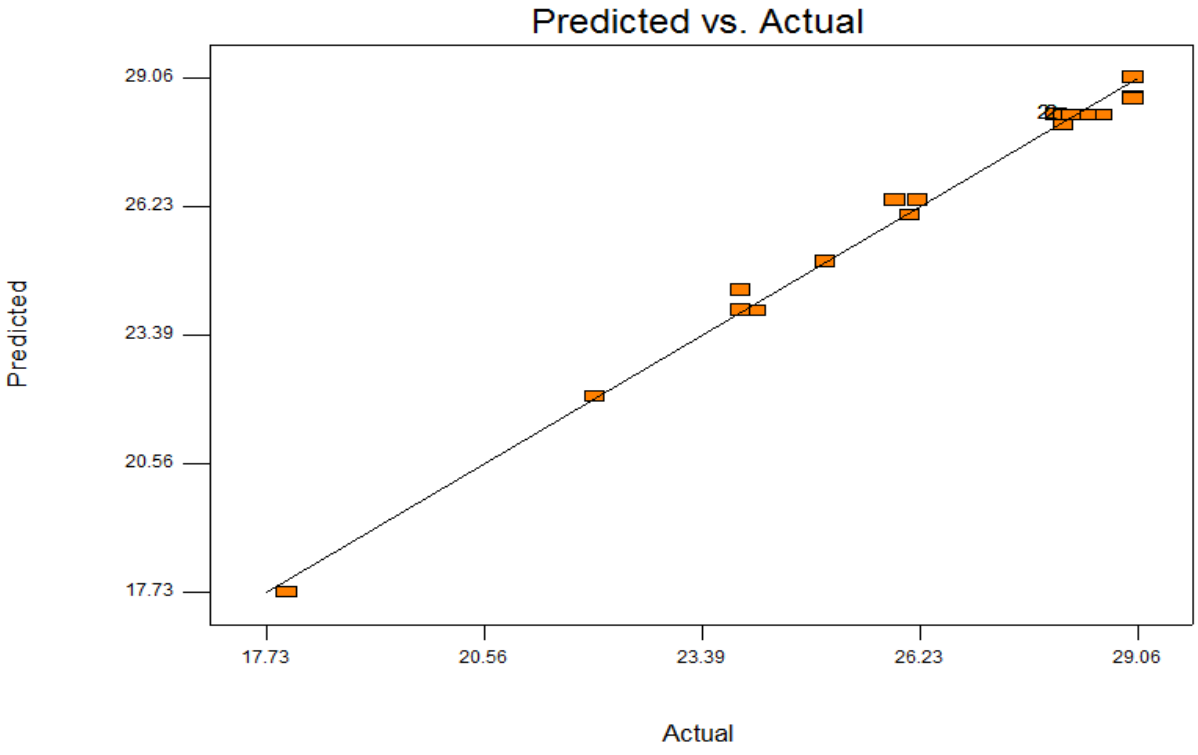
(b) Residual versus predicted plot



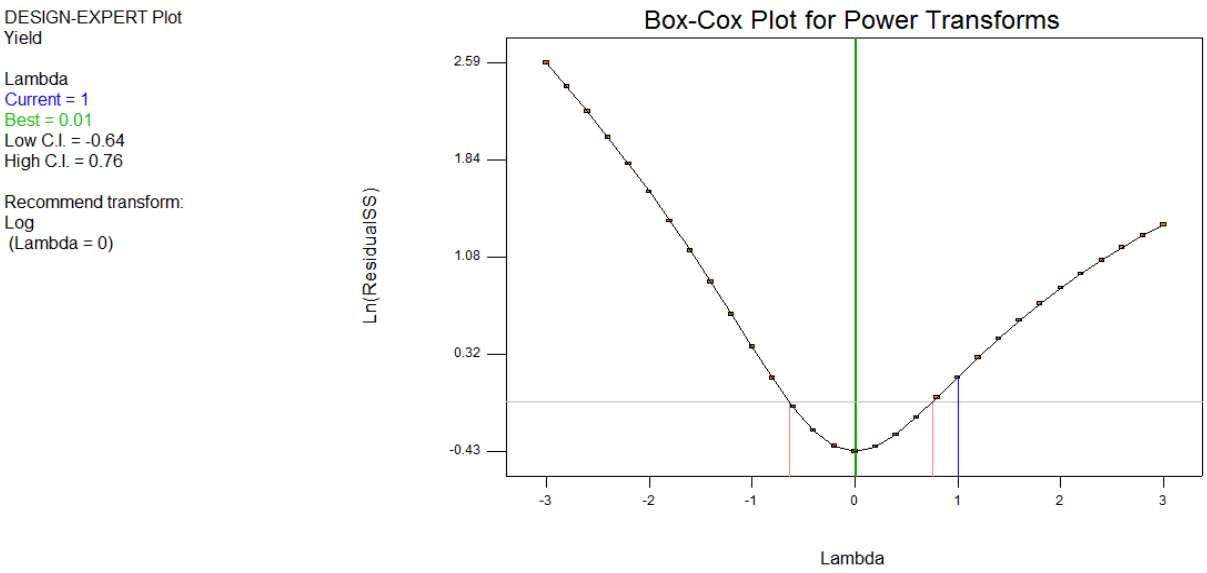
(c) Plot of outlier from normal distribution



(d) Plot of cook's distance



(e) Predicted versus actual residuals plot



(f) Plot of Box-cox for power transformation

Figure 4.1 Diagnostic plots (a),(b),(c),(d),(f) of adequacy checking for Washington peel

In general, moderate departure of residuals from normality is of little concern in the fixed effects analysis of variance. An error distribution that had considerably thicker or thinner tails than the normal is of more concern than a skewed distribution as shown in the figure 4.1 (f). Because the *F* test is only slightly affected, the analysis of variance (and related procedures such as multiple comparisons) is robust to the normality assumption. Departures from normality usually cause both the true significance level and the power to differ slightly from the advertised values, with the power generally being lower

Table 0.6 Diagnostics checking Statistics

Standard Order	Actual Vlue	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	18.00	17.73	0.27	0.793	1.748	1.171	1.989	13
2	24.10	23.92	0.18	0.793	1.165	0.521	1.189	14
3	22.00	22.04	-0.040	0.793	-0.259	0.026	-0.246	18
4	26.10	26.03	0.070	0.793	0.453	0.079	0.434	4
5	23.90	23.95	-0.050	0.793	-0.324	0.040	-0.309	6
6	28.10	28.04	0.060	0.793	0.388	0.058	0.371	16
7	26.20	26.36	-0.16	0.793	-1.036	0.411	-1.040	5

8	28.00	28.25	-0.25	0.793	-1.618	1.004	-1.787	10
9	25.00	25.02	-0.020	0.491	-0.083	0.001	-0.078	11
10	29.00	29.06	-0.060	0.491	-0.248	0.006	-0.236	7
11	25.90	26.36	-0.46	0.491	-1.898	0.347	-2.251	2
12	29.00	28.62	0.38	0.491	1.568	0.237	1.713	17
13	23.90	24.38	-0.48	0.491	-1.980	0.378	-2.410	12
14	29.00	28.60	0.40	0.491	1.650	0.263	1.835	1
15	28.10	28.24	-0.14	0.118	-0.439	0.003	-0.420	19
16	28.60	28.24	0.36	0.118	1.129	0.017	1.146	9
17	28.20	28.24	-0.040	0.118	-0.125	0.000	-0.119	20
18	28.10	28.24	-0.14	0.118	-0.439	0.003	-0.420	8
19	28.40	28.24	0.16	0.118	0.502	0.003	0.482	3
20	28.20	28.24	-0.040	0.118	-0.125	0.000	-0.119	15

4.2.2. Extraction Yield from Valencia Peel

Similarly, 20 experimental runs were performed and their corresponding extraction yields were demonstrated in the table 4.7. The extraction yield was ranged 14.4-25.9%. The minimum extraction yield was obtained on the experiment run number (15) at low levels of extraction parameters. On other hand, the maximum extraction yield was obtained on the two different experiment run numbers (4 and 13). Operating extraction at center level of temperature and concentration and high level of extraction time gave highest yield. Similarly, operating extraction at center level of both extraction temperature and time and high level of concentration gave the maximum yield as shown in the table 4.7. According to the investigation by Singh (2014), 23.9% of ethanol extraction yield was obtained from orange peel and the maximum ethanol extract yield of present study was compared with the result reported by (Singh, 2014). The ethanol extraction yield of the present study was significantly higher than the result shown by Singh (2014). According to the study Ibrahium (2012), the maximum yields obtained from Baladi orange peel using methanol and ethanol as a solvent were 28.32% and 27.96%. As compared with the present study, the maximum yield of the present study was significantly lower than the result obtained by Ibrahium (2012).

Table 4.7 Number of runs and corresponding extraction yields for Valencia peel

Std	Run	Block	Factor1 A: Temperature (^{0}c)	Factor2 B:Concentration (% v/v)	Factor3 C: Time (hr)	Response1 Yield (%)
20	1	Block 1	42.50	60.00	48.00	25.8
18	2	Block 1	42.50	40.00	48.00	25.2
19	3	Block 1	42.50	60.00	48.00	25.8
12	4	Block 1	42.50	80.00	48.00	25.9
17	5	Block 1	42.50	60.00	48.00	25.6
7	6	Block 1	25.00	80.00	72.00	22.8
5	7	Block 1	25.00	40.00	72.00	22.3
8	8	Block 1	60.00	80.00	72.00	25.8
6	9	Block 1	60.00	40.00	72.00	22.8
4	10	Block 1	60.00	80.00	24.00	24.4
11	11	Block 1	42.50	40.00	48.00	23.2
3	12	Block 1	25.00	80.00	24.00	17.6
14	13	Block 1	42.50	60.00	72.00	25.9
13	14	Block 1	42.50	60.00	24.00	21.8
1	15	Block 1	25.00	40.00	24.00	14.4
15	16	Block 1	42.50	60.00	48.00	25.6
9	17	Block 1	25.00	60.00	48.00	21.8
10	18	Block 1	60.00	60.00	48.00	25.8
2	19	Block 1	60.00	40.00	24.00	17.8
16	20	Block 1	42.50	60.00	48.00	25.7

4.2.2.1 Results and discussion on experimental design analysis

The response selected for analysis of variance for current study was extraction yield. The response yield was ranged from 14.4 to 25.9 % as observed in table 4.7. The ratio of maximum to minimum yield was 1.79861. A ratio greater than 10 usually indicates a transformation is required. For a ratio less than 3, the power transformations have little effect. The focus of Model fit summery was maximizing the "adjusted R-squared" and the "predicted R-squared". The

process order of the model was response surface quadratic model for analysis of variance. The model assumed for analysis of variance was response surface quadratic model and ANOVA was performed using Partial sum of squares methods. Model significance was checked for both model and specifically for model factors, linear model factors temperature (A), concentration (B) and time (C) and, quadratic model factors; pure quadratic terms (A^2, B^2, C^2) and interaction quadratic terms (AB, AC, BC) depending on the F-value and P-value.

Table 4.8 Analysis of variance for Valencia extract

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Model significance
Model	**206.76**	**9**	**22.97**	**261.50**	**< 0.0001**	**Significant**
A	31.33	1	31.33	356.61	< 0.0001	
B	25.60	1	25.60	291.40	< 0.0001	
C	55.70	1	55.70	633.97	< 0.0001	
A^2	9.00	1	9.00	102.45	< 0.0001	
B^2	3.08	1	3.08	35.11	0.0001	
C^2	8.51	1	8.51	96.86	< 0.0001	
AB	4.35	1	4.35	49.53	< 0.0001	
AC	5.61	1	5.61	63.87	< 0.0001	
BC	4.96	1	4.96	56.47	< 0.0001	
Residual	**0.88**	**10**	**0.088**			
Lack of Fit	0.63	5	0.13	2.54	0.1649	not significant
Pure error	0.25	5	0.050			
Cor total	**207.64**	**19**				

As illustrated in table 4.8, the Model F-value of 261.50 implies the model was significant. There was only a 0.01% chance that a "Model F-value" this large could be occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2, B^2, C^2, AB, AC, BC were significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 2.54 implies the Lack of Fit was not significant relative to the pure error. There was a 16.49% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good so the model can be fitted. The model fit summary statistiscs were listed as follows.

Std. Dev.	0.30	R-Squared	0.9958
Mean	23.30	Adj R-Squared	0.9920
C.V.	1.27	Pred R-Squared	0.9673
PRESS	6.79	Adeq Precision	56.520

The "Pred R-Squared" of 0.9673 was in reasonable agreement with the "Adj R-Squared" of 0.9920. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case, the ratio of 56.520 indicates an adequate signal. This model can be used to navigate the design space.

4.2.2.1.1 Development of Model equation

The application of RSM offers an empirical relationship between the response function and the independent variables. The mathematical relationships between the response and the independent variables temperature (A), concentration (B) and time (C) in terms of coded and actual factors can be determined by Design Expert software. The model equation that correlates the response (Y) to the extraction process variables in terms of coded factors after excluding the insignificant terms was given in equation.

Table 4.9 Estimated model equation coefficients for Valencia extract

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	25.61	1	0.10	25.39	25.84	
A-Temperature	1.77	1	0.094	1.56	1.98	1.00
B-Concentration	1.60	1	0.094	1.39	1.81	1.00
C-Time	2.36	1	0.094	2.15	2.57	1.00
A^2	-1.81	1	0.18	-2.21	-1.41	1.82
B^2	-1.06	1	0.18	-1.46	-0.66	1.82
C^2	-1.76	1	0.18	-2.16	-1.36	1.82
AB	-0.74	1	0.10	0.50	0.97	1.00

AC	-0.84	1	0.10	-1.07	-0.60	1.00
BC	-0.79	1	0.10	-1.02	-0.55	1.00

Final Equation in Terms of Coded Factors:

$$\begin{aligned}\text{Yield} = & \\ & +25.61 \\ & +1.77 * A \\ & +1.60 * B \\ & +2.36 * C \\ & -1.81 * A^2 \\ & -1.06 * B^2 \\ & -1.76 * C^2 \\ & +0.74 * A * B \\ & -0.84 * A * C \\ & -0.79 * B * C\end{aligned}$$

From the regression model equation developed in terms of coded factors, the response yield was affected by linear terms temperature (A), concentration (B) and time (C) and, quadratic terms, pure quadratics terms (A^2, B^2, C^2) and interaction quadratic terms (AB, AC, BC). On the basis of the coefficients in equations, it was evident that the percentage of response yield increases with the temperature (A), extraction time (B), concentration (C). Temperature, concentration and time have positive linear effect on extraction yield but concentration has a more weighty linear effect on yield as compared to extraction temperature and time. Pure quadratic terms (A^2, B^2, and C^2) also has negative effect on the response yield but the effect of pure quadratic term (A^2) has substantial effect than the other quadratic terms. Interaction of temperature and concentration (AB) has positive quadratic effect on response yield. Interaction of temperature and time (AC) and interaction of concentration and time (BC) have negative quadratic effect on yield.

Final Equation in Terms of Actual Factors:

$$\begin{aligned}\text{Yield} = & \\ & -18.86270 \\ & +0.57254 * \text{Temperture} \\ & +0.38692 * \text{Concentration} \\ & +0.57470 * \text{Time}\end{aligned}$$

-5.90724E-003 * Temperture^2

-2.64773E-003 * Concentration^2

-3.05398E-003 * Time^2

+2.10714E-003 * Temperture * Concentration

-1.99405E-003 * Temperture * Time

-1.64062E-003 * Concentration * Time

4.2.2.1.2 Model Adequacy Checking

A check of the normality assumption could be made by plotting a histogram of the residuals. If the NID (0, σ^2) assumption on the errors is satisfied, the plot should look like a sample from a non-normal distribution centered at zero. Unfortunately, with small samples, considerable fluctuation often occurs, so the appearance of a moderate departure from normality does not necessarily imply a serious violation of the assumptions. Gross deviations from normality are potentially serious and require further analysis.

An extremely useful procedure is to construct a normal probability plot of the residuals. A normal probability plot of the raw data is used to check the assumption of normality using the t-test. In the analysis of variance, it is usually more effective (and straightforward) to do this with the residuals. If the underlying error distribution is normal, the plot will resemble a straight line. In visualizing the straight line, place more emphasis on the central values of the plot than on the extremes.

As shown in the figure 4.2 (a), the error distribution was similar with normal error distribution at both right and left tails. The tendency of the normal probability plot was tend to straight line implies that the error distribution was somewhat similar with the anticipated normal distribution; that was both positive and negative residuals were quite as large (in absolute value) as expected. This plot was normal in terms of error distribution.

The other parameter used to check the adequacy of plot is the presence absence of outliers. Outlier is a very common defect that often shows up on normal probability plots in which one residual that is very much larger than any of the others. The presence of one or more outliers can seriously distort the analysis of variance, so when a potential outlier is located, careful investigation is mandatory. Frequently, the cause of the outlier is a mistake in calculations or a

data coding or copying error. If this is not the cause, the experimental circumstances surrounding this run must be carefully studied. If the outlying response is a particularly desirable value, the outlier may be more informative than the rest of the data. It should be careful not to reject or discard an outlying observation unless we have reasonably non-statistical grounds for doing so. At worst, it may end up with two analyses; one with the outlier and one without. A rough check for outliers may be made by examining the standardized residuals**.** If the errors are N (0, σ2*)*, the standardized residuals should be approximately normal with mean zero and unit variance. Thus, about 68 percent of the standardized residuals should fall within the limits ± 1, about 95 percent of them should fall within ±2, and fundamentally all of them should fall within ± 3. A residual bigger than 3 or 4 standard deviations from zero is a potential outlier (Montgomery, 2001).

As illustrated in the table 4.10 or figure 4.2 (d), 55 percent of the standard residuals were fallen within ± 1 standard deviation from zero, 90 percent of the standardized residuals were fall within ± 1.75 standard deviation from zero, 90 percent of the standardized residuals were fallen with in ± 2 standard deviation from zero, 90 percent of the standardized residuals were fallen with in ± 3 standard deviation from zero and 100 percent of standard residuals were fallen in the range of ± 3.5. As shown in the figure 4.2 (c), absolute value of a residual bigger than 3.5 standard deviations from zero was a potential outlier. But all of the standard residuals were fallen in the interval of ±3.5 standard deviation from zero and constitute 100 percent of standard residuals, indicates 100 percent of the residuals were less than ±3.5 and all were closely lying in the normal % probability plot. The adequacy of the model depends on the percent of standard residuals lying normal % probability curve. Based on this information, the assumed regression model was 100 percent adequate in terms of outlier. The holistic approach of the plot was normal.

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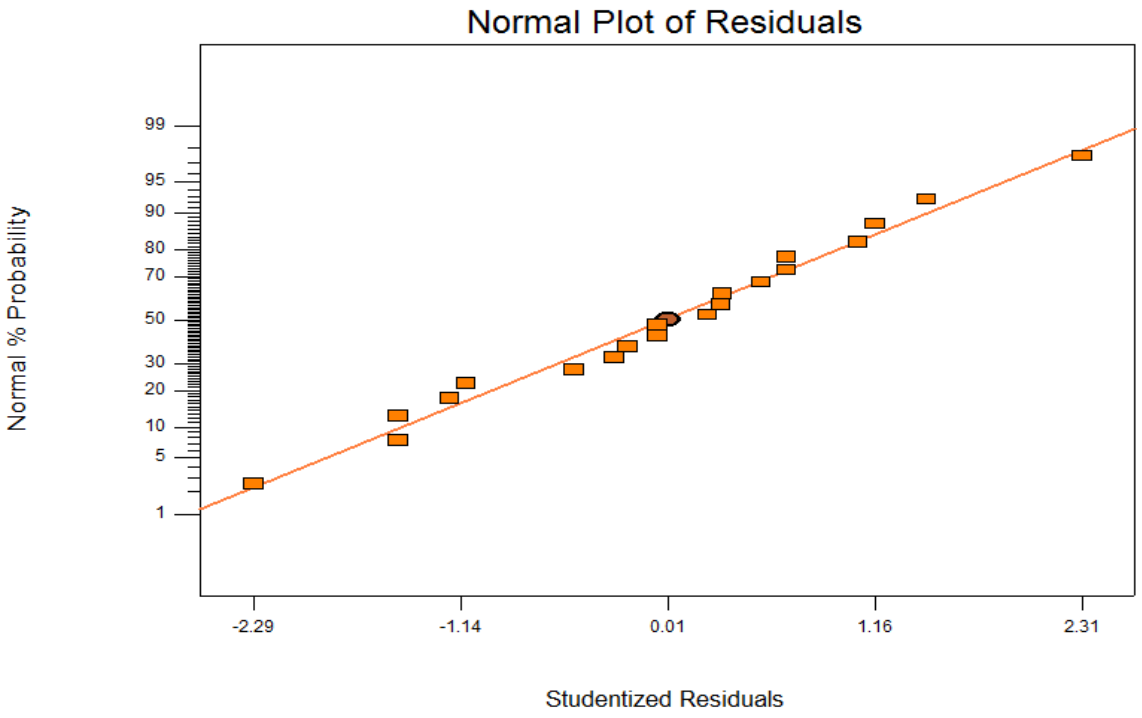
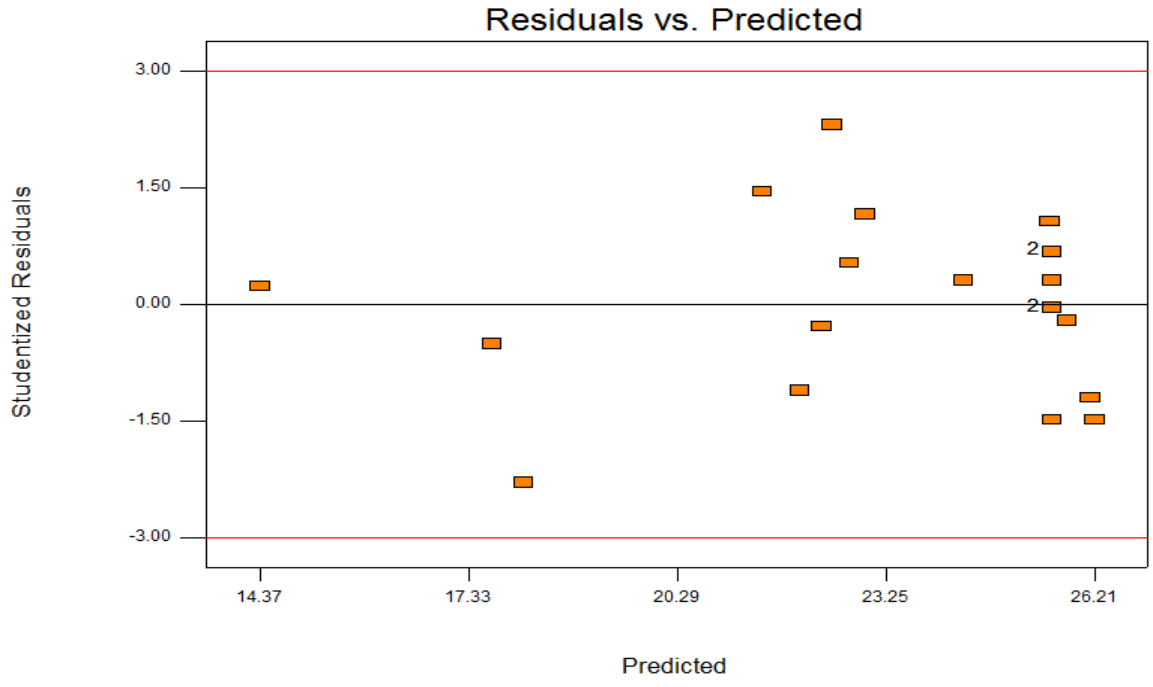
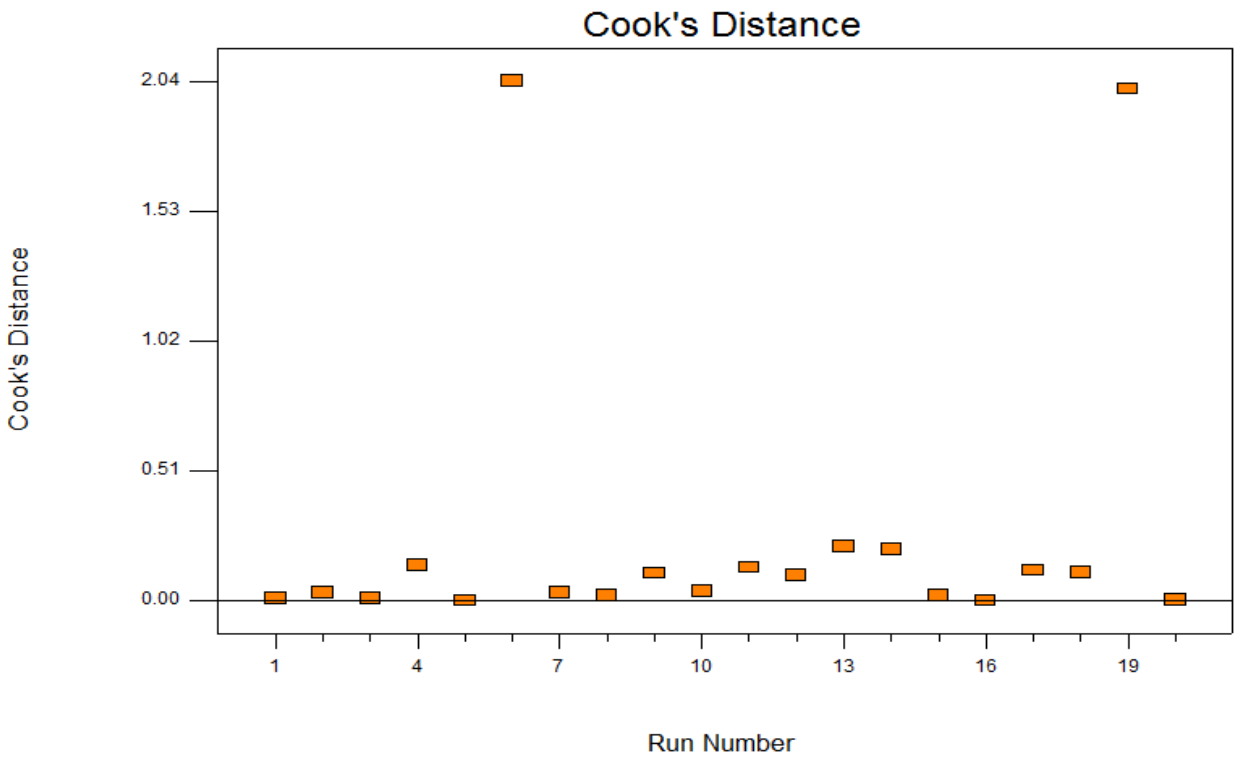


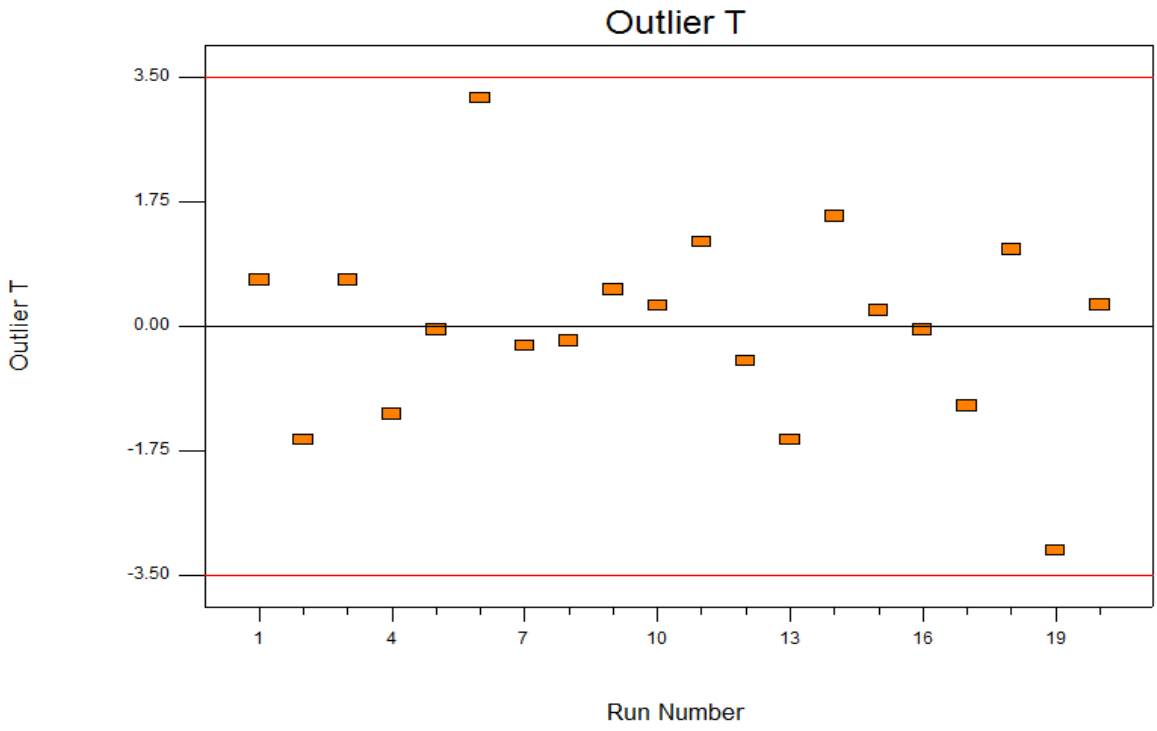
(a) Normal plot of residuals



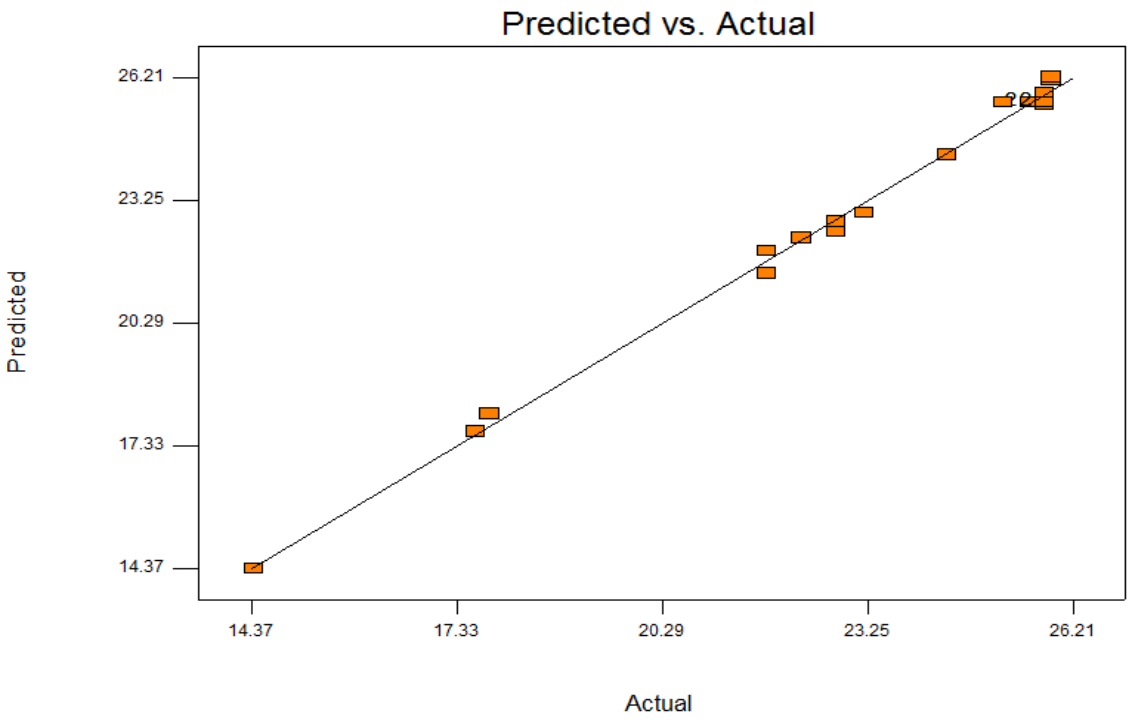
(b) Plot of residual versus predicted



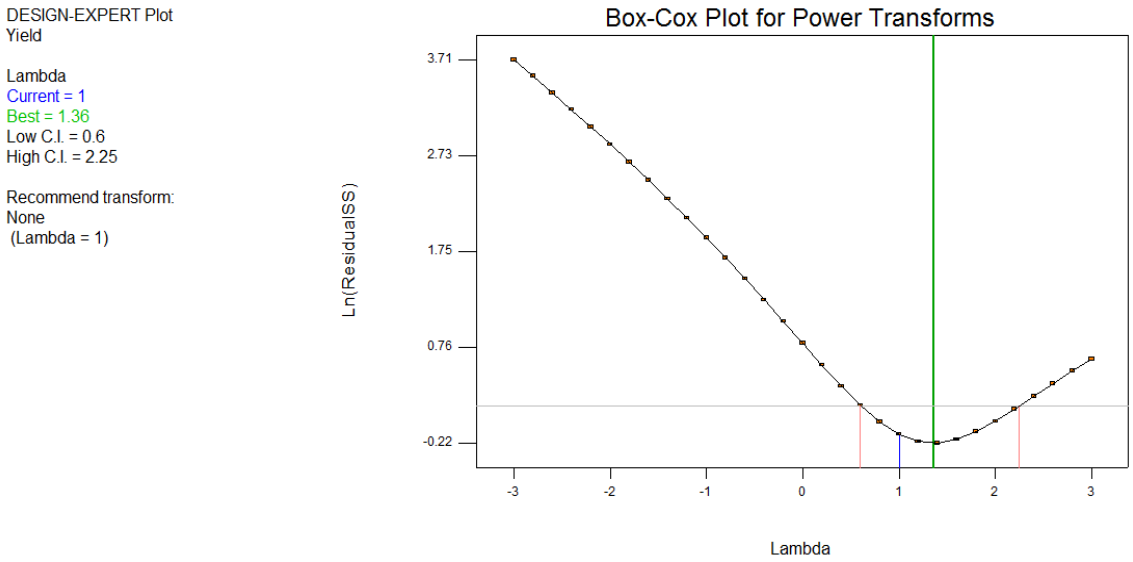
(c) Polt of cook's distance



(d) Plot of outliers from the normal distribution



(e) Plot of predicted versus actual residuals.



(f) Box-cox plot for power transformation

Figure 4.2 plots of diagnostics (a) (b), (c), (d), (e) (f) for adequacy checking for Valencia peel

Sometimes the plot of residuals versus predicted has a curvilinear shape; there may be a tendency for negative residuals to occur with low predicted values, positive residuals with intermediate predicted values, and negative residuals with high predicted values. This type of

pattern is suggestive of interaction between blocks and treatments. If this pattern occurs, a transformation should be used in an effort to eliminate or minimize the interaction

Table 4.10 Diagnostics checking Statistics for Valencia extract

Standard Order	Actual Vlue	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	14.40	14.37	0.031	0.793	0.231	0.020	0.220	15
2	17.80	18.11	-0.31	0.793	-2.291	2.014	-3.154	19
3	17.60	17.67	-0.069	0.793	-0.511	0.100	-0.491	12
4	24.40	24.36	0.041	0.793	0.305	0.036	0.291	10
5	22.30	22.34	-0.039	0.793	-0.288	0.032	-0.275	7
6	22.80	22.73	0.071	0.793	0.528	0.107	0.508	9
7	22.80	22.49	0.31	0.793	2.308	2.043	3.204	6
8	25.80	25.83	-0.029	0.793	-0.214	0.018	-0.204	8
9	21.80	22.03	-0.23	0.491	-1.109	0.119	-1.124	17
10	25.80	25.57	0.23	0.491	1.066	0.110	1.074	18
11	23.20	22.95	0.25	0.491	1.161	0.130	1.184	11
12	25.90	26.15	-0.25	0.491	-1.204	0.140	-1.235	4
13	21.80	21.49	0.31	0.491	1.444	0.201	1.540	14
14	25.90	26.21	-0.31	0.491	-1. 487	0.213	-1.599	13
15	25.60	25.61	-0.014	0.118	-0.049	0.000	-0.046	16
16	25.70	25.61	0.086	0.118	0.310	0.001	0.296	20
17	25.60	25.61	-0.014	0.118	-0.049	0.000	-0.046	5
18	25.20	25.61	-0. 41	0.118	-1.486	0.030	-1.597	2
19	25.80	25.61	0.19	0.118	0.670	0.006	0.650	3
20	25.80	25.61	0.19	0.118	0.670	0.006	0.650	1

4.2.3 Effect of Individual Factors on Yield

4.2.3.1 Effect of Concentration on Yield

Generally, the effect of ethanol concentration on the extraction yield was increasing with increasing concentration for wide range, remained constant for small range and started decreasing as further increased in concentration as demonstrated in the figure 4.3 below, by fixing both temperature and time at their center levels. As shown in the figure 4.3 (a) and (b), it was evident that phytochemical compound extraction from both Washington and Valencia orange peels were highly increased with increased concentration from low to center level due to increase in hydrolysis rate of ethanol soluble compounds, and was slightly increased with increased concentration from center level to 70% due to decrease in extraction of water soluble compounds. But as concentration increased from 70% to high level, the extraction yield remained constant and as concentration approached to high level, the yield was tended to decrease. As the concentration of ethanol increased above high level, the diffusion of water soluble towards the solvent was decreased due decreased in concentration water in the solvent. In addition, as the concentration of ethanol further increased, concentration of OH^- present in the solvent was increasing and led to the degradation of phytochemical compounds. The cumulative of the two led in decrease in extraction yield as concentration further increased. According to Hossain (2006), the total phenol content obtained at 85% ethanol concentration was 70.43 ± 6.63 mg GAE/100g and as ethanol concentration increased to 95%, the total phenol content was reduced to 58.59 ± 3.69mg GAE/100g.

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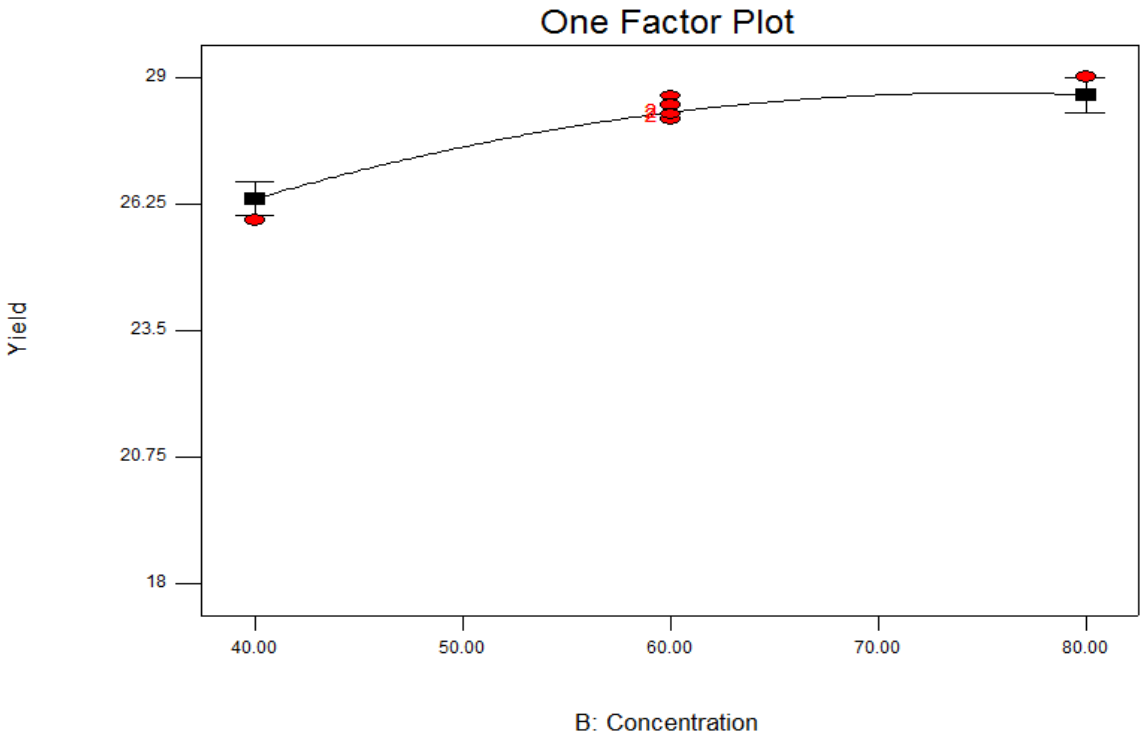
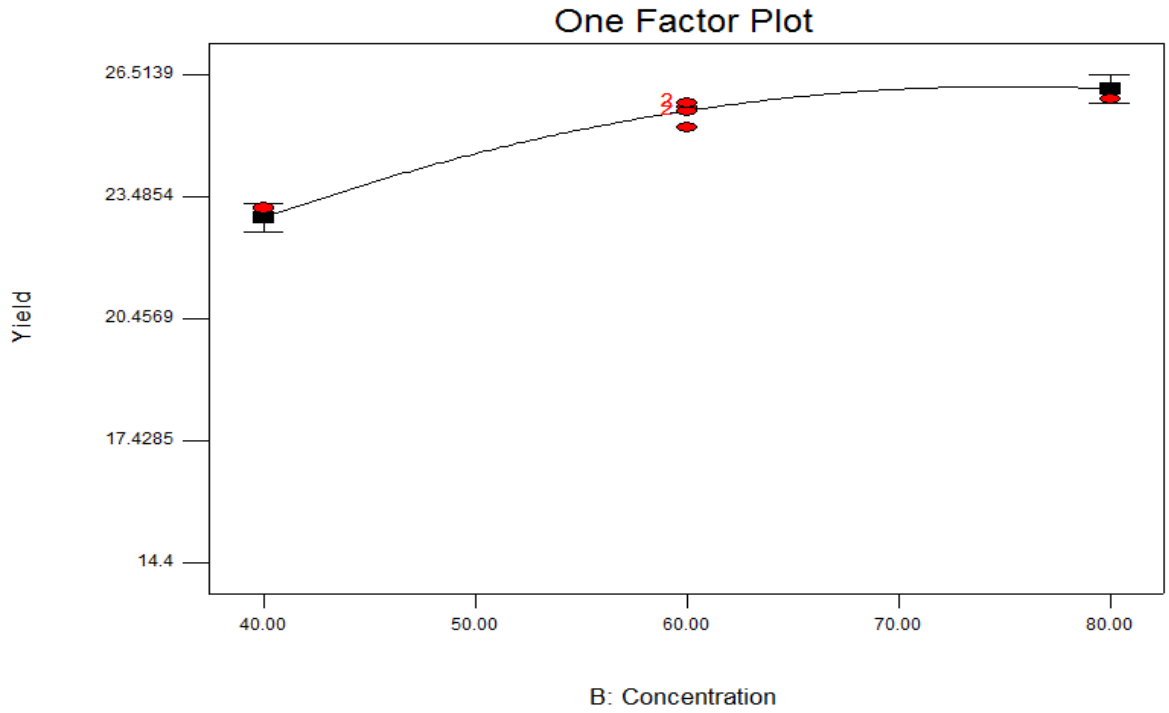


(a) Effect of concentration on yield at fixed temperature and time for Washington variety

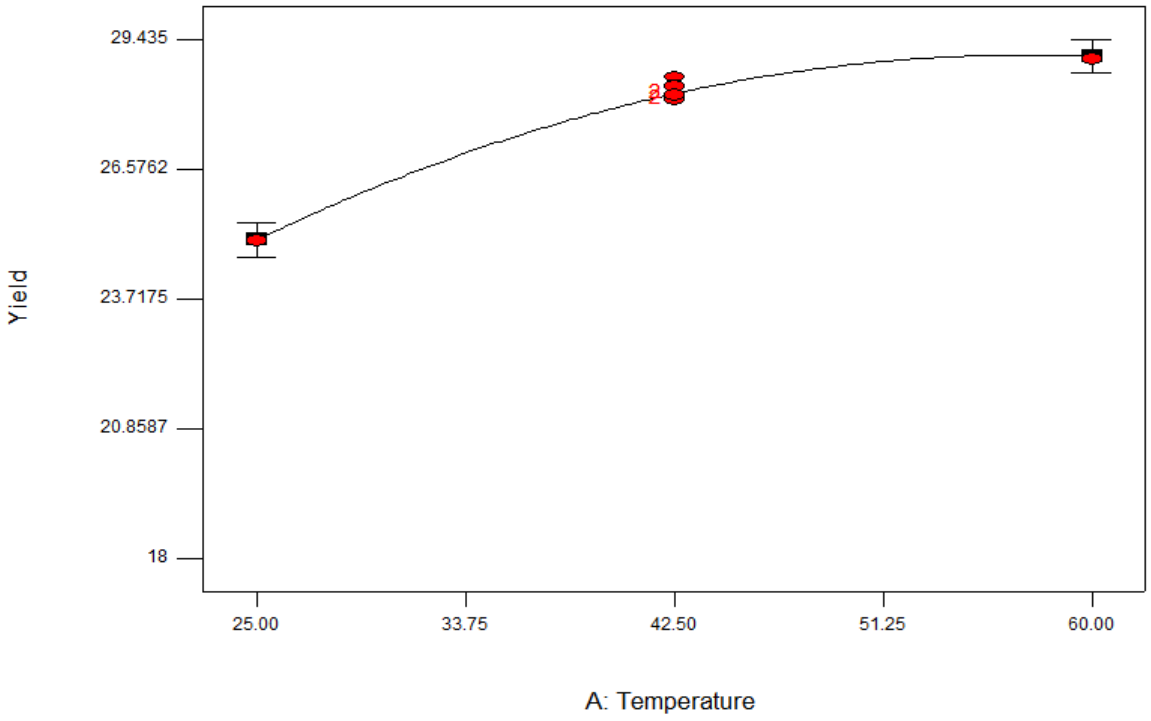


(b) Effect of concentration on yield at fixed temperature and time for valencia variety

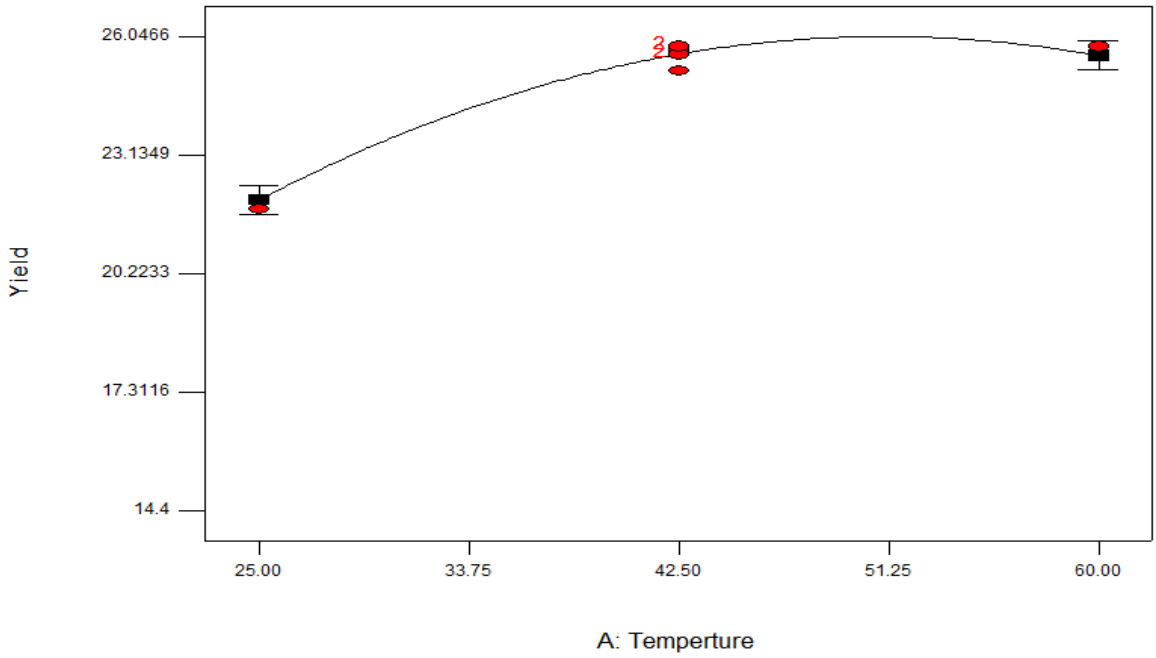
Figure 4.3 (a) and (b) effect of concentration on yield at fixed temperature and time for Washington and Valencia extract respectively

4.2.3 2 Effect of temperature on yield

As shown in Figure 4.4, extraction of total phytochemical compounds were significantly affected by temperature at fixed center levels both concentration and time. Generally, as observed from the figure, with rising extraction temperature, extraction yield was increasing. The reason is that as the extraction temperature increased, the rate of solubility of phytochemical compounds was increasing and gave high rate of extraction. Hence, the extraction yield was increased as extraction temperature increased. At low temperature, hydrolysis or solubility of phytochemical compounds in the solvent was not sufficient and the rate of diffusion of phytochemical compounds towards the solvent was low. Therefore, the extraction yield was low at low temperature. As illustrated in the figure 4.4 (a), the extraction yield was increasing as the temperature increased from low to center level, was slightly increasing for wide range interval and remained constant for small interval as temperature increased from center level to high level for Washington peel. Similarly, extraction yield was increasing as temperature increased from low to center level and was slightly increasing for some interval, remaining constant for another some interval and finally decreasing for last interval as temperature increased from center to high level for Valencia peel as shown in the figure 4.4 (b).



(a) Effect of temperature on yield at fixed concentration and time for Washington variety



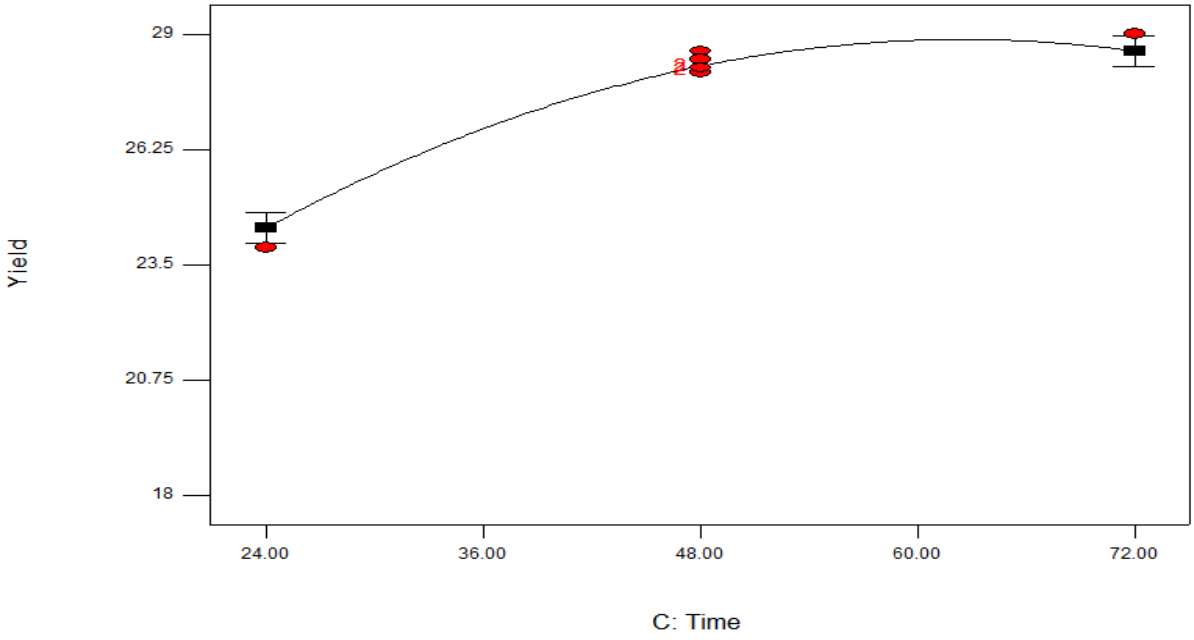
(b) Effect of temperature on yield at fixed concentration and time for Valencia variety

Figure 4.4 (a) and (b) effect of temperature on yield at fixed concentration and time for Washington and Valencia extracts respectively

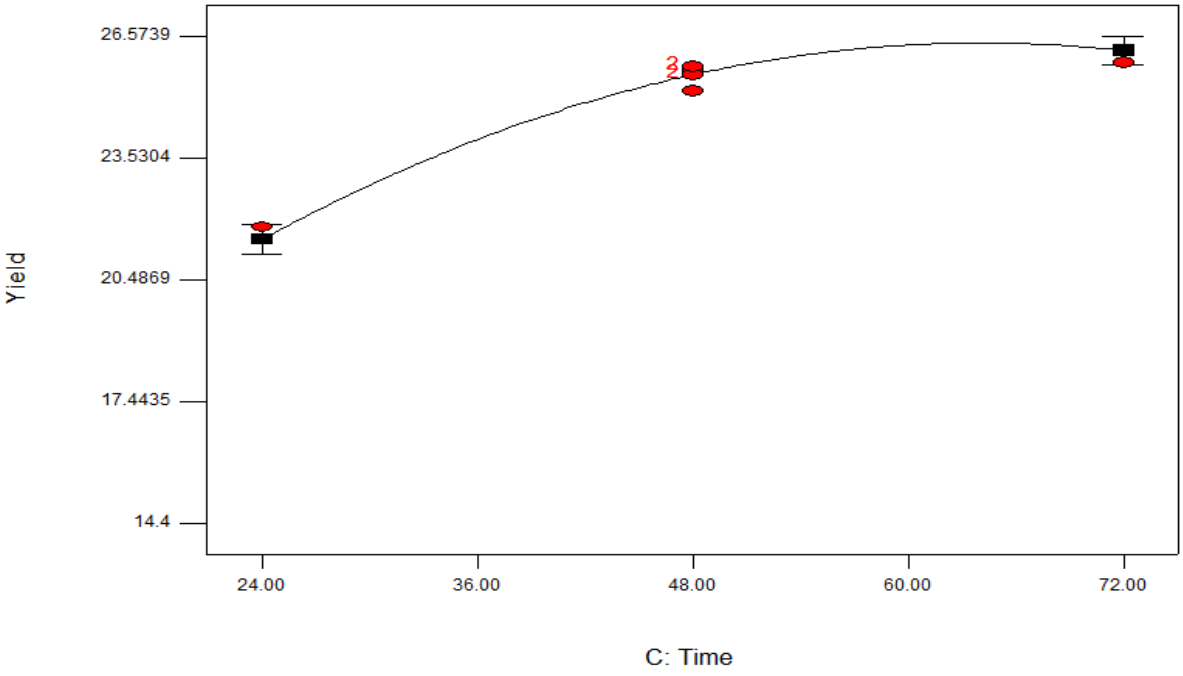
4.2.3.3 Effect of time on yield

The extraction yield was increased significantly with the increasing in the extraction time at fixed levels of both temperature and concentration as illustrated in the Figure 4.5. At low extraction time, hydrolyze time of soluble phytochemical compounds in the solvent was low, so the total diffused compounds towards the solvent were low. Hence, the extraction yield was low. As the extraction time increased from low to high level, the contact time of solvent and solute was increased and the total amount of phytochemical compounds diffused to the solvent increased, for this reason, the extraction yield was increased as increase in extraction time from low to center level and slightly increased as extraction time increased from center level to 60 h. As extraction time further increased from 60 h, the extraction yield was remained constant for some interval of time and started decreasing as the extraction time approaching to high level because long period of extraction would cause for the thermal degradation of soluble phytochemical compounds as shown in the figure 4.5 (a) and (b). But as illustrated in the figure, as the extraction time increased from center to high level, the time interval in which extraction yield remained constant was slightly wider in Valencia than Washington extract but the time

interval in which reduction in extraction yield was slightly wider in Washington than Valencia extract.



(a) Effect of time on yield at fixed temperature and concentration for Washington variety



(c) Effect of time on yield at fixed temperature and concentration for Valencia variety

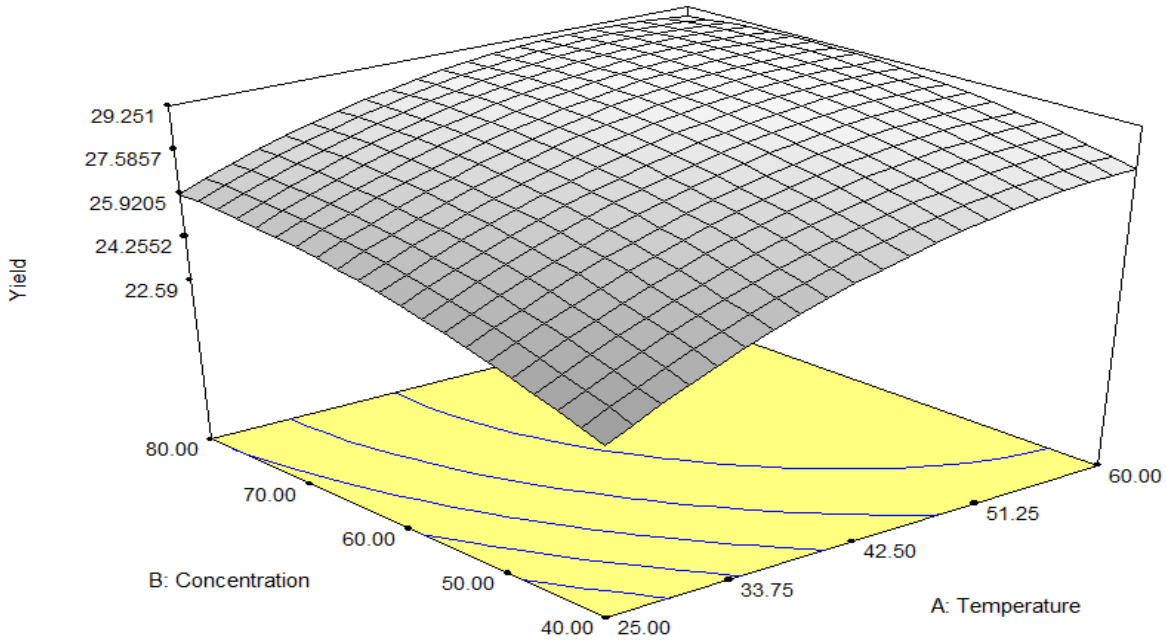
Figure 4.5 (a) and (b) effect of time at fixed temperature and concentration for Washington and Valencia extracts respectively4.2.4. Effect of Interaction of Factors on Yield

4.2.4. Effect of Interaction of Factors on Yield

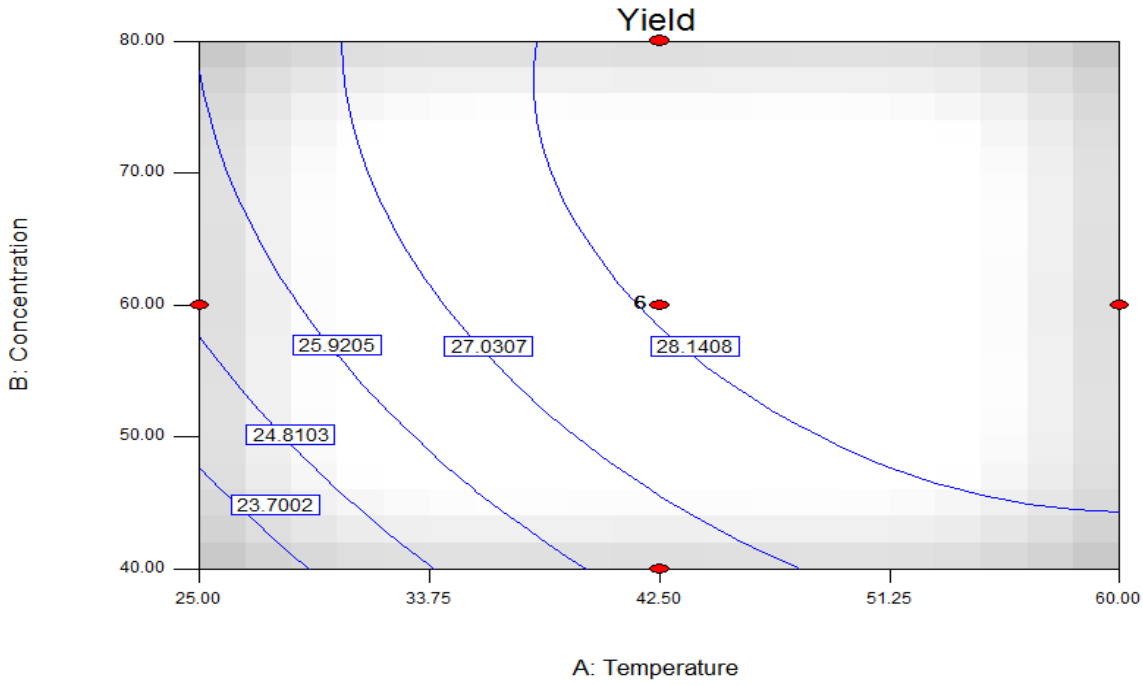
The extraction response was also significantly affected by two interactive variables at fixed third variable. The interaction between of the two factors was observed using three-dimensional response surface curves (plotted in order to understand the interactions between the variables and the optimum levels of each variable for maximum yield) and contour curve (presented the effect of two variables) on the yield holding the third variable at constant level. The interaction between two variables namely, temperature and concentration at fixed time, temperature and time at fixed concentration, concentration and time fixed temperature. The significance of interaction between the corresponding variables was indicated by saddle nature of the contour plots.

4.2.4.1 Effect of Temperature and Concentration on Yield

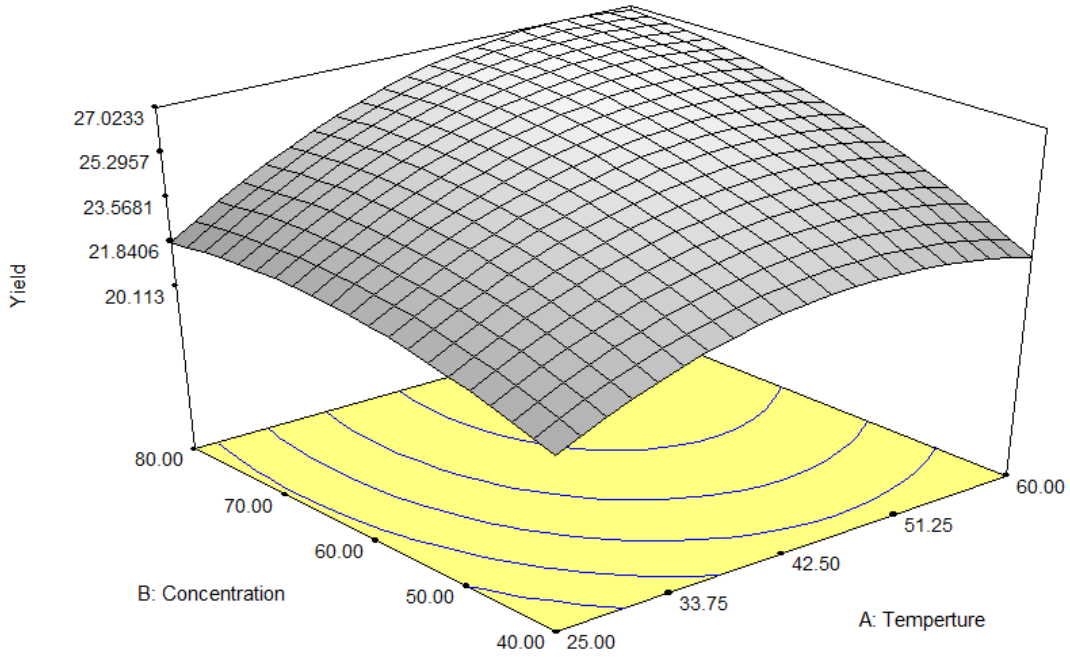
The interactive effects of temperature and concentration on extraction yield of the two varieties were shown in the form of 3D plots and surface contour. The influence of temperature and concentration on the response yield at fixed center level of extraction time was significant for both Washington and Valencia extract as demonstrated in the figure 4.6 below. The response yield on the contour plots of second order predicted model indicated that at lower temperature and concentration, the amount of phytochemical compounds diffused towards the solvent was low due to low hydrolyzing rate of soluble phytochemical compounds at both low temperature and concentration. Hence, the extraction yield was low at low temperature and concentration. The extraction yield was increased as increased in both temperature and concentration as shown in the figure 4.6 (a), (b). (b), (c) and (d). Solubility of solute in solvent or mass transfer of solid to solvent is directly proportional with temperature. Increase in temperature could affect the yield by increasing the rate of solubility of phytochemicals (both water and ethanol soluble compounds) in ethanol-water mixture binary solvent. Hydrolysis of solid in solvent is also proportional with solvent concentration. Extraction yield was affected by increased in ethanol concentration by facilitating hydrolysis of ethanol soluble phytochemical compounds (water soluble phytochemicals decreased). Extraction yield was limited to some interval of both temperature and concentration due to degradation started at high temperature and concentration. At single contour surface line, the same response could be obtained by increasing the temperature and decreasing concentration or vice versa.



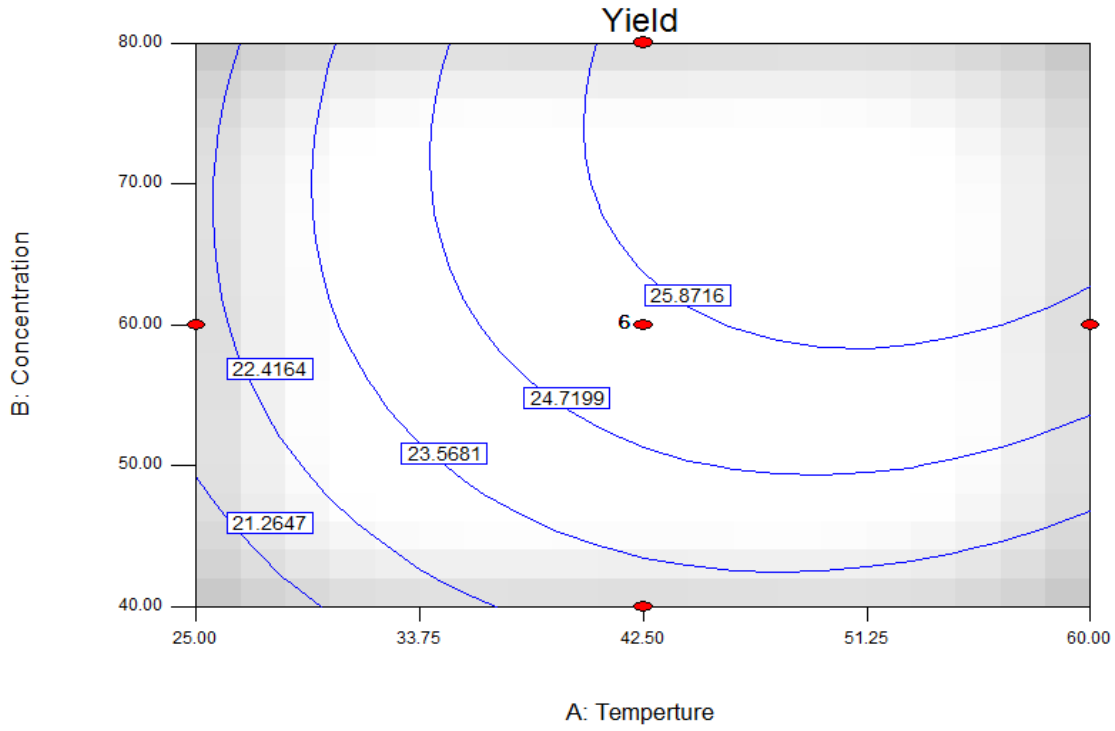
(a) 3D surface showing effect of Temperature and concentration on yield at fixed time for Washington extract



(b) Contour plot showing effect of Temperature and concentration on Yield at fixed time for Washington extract



(c) 3D surface showing effect of Temperature and concentration on Yield at fixed time for Valencia extract

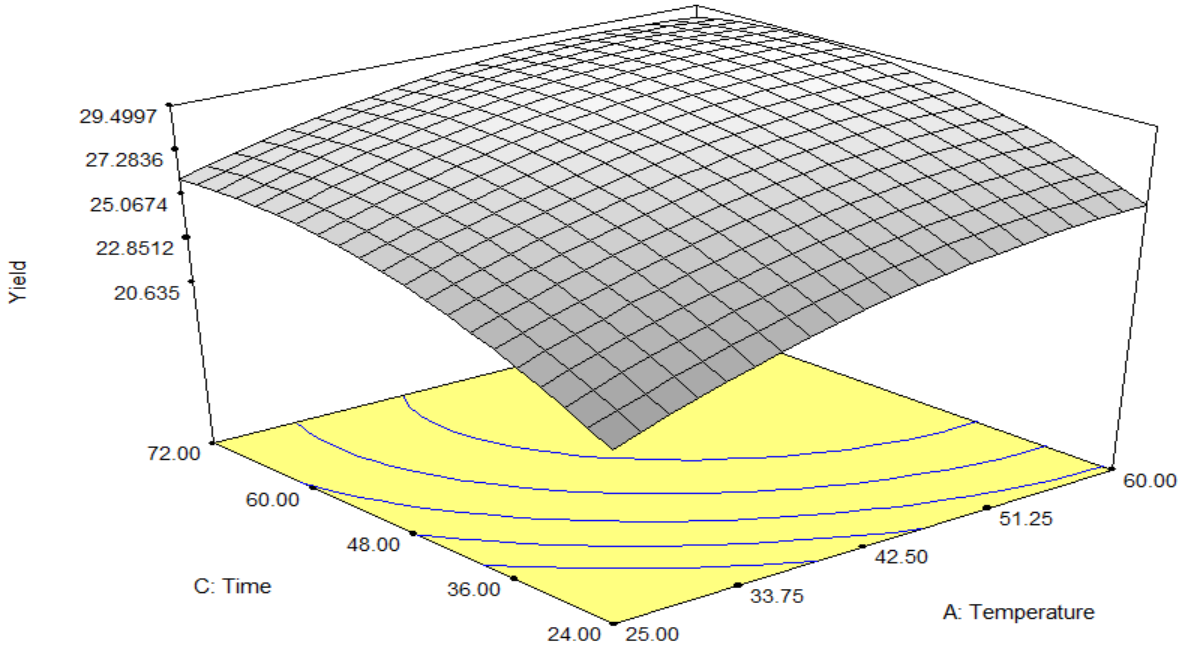


(d) Contour plot showing effect of Temperature and concentration on Yield at fixed time for Valencia extract

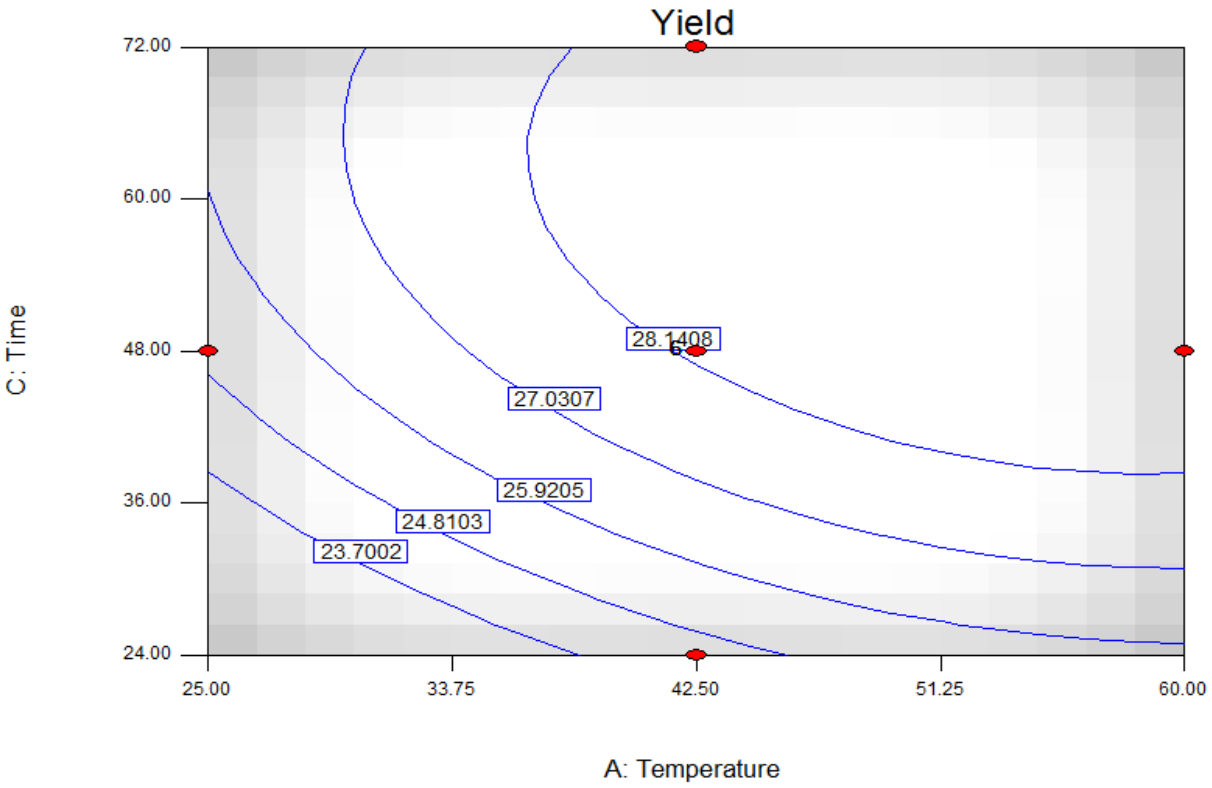
Figure 4.6 (a), (b), (c) and (d) 3D surface and Contour plot showing effect of temperature and concentration on yield at fixed time for Washington and Valencia extracts respectively

4.2.4.2 Effect of Temperature and Time on Yield

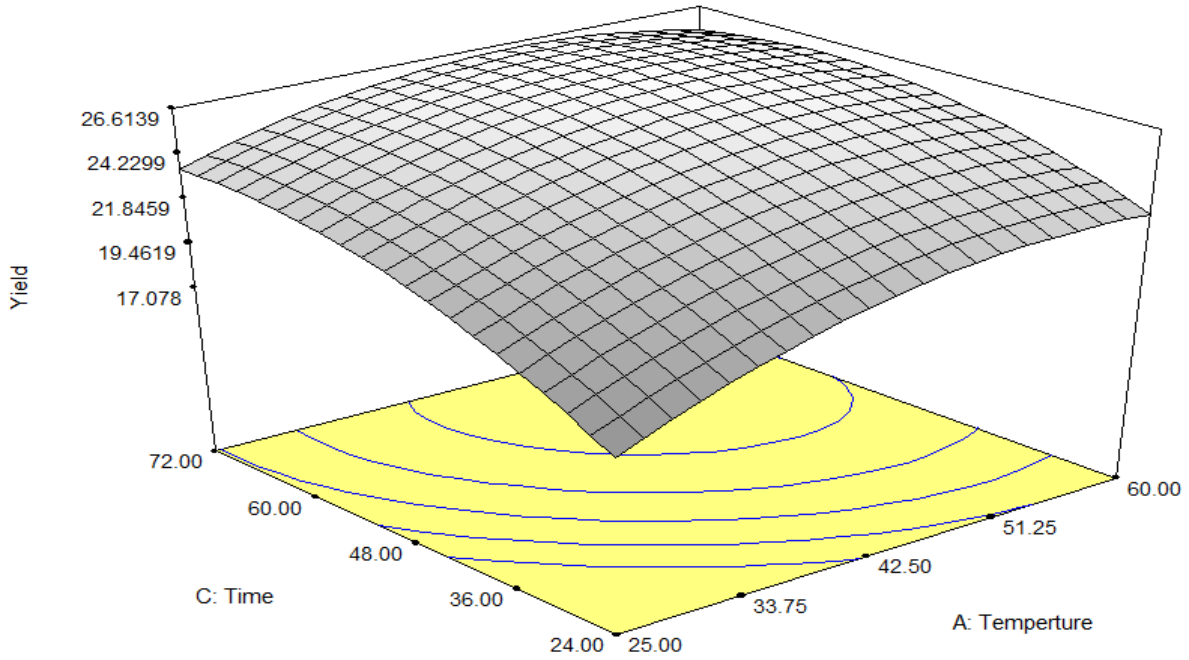
The effects of temperature and time on the extraction yield of antioxidant from the two varieties of orange peels were shown in the form of 3D plots and surface contour. As shown in the figure 4.7 below, with rising of the extraction temperature and time, the antioxidant yield was increased up to maximum value and remained constant for some interval but started declining slightly as further increased in temperature and extraction time due to soluble polyphenols started degrading and damaging as duration of extraction time proceeded at high temperature. As illustrated in the figure 4.7, the minimum yield was obtained at low levels of both temperature and time due to low diffusion rate of the soluble polyphenols towards the solvent at low temperature and the duration of contact time between solute and solvent is short. The maximum extraction yield was obtained at contour surface line nearly below center point for Washington and at contour surface line slightly above center point for Valencia extract. Similar yield was also obtained at that contour surface line by decreasing the extraction time from the center point and increasing extraction temperature from the center point or vice versa. As further increased in level of both temperature and time, the amount of degradable polyphenols increased and it was an evident that at high extraction temperature and high extraction time, the extraction yield was declined.



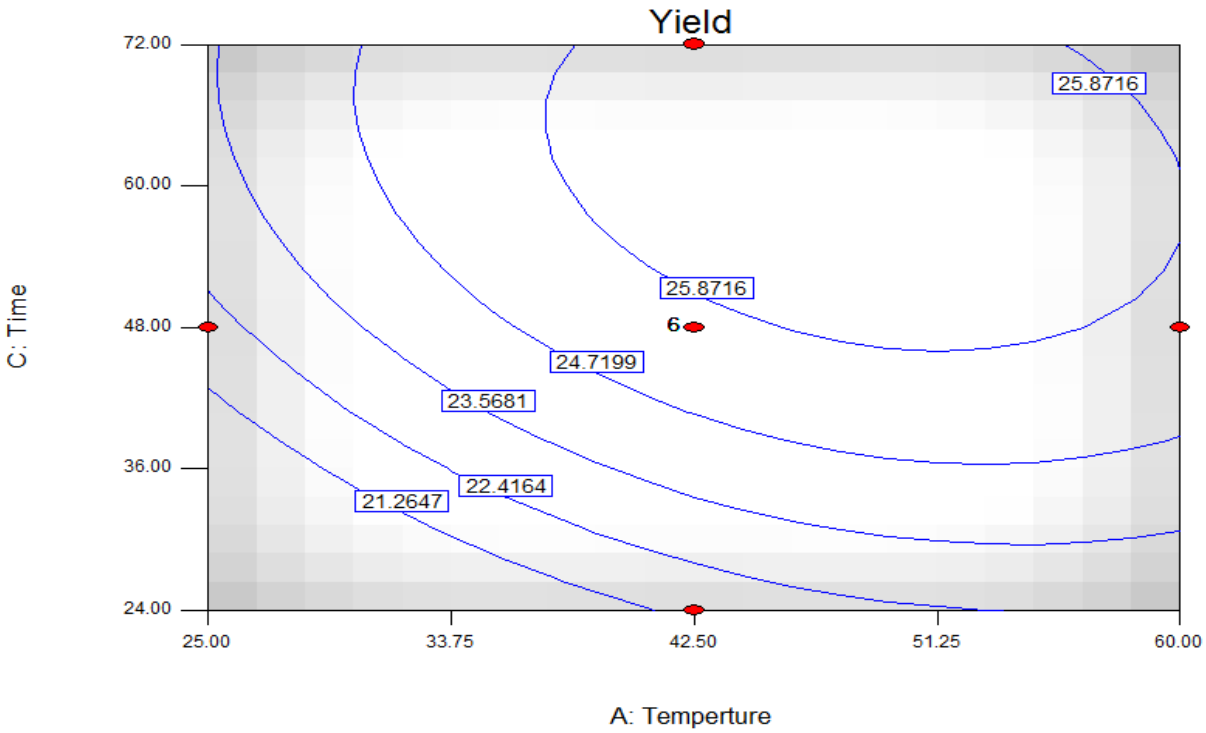
(a) 3D surface showing effect of Temperature and Time on Yield at fixed concentration for Washington extract



(b) Contour plot showing effect of Temperature and Time on Yield at fixed concentration for Washington extract



(c) 3D surface showing effect of Temperature and Time on Yield at fixed concentration for Valencia extract



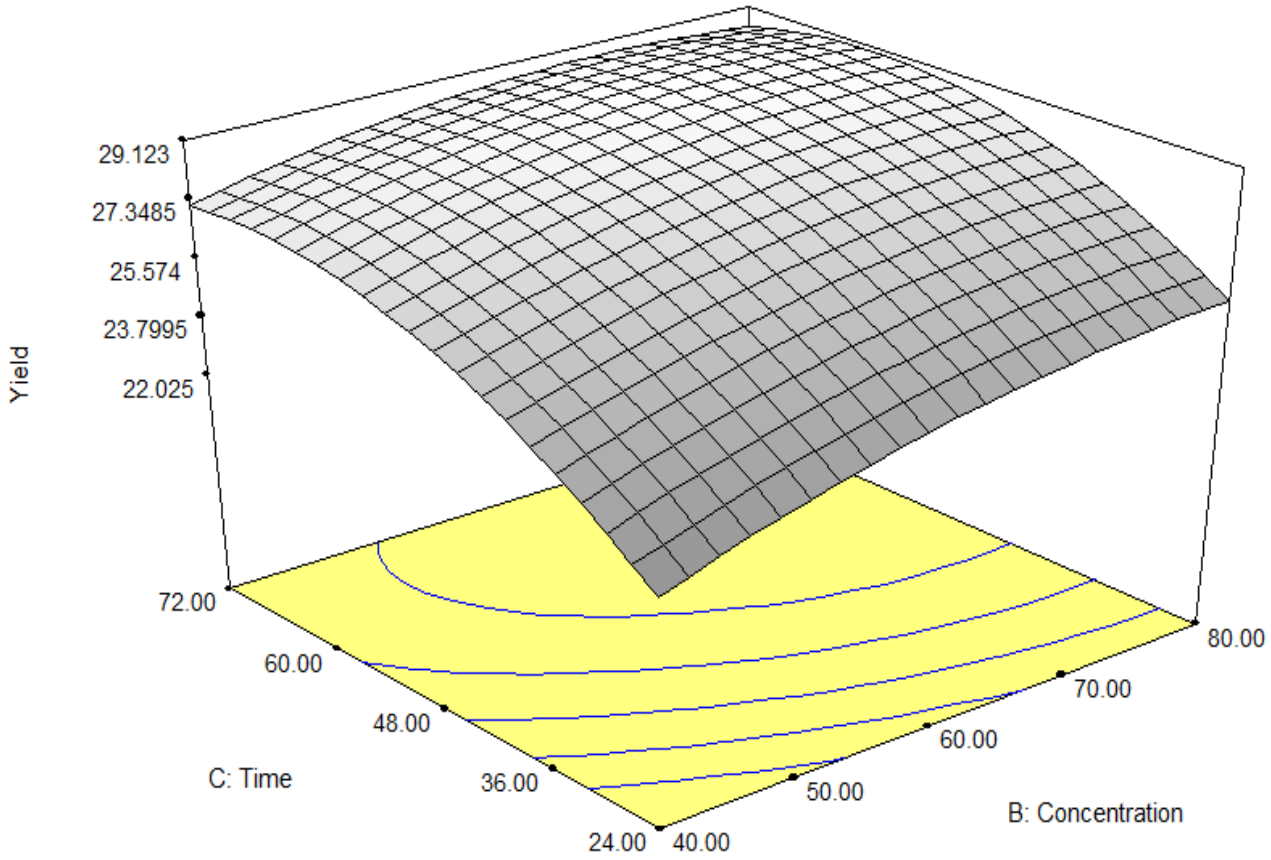
(d) Contour plot showing effect of Temperature and Time on Yield at fixed concentration for Valencia extract

Figure 4.7 (a), (b), (c) and (d) 3D surface and Contour plot showing effect of Temperature and Time on Yield at fixed concentration for Washington and Valencia extracts respectively

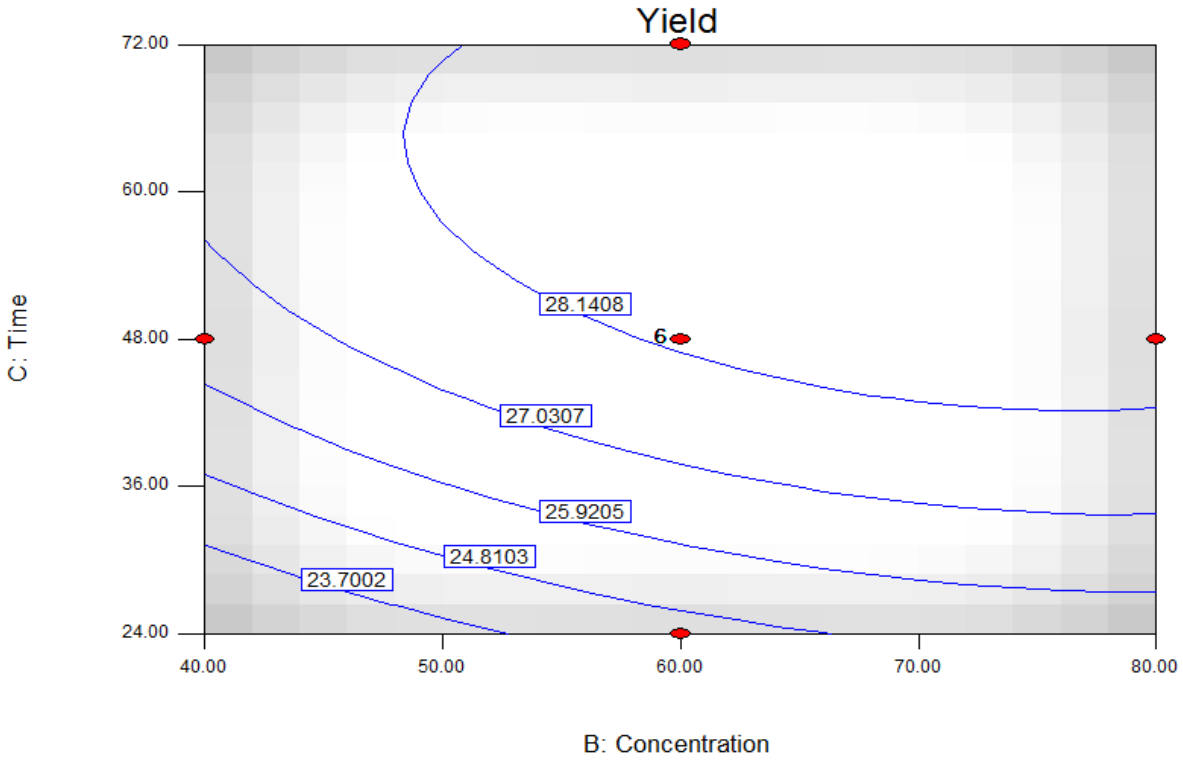
4.2.4.3 Effect of Concentration and Time on Yield

The effects of extraction time and concentration on the response yield of extract by fixing the temperature at its center point was shown in the form of 3D plots and surface contours as illustrated in the figure 4.8. At both low concentration and time, the extraction yield was low because of poor diffusion of phytochemical compounds (phenols, flavonoids etc.) at low concentration due to less effect of the hydrolysis treatment and, the contact duration of soluble phytochemical compounds with solvent was low at low extraction time. Hence, the extraction yield was low at both low concentration and time as shown in the figure 4.8. But the extraction yield was increased as both ethanol concentration and extraction time were increased due to increase in efficiency of hydrolysis rate of phytochemical compounds by solvent and the duration of contact between the solute and solvent was increased. As ethanol concentration increased, OH^- concentration was increasing and the rate hydrolyzing the phytochemical compounds was increasing. Side by side, as the extraction time increased, the duration of contact between the solute and solvent was increased and the amount of hydrolyzed solutes diffused

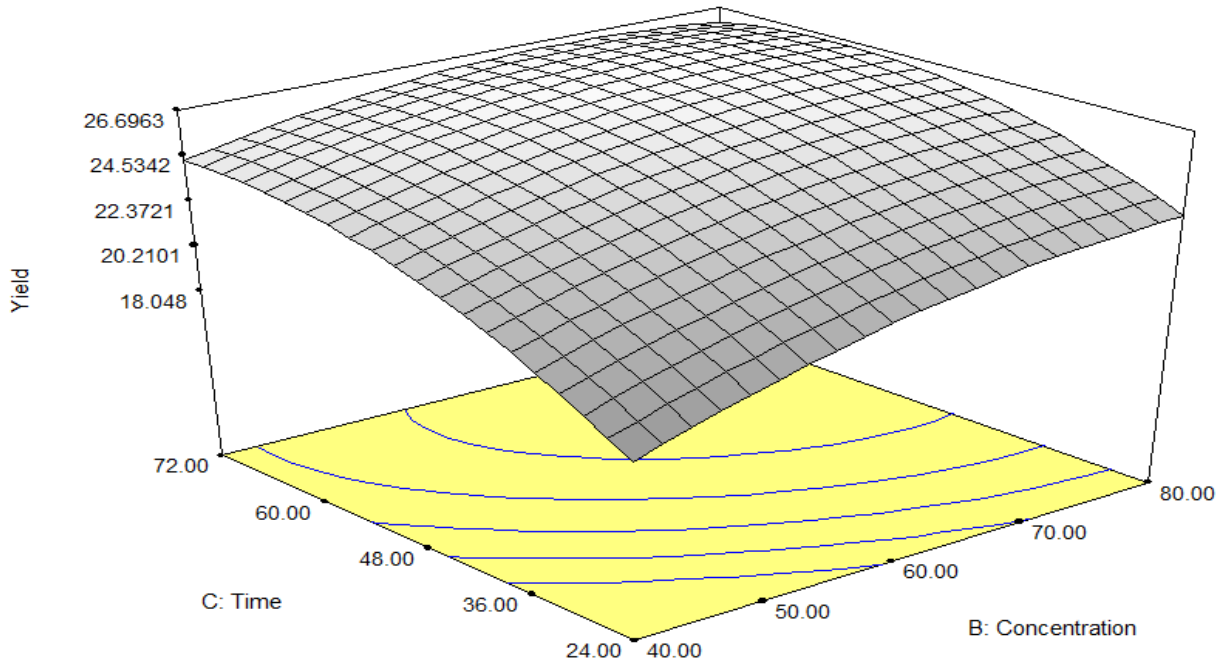
towards the solvent was increased. For Washington extract, the optimal response value was obtained at the entire contour surface line found nearly below the center point. Operating at center level of concentration and nearly below the center point of extraction time gave the optimal value. Similar value was also obtained operating by increasing the concentration from center level and side by side decreasing the extraction time from the center level and vice versa as illustrated in the figure 4.8 (a) and (b). The optimal response value was obtained at the entire contour surface line found nearly below the center point. For Valencia extract, the optimal yield was obtained operating at center level of concentration and at the nearly above the center level of time. Optimal value was also obtained operating by increasing the concentration from the center point and at the same time decreasing extraction time from the center level or vice versa at entire contour surface line slightly above the center point as shown in the figure 4.8 (c) and (d).



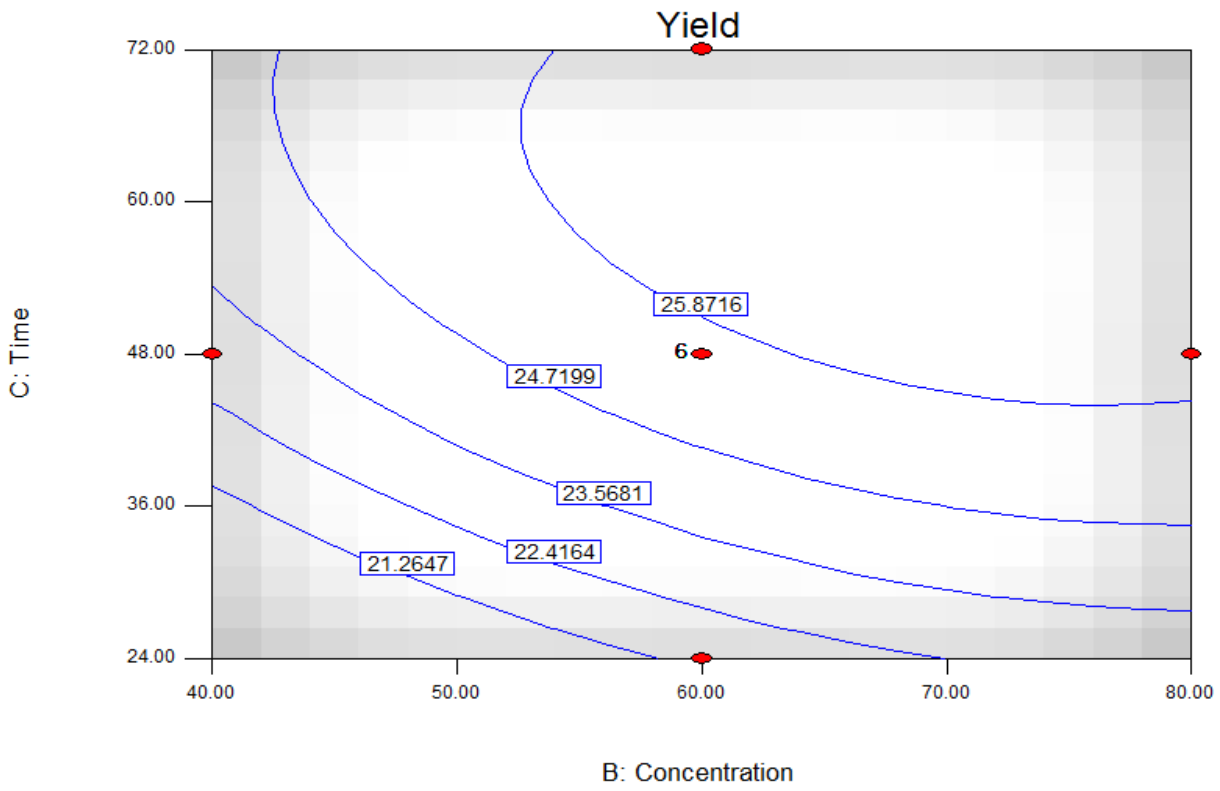
(a) 3D surface showing effect of Concentration and Time on Yield at fixed temperature for Washington extract



(b) Contour plot showing effect of concentration and time at fixed temperature for Washington extract



(c) 3D surface showing effect of Concentration and Time on Yield at fixed temperature for Valencia extract.



(d) Contour plot showing effect of time and concentration at fixed temperature for Valencia extract

Figure 4.8 (a), (b), (c) and (d) 3D surface and Contour plot showing effect of time and concentration at fixed temperature for Washington and Valencia extracts respectively

4.2.5 Optimization of Extraction Factors

The response yield was influenced by individual effects of factors by fixing other two factors constant as demonstrated by single factor plot and, interactive effect of factors by fixing the other third factor constant as illustrated by 3D plots and contour surface as shown in the figures 4.3, 4.4, 4.5, 4.6, 4.7, 4.8 above. Once the individual and interactive effects of factors were investigated, optimization of the process variables in order to obtain the highest yield was conducted using the model regression developed. The expert design gives three different optimization choices, numerical optimization (set goal for each response), graphical optimization (set minimum and maximum limits for each response and then create an overlay highlighting an area of operability) and point prediction optimization (enter desired operating conditions and discover predicted response values with confidence intervals). In numerical optimization choice, depending on constraints (criteria) selected, different alternative solutions of optimization was

given by expert design. For this study, numerical optimization was selected to obtain better highest response yield for the varieties of the extracts.

4.2.5.1 Optimization of extraction factors for Washington extract

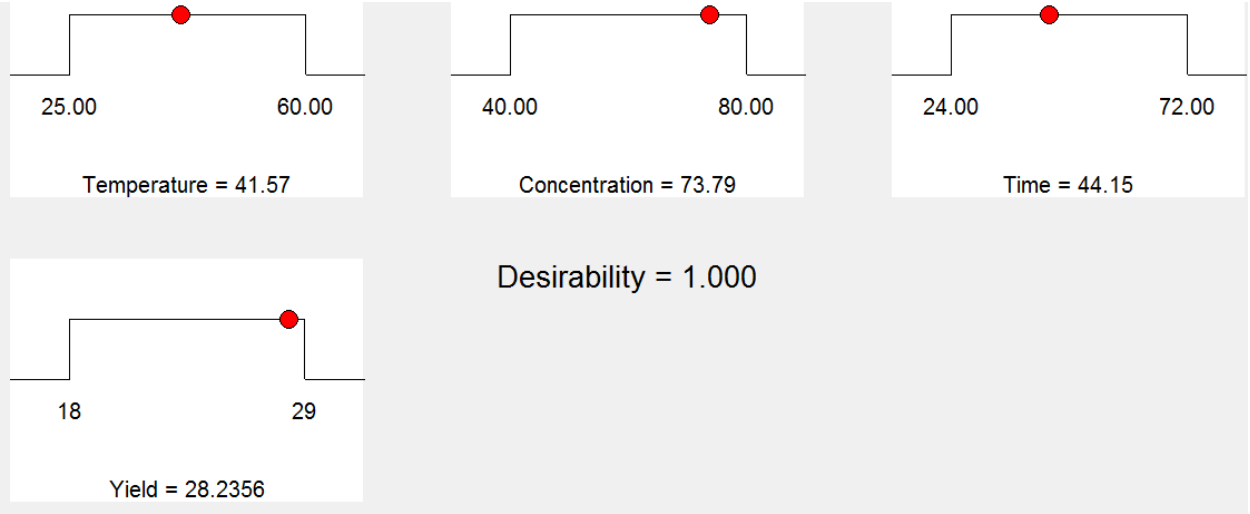
In numerical optimization, there are criteria's and constraints to be specified for process variables (temperature, concentration and time) and response yield. The goal of optimization should be set (in range, minimum, maximum, target and equal to) for factors and response variable. The limit (upper and lower), weight (upper and lower) and importance of all factors and response should be specified.

Table 4.11 Optimization constraints for Washington extract

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Temperature	Is in range	25	60	1	1	1
Concentration	Is in range	40	80	1	1	1
Time	Is in range	24	72	1	1	1
Yield	Is in range	14.4	25.9	1	1	1

Table 4.12 Different alternative optimization solutions for Washington extract

Number	Temperature	Concentration	Time	Yield	Desirability	
1	30.68	46.82	38.66	23.6664	1.000	
2	27.34	73.80	30.62	23.8034	1.000	
3	40.28	53.33	61.85	28.2456	1.000	
4	57.15	69.92	71.11	28.9966	1.000	
5	41.57	73.79	44.15	28.2356	1.000	Selected
6	40.30	49.09	53.82	27.5763	1.000	
7	52.11	76.96	38.53	28.3183	1.000	
8	32.60	48.92	60.16	26.5882	1.000	
9	47.27	68.62	40.52	28.2655	1.000	
10	32.27	72.77	49.01	27.3563	1.000	



The goals of temperature, concentration, time and response yield all were set <in range> from lower and upper limits of their values. The upper and lower limits of the variables were specified and all variables were very significant and important due to their positive values from the developed regression model equation. The expert design under numerical optimization gave 10 different alternative optimizing solutions. Depending on the parameters by compromising yield, antioxidant activity, economy and energy carrying, the best solution was selected among alternatives. Low temperature is more potent for antioxidant activity but not good to obtain high yield, intermediate temperature is good at yield, high temperature is not good for both yield and antioxidant activity. Operating extraction at low temperature for long time, increases yield without affecting antioxidant activity but are reduced at high temperature. Concentration more than 80% reduce both yield and antioxidant activity. Under taking the consideration of these constraints, 41.57 ºC, 73.79% and 44.15 h were selected which gave 28.2356% yield among optimizing alternatives given by expert design software.

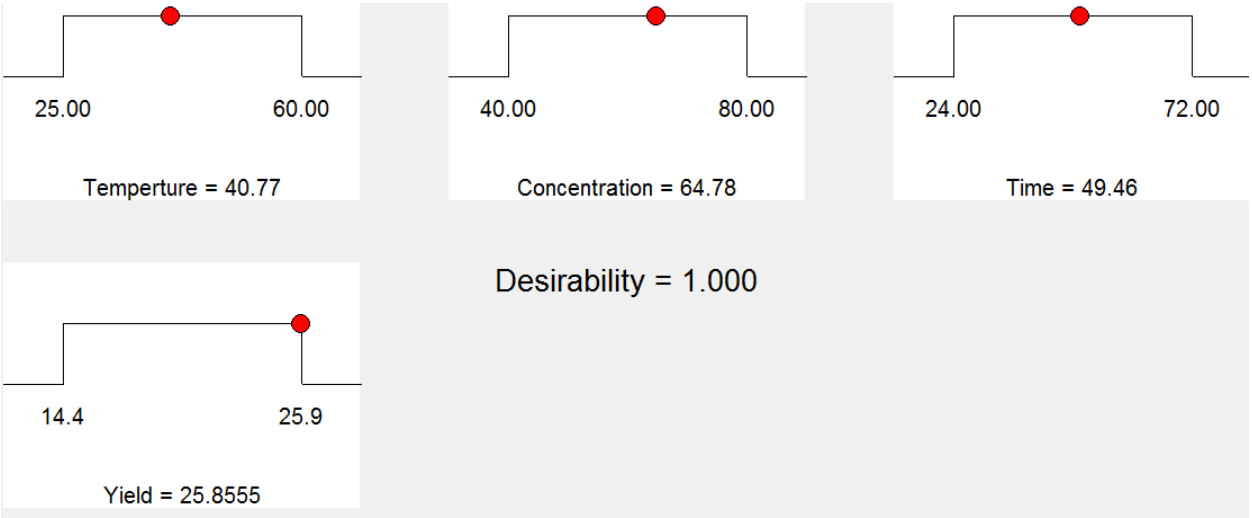
4.2.5.2 Optimization of extraction factors for Valencia extract

Table 4.13 Optimization Constraints for Valencia extract

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Temperature	Is in range	25	60	1	1	1
Concentration	Is in range	40	80	1	1	1
Time	Is in range	24	72	1	1	1
Yield	Is in range	14.4	25.9	1	1	1

Table 4.14 Different alternative optimization solutions for Valencia extract

Number	Temperature	Concentration	Time	Yield	Desirability	
1	56.13	74.20	33.33	25.5456	1.000	
2	58.48	54.34	47.77	24.9755	1.000	
3	36.71	69.48	36.56	23.7548	1.000	
4	40.77	64.78	49.46	25.8555	1.000	Selected
5	40.37	48.19	47.06	23.993	1.000	
6	28.12	79.81	46.31	22.7144	1.000	
7	59.82	52.96	53.10	24.9473	1.000	
8	51.53	64.33	27.95	23.7277	1.000	
9	48.51	44.49	68.81	24.9377	1.000	
10	33.18	78.53	53.79	24.7651	1.000	



From the given 10 alternative solutions in the table 24, nearly upper limit yield (25.8555) was obtained at temperature 40.77 ºC, ethanol concentration 64.78% and extraction time 49.46 h. The yield obtained in this solution was best from other alternative solutions given in the table 24. The operating temperature, ethanol concentration and extraction time were also reasonable with regard to antioxidant activity, economy and energy carrying in addition to highest extraction yield obtained. Hence, the maximum yield (25.8555%) was under optimum operating conditions, 40.77 ºC extraction temperatures, 64.78% ethanol concentration and 49.46 h extraction time.

4.3 Results and Discussion on Characterization of Extracts

4.3.1 Color of extract

In this study, the color value of extract was identified in the form of the color absorbance of stock solution of extract sample. Its color absorbance was measured using spectrophotometer at 517nm at room temperature. The absorbance of a sample is directly proportional to the amount absorbing material through which the light must travel at 517nm wave length. The color absorbance of Washington and Valencia extracts were 2.333 and 2.634 respectively. These colors absorbance were the amount colors of extracts through which the light must travel respectively. The color values were calculated using equation (5) and the values were 20.29 and 37.63 for Washington and Valencia extract respectively. Its value indicates a relative degree of intensity of color (lightness or darkness). The Valencia extract was darker than Washington extract. The darker had highest antioxidant activity and higher phenolic and flavonoid contents.

4.3.2 pH value of extract

The pH values of both Valencia and Washington variety extracts were 4.30 and 4.80 respectively. The term “pH” is a measure of acidity. The lower pH values, the more acid of the extracts. Low- acid foods have pH values higher than 4.6 to 6.9. The non-acidic or alkaline foods have pH values 7.0 or greater. From the values, it deduced that both the extract solutions were acidic and the Valencia extract was high- acidic and better in antioxidant activity than Washington variety extract.

4.3.3 Specific gravity of extract

The weight of 1ml of extract was 1mg similarly; the weight of 1ml of water was 1mg. The specific density of extract was calculated by dividing the density of 1ml of extract to the density of 1ml of water using the relationship written in equation (4). The specific gravity obtained was 1.00. This result shows evidence that the density of solid extract is similar with the density of water at room temperature.

4.3.4 Total Phenol Content of extract

A colorimetric assay using the Folin-Ciocalteu reagent was referenced for determination of phenolic compounds. The reaction between the Folin-ciocalteu reagent and phenolic compounds

results in the formation of a blue color complex that absorbs radiation and allows quantification of phenolic compounds by UV-spectrophotometer. The reaction forms a blue chromophore constituted by a phosphotungstic-phosphmolbdenum complex where the maximum absorption of the chromophores depends on the concentration of phenolic compounds. The more rapidly increases in absorbance, the more potent the antioxidant activity of the extract. The regent rapidly decomposes in alkaline solution, which makes it necessary to use an enormous excess of reagent to obtain a complete reaction. This excess can be result in precipitates and high turbidity, making spectrophotometer analysis impossible. The addition of NaCO3 salt neutralizes and prevents this problem.

Table 4.15 Gallic acid standard solution preparation and corresponding absorbance

Gallic acid(µL)	Methanol (µL)	Concentration (mg/ml)	FC (ml)	Na_2NO_3 (ml)	Distil water (ml)	absorbance	Absorbance Mean ± SD
0	1000	0	1	1	7	0.002	
20	980	1.0	1	1	7	1.040	1.041±0.002
20	980	1.0	1	1	7	1.040	
20	980	1.0	1	1	7	1.043	
40	960	2.0	1	1	7	1.203	1.201±0.006
40	960	2.0	1	1	7	1.195	
40	960	2.0	1	1	7	1.206	
80	920	4.0	1	1	7	1.335	1.363±0.027
80	920	4.0	1	1	7	1.366	
80	920	4.0	1	1	7	1.388	
120	880	6.0	1	1	7	1.544	1.540±0.005
120	880	6.0	1	1	7	1.542	
120	880	6.0	1	1	7	1.535	
160	840	8.0	1	1	7	1.662	1.675±0.011
160	840	8.0	1	1	7	1.680	
160	840	8.0	1	1	7	1.682	
200	800	10.0	1	1	7	1.853	1.864±0.011
200	800	10.0	1	1	7	1.874	

200	800	10.0	1	1	7	1.865	
240	760	12.0	1	1	7	2.054	2.068±0.018
240	760	12.0	1	1	7	2.062	
240	760	12.0	1	1	7	2.089	

Table 4.16 Concentrations of Gallic acid standard solution and their corresponding absorbance

Concentration (mg/ml)	Absorbance Mean ± SD
0	0.002
1.0	1.041 ± 0.002
2.0	1.201 ± 0.006
4.0	1.363 ± 0.027
6.0	1.540 ± 0.005
8.0	1.675 ± 0.011
10.0	1.864 ± 0.011
12.0	2.068 ± 0.018

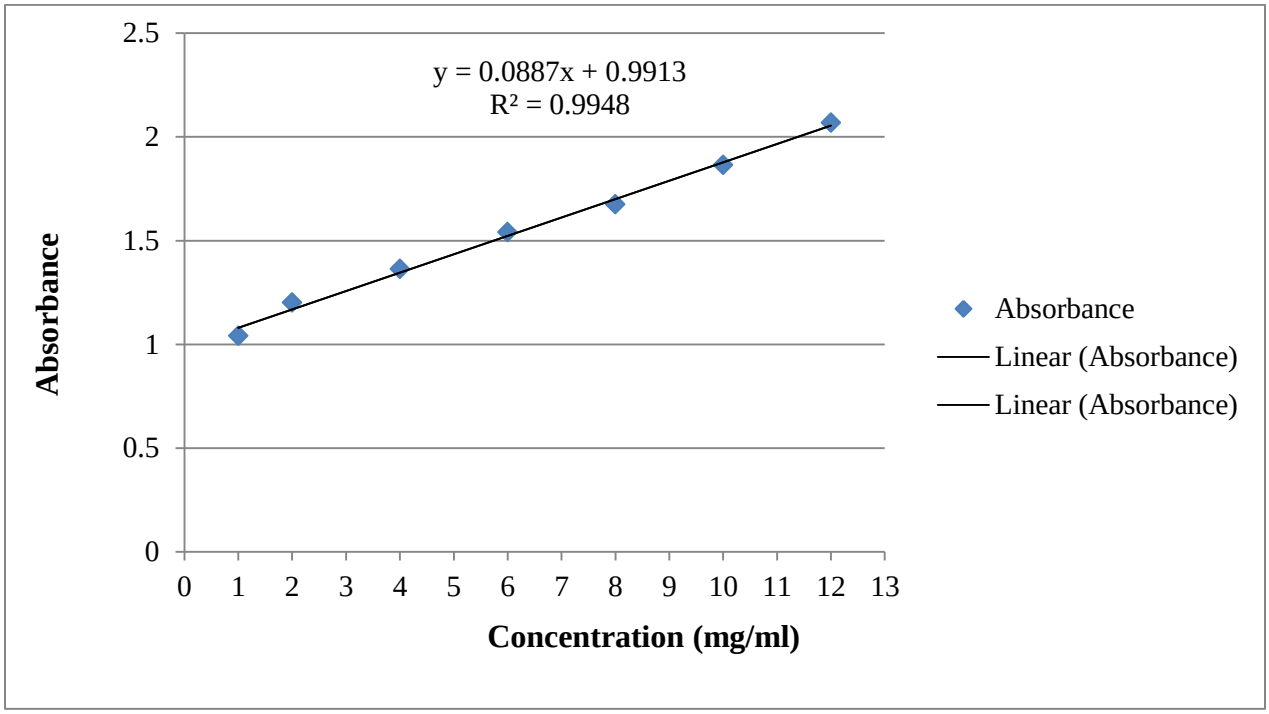


Figure 4.9 Gallic acid standard calibration curve

Table 4.17 Sample solution preparation and corresponding absorbance for Washington extract

Sample (µL)	Methanol (µL)	Concentration (mg/ml)	FC (ml)	Na_2CO_3 (ml)	Distill Water (ml)	Absorbance	Absorbance Mean ± SD
20	980	1.0	1	1	7	0.403	0.403±0.001
20	980	1.0	1	1	7	0.404	
20	980	1.0	1	1	7	0.402	
40	960	2.0	1	1	7	0.698	0.712±0.015
40	960	2.0	1	1	7	0.728	
40	960	2.0	1	1	7	0.709	
80	920	4.0	1	1	7	1.133	1.121±0.013
80	920	4.0	1	1	7	1.107	
80	920	4.0	1	1	7	1.124	
120	880	6.0	1	1	7	1.285	1.320±0.034
120	880	6.0	1	1	7	1.322	
120	880	6.0	1	1	7	1.353	
160	840	8.0	1	1	7	1.388	1.411±0.021
160	840	8.0	1	1	7	1.430	
160	840	8.0	1	1	7	1.415	
200	800	10.0	1	1	7	1.501	1.521±0.019
200	800	10.0	1	1	7	1.523	
200	800	10.0	1	1	7	1.539	
240	760	12.0	1	1	7	1.579	1.591±0.014
240	760	12.0	1	1	7	1.607	
240	760	12.0	1	1	7	1.587	

Table 4.18 Amount of phenol content from a gram of dry Washington extract

Concentration of sample mg/ml	Absorbance Mean ± SD	Gallic acid equivalent Concentration (mg/ml)	Total phenol content (mg GAE/g extract)
1.0	0.403 ± 0.001	-	-
2.0	0.712 ± 0.015	-	-
4.0	1.121 ± 0.013	1.462	29.24
6.0	1.320 ± 0.034	3.706	74.12
8.0	1.411 ± 0.021	4.732	94.64
10.0	1.521 ± 0.019	5.972	119.44
12.0	1.591 ± 0.014	6.761	135.22

As observed in the table 4.18, only concentrations (4.0, 6.0, 8.0, 10.0 and 12.0mg/ml) and their corresponding absorbance (1.121 ± 0.013, 1.320 ± 0.034, 1.411 ± 0.021, 1.521 ± 0.019 and 1.591 ± 0.014 were satisfying the standard Gallic acid calibration curve (linear regression curve) for of Washington extract. The Gallic acid equivalent concentrations at these absorbance were obtained using the Gallic acid standard calibration curve ($y = 0.0887x + 0.9913$, $R^2 = 0.9948$) as shown in the figure 4.9. The Gallic acid equivalent concentrations (C) obtained from the calibration curve were 1.462, 3.706, 4.732, 5.972 and 6.761mg/ml. Total phenol contents (T) expressed in Gallic acid equivalent (mg of Gallic acid/g dry extract) was calculated by direct substitution in the relationship expressed in equation (6). Finally, the total phenol contents were 29.24 mg of Gallic acid equivalent per gram of dry extract (29.24 mg GAE/g extract), 74.12 mg of Gallic acid equivalent per gram of dry extract (74.12 mg GAE/g extract), 94.64 mg of Gallic acid equivalent per gram of dry extract (94.64 mg GAE/g extract), 119.44 mg of Gallic acid equivalent per gram of dry extract (119.44 mg GAE/g extract) and 135.22 mg of Gallic acid equivalent per gram of dry extract (135.22 mg GAE/g extract) for Washington extract. From the results, total phenol content was depend on the concentration of sample, as concentration of sample increases, the absorbance increases and indicates the potent of antioxidant activity. The minimum concentration of Washington extract sample which met the standard Gallic acid calibration curve was 3.366mg/ml. The concentration of sample below 3.366mg/ml were outlying the calibration curve. This was minimum limit of concentration of Washington extract which met the

requirements to obtain total phenol content. The maximum limit of extract concentration was not determined due to increase in concentration increases the absorbance and indicates potential of antioxidant activity increases. 135.22 mg GAE/g extract, the maximum amount of phenol content under this study obtained at the concentration 12.0mg/ml.

Table 4.19 Sample solution preparation and corresponding absorbance for Valencia extract

Sample (µL)	Methanol (µL)	Concentration (mg/ml)	FC (ml)	Na_2CO_3 (ml)	Distill Water (ml)	Absorbance	Absorbance Mean ± SD
20	980	1.0	1	1	7	0.585	0.560±0.023
20	980	1.0	1	1	7	0.539	
20	980	1.0	1	1	7	0.556	
40	960	2.0	1	1	7	0.854	0.884±0.031
40	960	2.0	1	1	7	0.883	
40	960	2.0	1	1	7	0.915	
80	920	4.0	1	1	7	1.256	1.266±0.011
80	920	4.0	1	1	7	1.278	
80	920	4.0	1	1	7	1.264	
120	880	6.0	1	1	7	1.374	1.374±0.019
120	880	6.0	1	1	7	1.355	
120	880	6.0	1	1	7	1.393	
160	840	8.0	1	1	7	1.572	1.579±0.007
160	840	8.0	1	1	7	1.586	
160	840	8.0	1	1	7	1.578	
200	800	10.0	1	1	7	1.690	1.711±0.019
200	800	10.0	1	1	7	1.728	
200	800	10.0	1	1	7	1.716	
240	760	12.0	1	1	7	1.801	1.801±0.001
240	760	12.0	1	1	7	1.802	
240	760	12.0	1	1	7	1.800	

Table 4.20 Amount of phenol contents from a gram of dry Valencia extract

Concentration of sample (mg/ml)	Absorbance Mean ± SD	Gallic acid equivalent Concentration (mg/ml)	Total phenol content (mg GAE/g extract)
1.0	0.560 ± 0.023	-	-
2.0	0.884 ± 0.031	-	-
4.0	1.266 ± 0.011	3.097	61.94
6.0	1.374 ± 0.019	4.315	86.30
8.0	1.579 ± 0.007	6.626	132.52
10.0	1.711 ± 0.019	8.114	162.28
12.0	1.801 ± 0.001	9.129	182.58

Similarly, as illustrated in the table 4.20, only the sample concentrations (4.0, 6.0, 8.0, 10.0 and 12.0mg/ml) with their respective absorbance (1.266 ± 0.011, 1.374 ± 0.019, 1.579 ± 0.007, 1.711 ± 0.019 and 1.801 ± 0.001) were satisfying the standard Gallic acid calibration curve (linear regression curve) for Valencia extract. The Gallic acid equivalent concentrations at these absorbance were obtained using the Gallic acid standard calibration curve ($y = 0.0887x + 0.9913$, $R^2 = 0.9948$) as shown in the figure 4.9. The Gallic acid equivalent concentrations (C) obtained were 3.097, 4.315, 6.626, 8.114 and 9.129mg/ml. All variables, V, W and C were known, total phenol contents expressed in equivalent (mg of Gallic acid/g dry extract) was calculated by direct substitution in the above relationship. Finally, the total phenol contents were 61.94 mg of Gallic acid equivalent per gram of dry extract (61.94 mg GAE/g extract), 86.30 mg of Gallic acid equivalent per gram of dry extract (86.30 mg GAE/g extract), 132.52 mg of Gallic acid equivalent per gram of dry extract (132.52 mg GAE/g extract), 162.28 mg of Gallic acid equivalent per gram of dry extract (162.28 mg GAE/g extract) and 182.58 mg of Gallic acid equivalent per gram of dry extract (182.58 mg GAE/g extract) for Valencia extract.

In the present study, the two varieties were investigated and compared each other in terms of their total phenol contents in a gram of dry extract. The highest phenol content of Washington extract was 135.22 mg GAE/g extract and the highest phenol content of Valencia extract was 182.58 mg GAE/g extract. The amount of total phenol content in a gram of Valencia extract was significantly greater than that of total phenol content of Washington extract. It was evidence that

Citrus sinensis variety Valencia extract was potent in antioxidant activity than Citrus sinensis var. Washington extracts.

Similarly, the results both extracts the present study in terms of total phenol contents were compared with various past investigations on different varieties of orange peels.

According to the investigation of Asad *et al*., (2013), four different varieties of orange peels, citrus sinensis var. Washington Navel, citrus sinensis var. Valencia, citrus sinensis var. Sungin and citrus reticulate var. Page were studied and their total phenol contents were reported as 148.5, 128.5, 145.9 and 99.4 mg GAE/g extract respectively.

According to the study of Ebrahimzadeh *et al*., (2009), similarly four different varieties of orange peels such as citrus sinensis var. Washington Navel, citrus sinensis var. Valencia, citrus sinensis var. Sungin and citrus reticulate var. Clementine were also investigated and results of total phenol contents were reported as 160.3, 132.9, 153.8 and 161.7mg GAE/g extract.

Study Fidrianny *et al*., (2014) obtained the total phenol contents on three different varieties of orange peels. The varieties were Kintamani, Banyuwangi and Jember orange peels and their total phenol contents reported were 100.8, 88.5 and 95.4mg GAE/g extract respectively.

Other two studies also reported the total contents of extracts certain varieties of orange peels. According to the study Ibrahium (2012), the result of total phenol content obtained was 169.56mg GAE/g extract. Similarly, 169.56mg GAE/g extract of total phenol content of a certain orange peel was investigated by Singh (2014).

The results of present study in terms of total phenol contents were satisfactory as compared to various past investigations on different varieties of orange peel extracts. The total phenol contents of this study were higher than the total phenol contents of all varieties of the extracts reported by Fidrianny *et al*., (2014).

The total phenol content of Washington extract of this study was lower than citrus sinensis var. Washington Navel and the total phenol content of Valencia extract of this study was higher than citrus sinensis var. Valencia extract as reported by Ebrahimzadeh *et al*., (2009).

The total phenol content of Washington extract of the present investigation was slightly lower than sinensis var. Washington Navel extract and the total phenol content of Valencia extract of was meaningfully higher than all citrus sinensis var. Valencia extract as studied by Asad *et al*., (2013). Generally, the total phenol content of Valencia extract of this study obtained was significantly higher than all variety extracts of all past investigations.

4.3.5 Total Flavonoid Content of extract

The colorimetric method using aluminum chloride for quantification of total flavonoids was lead to formation of a complex between the aluminum ion, Al (III) and the carbonyl and hydroxyl groups of flavones, flavonols and flavanones that produce a yellowish color complex which used for quantification of total flavonoids by UV- Spectrophotometer and resulting in an increase in absorbance due to increase in concentration of flavonoid compounds.. Hence, the more rapidly the absorbance increases, the more potent the antioxidant activity of the extract.

Table 4.21 Catechin standard solution preparation and the corresponding absorbance

Catechin (μL)	Methanol (μL)	Concentration (mg/ml)	$NaNO_2$ (μL)	$AlCl_3.6H_2O$ (μL)	NaOH (ml)	absorbance	Absorbance Mean ± SD
0	1000	0	75	150	0.5	0.005	0.0050 ± 0
100	900	0.05	75	150	0.5	0.089	0.090 ± 0.000002
100	900	0.05	75	150	0.5	0.091	
200	800	0.10	75	150	0.5	0.149	0.1510 ± 8E-06
200	800	0.10	75	150	0.5	0.153	
300	700	0.15	75	150	0.5	0.211	0.2220 ± 0.000242
300	700	0.15	75	150	0.5	0.233	
400	600	0.20	75	150	0.5	0.302	0.3015 ± 5E-07
400	600	0.20	75	150	0.5	0.301	
500	500	0.25	75	150	0.5	0.387	0.3775 ± 0.000181
500	500	0.25	75	150	0.5	0.368	
600	400	0.30	75	150	0.5	0.417	0.4370 ± 0.00080
600	400	0.30	75	150	0.5	0.457	
700	300	0.35	75	150	0.5	0.543	0.5320 ±

700	300	0.35	75	150	0.5	0.521	0.000242
800	200	0.40	75	150	0.5	0.604	0.6015 ± 0.0000125
800	200	0.40	75	150	0.5	0.599	
900	100	0.45	75	150	0.5	0.672	0.6755 ± 0.0000245
900	100	0.45	75	150	0.5	0.679	
1000	0	0.5	75	150	0.5	0.751	0.7505 ± 5E-07
1000	0	0.5	75	150	0.5	0.750	

Table 4.22 Concentrations and Absorbance for catechin standard calibration curve

Catechin Concentration (mg/ml)	Absorbance Mean ± SD
0	0.0050
0.05	0.0900 ± 0.000002
0.10	0.1510 ± 8E-06
0.15	0.2220 ± 0.000242
0.20	0.3015 ± 5E-07
0.25	0.3775 ± 0.000181
0.30	0.4370 ± 0.00080
0.35	0.5320 ± 0.000242
0.40	0.6015 ± 0.0000125
0.45	0.6755 ± 0.0000245
0.5	0.7505 ± 5E-07

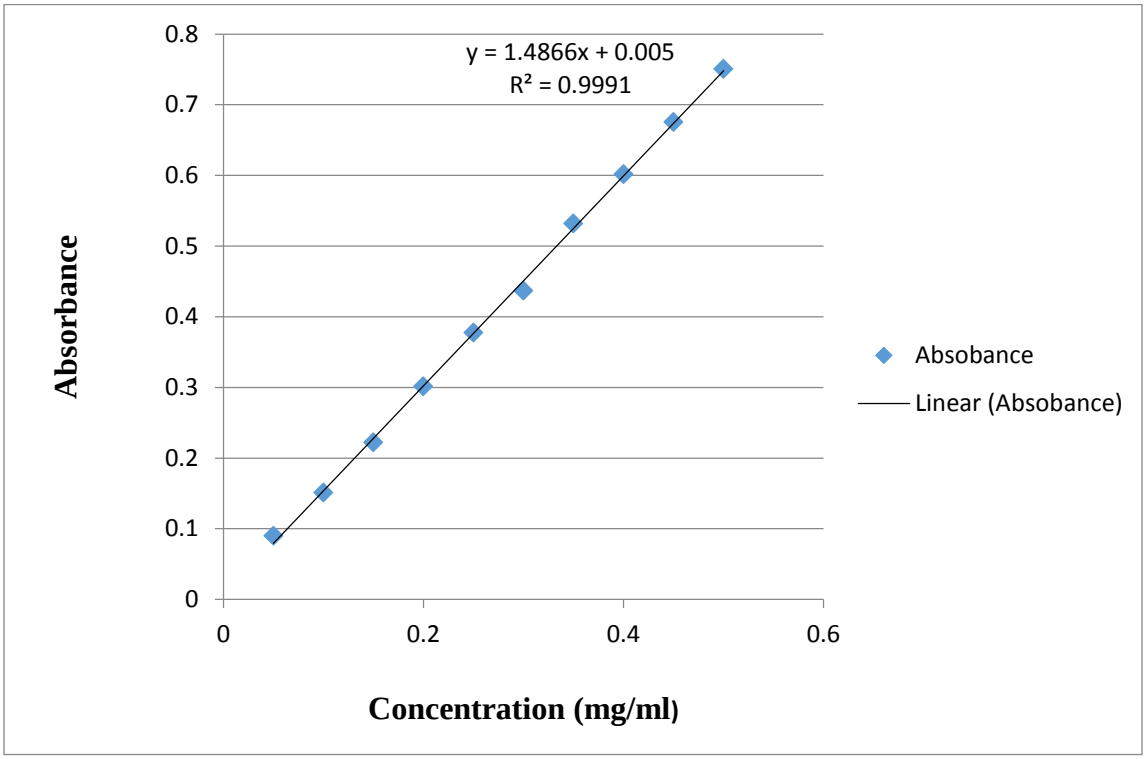


Figure 4.10 Catechin Standard linear calibration curve

Table 4.23 Washington extracts sample preparation and respective absorbance

Sample (μL)	Methanol (μL)	Concentration (mg/ml	$NaNO_2$ (μL)	$AlCl_3.6H_2O$ (μL)	NaOH (ml)	absorbance	Absorbance Mean± SD
250	750	0.9884	75	150	0.5	0.127	0.135 ± 7.25E-05
250	750	0.9884	75	150	0.5	0.135	
250	750	0.9884	75	150	0.5	0.144	

The total flavonoid content was calculated using the relationship written in the equation (8). The total flavonoid content was expressed as milligrams of catechin equivalents (mg of CAE/g dry extract) using the calibration curve of catechin ($y = 1.4866x + 0.005$, $R^2 = 0.9991$) where x is concentration, y is absorbance and R^2 is linear regression for Washington extract. The absorbance at this concentration of extract was 0.135 ± 7.25E-05 as illustrated in the table 4.23. The catechin equivalent concentration at this absorbance was determined using the catechin standard calibration curve ($y = 1.4866x + 0.005$) as shown in the figure 4.10. So the total catechin equivalent concentration (C) was 0.08745 mg/ml. Finally, the total flavonoid content expressed in catechin equivalent (mg of CAE/g dry extract) was calculated by substituting in the

values in the equation (8). The total flavonoid content was 22.12 mg of catechin equivalent per gram of dry Washington extract (22.12 mg CAT/g extract).

Table 4.24 Valencia extracts sample preparation and respective absorbance

Sample (μL)	Methanol (μL)	Concentration (mg/ml	$NaNO_2$ (μL)	$AlCl_3.6H_2O$ (μL)	NaOH (ml)	absorbance	Absorbance Mean± SD
250	750	0.8721	75	150	0.5	0.137	0.137 ± 2.5E-06
250	750	0.8721	75	150	0.5	0.135	
250	750	0.8721	75	150	0.5	0.138	

Similarly, the total flavonoid content for Valencia extract was also calculated using equation (8). The absorbance of 0.8721 mg/ml concentration of the extract solution was 0.137 ± 2.5E-06 as shown in table 4.24. The catechin equivalent concentration at this absorbance was determined using the catechin standard calibration curve (y = 1.4866x + 0.005) as shown in the figure 4.10, by substituting 0.137 for the value of y and calculating the value of x, that is the catechin equivalent concentration of extract. So the total catechin equivalent concentration (C) was 0.0888 mg/ml. The total flavonoid content for Citrus sinensis variety Valencia expressed in catechin equivalent (mg of CAE/g dry extract) was calculated by substituting in the values in equation (8). Finally, the total flavonoid content was 25.456 mg of catechin equivalent per gram of dry Valencia extract. In this study, the two varieties were compared in terms of the total flavonoid contents in a gram of dry extract. The amount of total flavonoid content from a gram of Valencia extract was greater than that of total Washington extract. It was evidence that Citrus sinensis variety Valencia extract was better in antioxidant activity than Citrus sinensis vat. Washington extracts.

According to the study investigation by Ibrahium (2012), the total amount of flavonoid content from the extract of orange peel was demonstrated as 29.75 µg Qurecetin equivalent per gram of dry extract(29.75 µg QE/g of extract).

Study Ebrahimzadeh *et al*., (2009) also investigated the total amount of flavonoid contents from different varieties of orange peels such as Citrus sinensis var. Washington Navel, Citrus sinensis var. Valencia, Citrus sinensis var. Sungin and Citrus reticulata var. Clementine. According to the

demonstration of Ebrahimzadeh *et al*., (2009), the total flavonoid content for each orange variety was investigated as 23.2 mg Quercetin equivalent/g of extract powder, 7.2 mg Quercetin equivalent/g of extract powder, 2.1 mg Quercetin equivalent/g of extract powder and 5.7 mg Quercetin equivalent/g of extract powder respectively.

According to Fidrianny *et al*., (2014), the total flavonoid content the extract was determined from the three varieties of sweet orange peels. Kintamani orange peel (12.2 mg QE/g), Banyuwangi orange peel (9.3 mg QE/g) and Jember orange peel (15 mg QE/g).

Similarly, the total flavonoid content was exposed as 0.3 mg QE/g dry extract by the study Singh (2014). Finally, according to the demonstration by Asad *et al*., (2013), four varieties of orange peels were studied and their total flavonoid content were investigated, Citrus sinensis var. Washington Navel, Citrus sinensis var. Valencia, Citrus sinensis var. Sungin and Citrus reticulata var. page and their flavonoid amounts were 25.7 mg QE/g, 2.5 mg QE/g, 6.9 mg QE/g, and 0.2 mg QE/g.

In this study, two varieties of sweet orange peels (Citrus sinensis var. Washington and Citrus sinensis var. Valencia) were studied and their total flavonoid contents were observed as 22.12 mg of catechin equivalent per gram of dry extract (22.12 mg CAT/g extract) for Washington and 25.456 mg of catechin equivalent per gram of dry extract (25.456 mg CAT/g extract) for Valencia variety. The total flavonoid content of ethanolic extract of the present study observed that Valencia variety orange peels had higher flavonoid compared to Washington variety orange peels but the difference was less significant.

4.3.6 Radical Scavenging Activity of Extract

The radical scavenging of extracts was tested using a methanolic solution of the “stable” free radical DPPH at room temperature and produce a violet solution in alcohol. It is reduced in the presence of antioxidant molecule. The evaluation of the antioxidant activity of extract was determined based on the scavenging activity against the free radical DPPH through IC_{50} parameter. The lower IC_{50} indicates a greater ability of neutralize the free radicals. Unlike laboratory generated free radical such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reaction such as metal ions chelation and enzyme inhibition. A freshly prepared DPPH solution exhibited as a deep purple color complex

with absorption medium. Thus antioxidants quenched DPPH free radicals (i.e. by providing hydrogen atom or by electron donation, conceiving via free radical attack on the DPPH molecule) & converted them to a color less/bleached product resulting in a decrease in absorbance. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract**.**

Table 4.25 Ascorbic acid standard curve preparation

Ascorbic acid (µL)	Methanol (µL)	Concentration (mg/ml)	DPPH (ml)	Absorbance	Average absorbance
0	1000	0	4	1.007	1.007
20	980	1.0	4	0.867	
20	980	1.0	4	0.858	0.8625 ± 4.05E-05
40	960	2.0	4	0.681	
40	960	2.0	4	0.677	0.6790 ± 8E-06
80	920	4.0	4	0.364	
80	920	4.0	4	0.399	0.3815 ± 0.000613
120	880	6.0	4	0.059	
120	880	6.0	4	0.067	0.0630 ± 3.2E-05
160	840	8.0	4	0.052	
160	840	8.0	4	0.051	0.0515 ± 5E-07
200	800	10.0	4	0.051	
200	800	10.0	4	0.051	0.0510 ± 0
240	760	12.0	4	0.051	
240	760	12.0	4	0.051	0.0510 ± 0

Table 4.26 Average absorbance of ascorbic acid and corresponding concentration

Ascorbic acid concentration (mg/ml)	Average absorbance
0	1.007
1.0	0.8625 ± 4.05E-05
2.0	0.6790 ± 8E-06
4.0	0.3815 ± 0.000613

6.0	0.0630 ± 3.2E-05
8.0	0.0515 ± 5E-07
10.0	0.0510 ± 0
12.0	0.0510 ± 0

Table 4.27 Preparation of DPPH absorbance for Washington extract

Sample (µL)	Methanol (µL)	Concentration (mg/ml)	DPPH (ml)	Absorbance	Absorbance Mean ± SD
20	980	1.0	4	0.534	0.5330 ± 0.000002
20	980	1.0	4	0.532	
40	960	2.0	4	0.303	0.3005 ± 0.0000125
40	960	2.0	4	0.298	
80	920	4.0	4	0.090	0.0895 ± 5E-07
80	920	4.0	4	0.089	
120	880	6.0	4	0.072	0.0725 ± 5E-07
120	880	6.0	4	0.073	
160	840	8.0	4	0.080	0.0810 ± 0.000002
160	840	8.0	4	0.082	
200	800	10.0	4	0.089	0.0905 ± 4.5E-06
200	800	10.0	4	0.092	
240	760	12.0	4	0.097	0.0990 ± 8E-06
240	760	12.0	4	0.101	

Table 4.28 DPPH absorbance of sample concentration for Washington extract

Sample Concentration (mg/ml)	Absorbance Mean ± SD
1.0	0.5330 ± 0.000002
2.0	0.3005 ± 0.0000125
4.0	0.0895 ± 5E-07
6.0	0.0725± 5E-07
8.0	0.0810 ± 0.000002

10.0	0.0905± 4.5E-06
12.0	0.0990 ± 8E-06

Table 4.29 Preparation of DPPH absorbance for Valencia extract

Sample (µL)	Methanol (µL)	Concentration (mg/ml)	DPPH (ml)	Absorbance	Absorbance Mean ± SD
20	980	1.0	4	0.448	0.4440 ± 3.2E-05
20	980	1.0	4	0.440	
40	960	2.0	4	0.264	0.2620 ± 8E-06
40	960	2.0	4	0.260	
80	920	4.0	4	0.070	0.0710 ± 2E-06
80	920	4.0	4	0.072	
120	880	6.0	4	0.066	0.0665 ± 5E-07
120	880	6.0	4	0.067	
160	840	8.0	4	0.071	0.0710 ± 0
160	840	8.0	4	0.071	
200	800	10.0	4	0.073	0.0735 ± 5E-07
200	800	10.0	4	0.074	
240	760	12.0	4	0.080	0.0795 ± 5E-07
240	760	12.0	4	0.079	

Table 4.30 DPPH absorbance of sample concentration for Valencia extract

Sample Concentration (mg/ml)	Absorbance Mean ± SD
1.0	0.4440 ± 3.2E-05
2.0	0.2620 ± 8E-06
4.0	0.0710 ± 2E-06
6.0	0.0665 ± 5E-07
8.0	0.0710 ± 0
10.0	0.0735 ± 5E-07
12.0	0.0795 ± 5E-07

Table 4.31 Comparison of antioxidant activity of sample extracts with standard ascorbic acid

Solution	Concentration (mg/ml)	Absorbance	% IA	IC_{50} (mg/ml)
Blank	0	1.0070	0	
Ascorbic Acid	1.0	0.8625	14.35	3.1816
	2.0	0.6790	32.5	
	4.0	0.3815	62.12	
	6.0	0.0630	93.74	
	8.0	0.0515	94.89	
	10.0	0.0510	94.94	
	12.0	0.0510	94.94	
Washington Extract	1.0	0.5330	47.07	1.1269
	2.0	0.3005	70.16	
	4.0	0.0895	91.12	
	6.0	0.0725	92.80	
	8.0	0.0810	91.96	
	10.0	0.0905	91.01	
	12.0	0.0990	90.17	
Valencia Extract	1.0	0.4440	55.91	0.6731
	2.0	0.2620	73.98	
	4.0	0.0710	92.95	
	6.0	0.0665	93.40	
	8.0	0.0710	92.95	
	10.0	0.0735	92.70	
	12.0	0.0795	92.11	

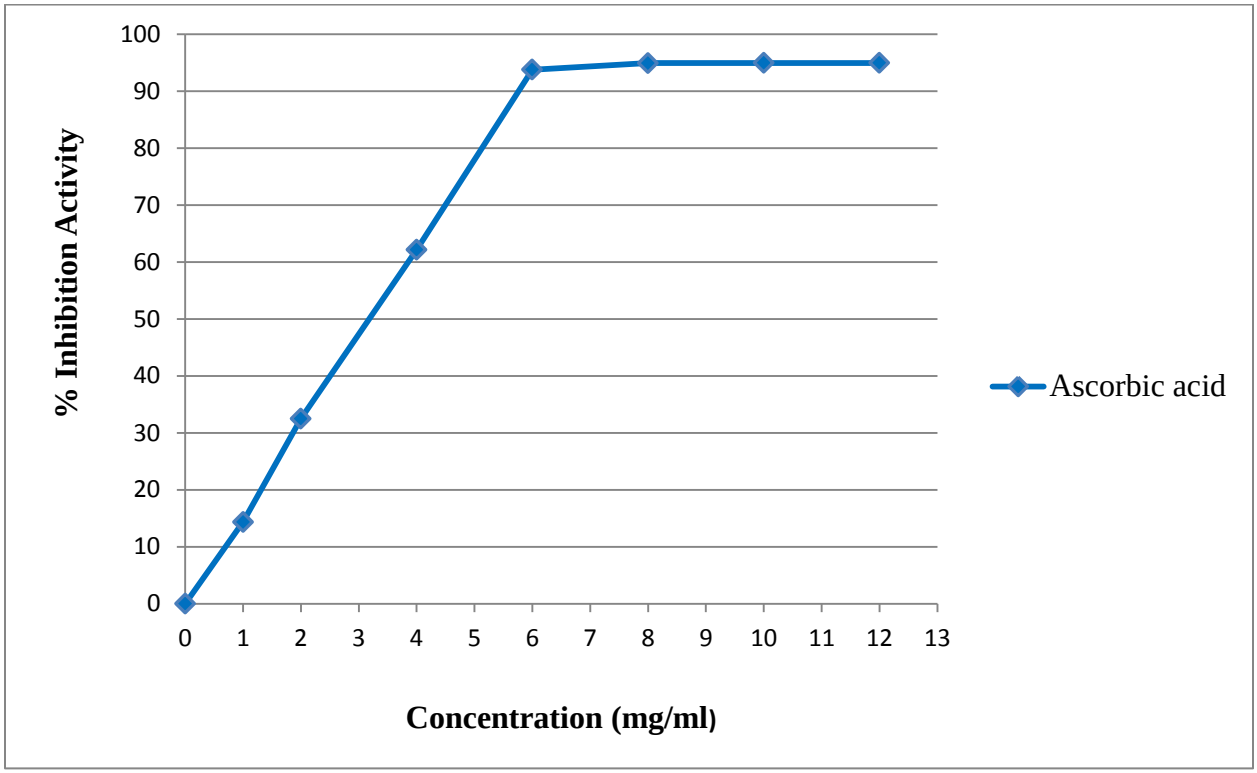


Figure 4.11 DPPH free radical percent inhibition activity curve for standard ascorbic acid

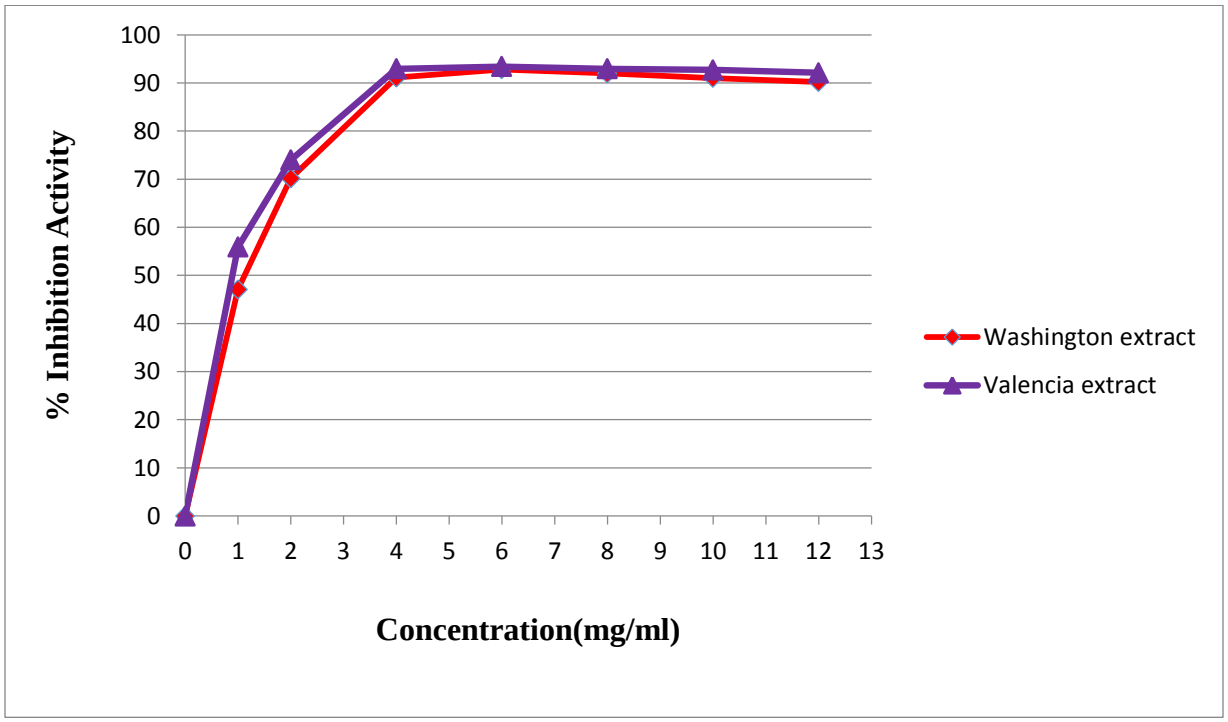


Figure 4.12 DPPH free radical inhibition activity curve for Washington and Valencia extracts

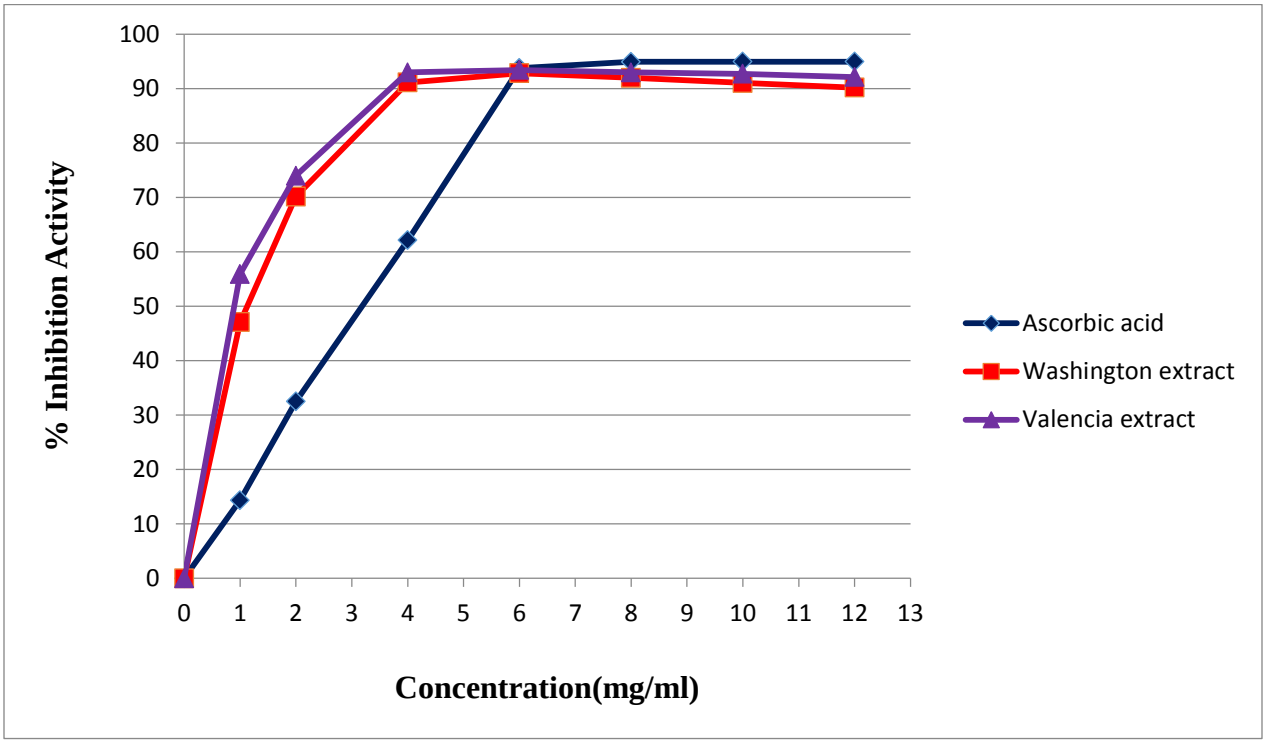


Figure 4.13 Comparison of percent DPPH free radical inhibition activities of ascorbic acid, Washington and Valencia extracts

Free radical scavenging activity or inhibition activity of free radical DPPH percentage (IA %) of standard Ascorbic acid, Washington extract and Valencia extract was calculated using the relationship written in the equation (9). As tabulated in the table 4.31, radical scavenging activity percentage of positive control Ascorbic acid was range from 14.35 to 94.94% with the corresponding concentration of ascorbic acid range from 1.0 to 12.0 mg/ml. The minimum inhibition activity (14.35%) was observed at the concentration (1.0mg/ml) and the maximum radical scavenging activity (94.94%) was obtained at the concentration (10.0mg/ml). As demonstrated in table 4.31 and figure 4.11 and 4.13, increased in the concentration to (12.0mg/ml), DDPH radical scavenging activity percentage was remained constant (94.94%).

The DPPH radical inhibition activity percentages of extracts (Washington and Valencia extract) were presented in the table 4.31 and also illustrated in the figure 4.12 and 4.13. The concentrations for both extracts were ranged in the same interval and their percentage of free radical scavenging activity was compared each other. In this study, different concentration was prepared and ranged from (1.0 to 12.0 mg/ml) for both extracts and their respective antioxidant activity or free radical inhibition activity was ranged (47.07 to 92.80 %) for Washington extract

and (55.91 to 93.40%) Valencia extracts as shown in the table 4.31. At the same concentration both varieties of the extract, they had a significant difference in capable of scavenging or removing the free radicals. At low concentration (1.0 mg/ml), Washington extract had the capacity to remove 47.07% of free radicals and similarly, at the same concentration (1.0 mg/ml), Valencia extract had an ability to scavenge 55.91% of free radicals. Comparison of both varieties of extracts in terms of antioxidant activity at the same low concentration, there was a significant difference in their oxidation preventing capacity. Valencia extract had higher capacity in removing free radicals or preventing the formation of radicals. At concentration (6.0mg/ml), the highest capacity of free radical scavenging activity (92.80 %) was achieved in the case of Washington extract and at similar concentration (6.0mg/ml) the highest DPPH free radical scavenging activity (93.40%) was obtained in case of Valencia extract. Likewise, Valencia extract at low concentration was significantly higher in radical scavenging capacity at high concentration as compared to Washington extract at the same concentration. Further increased in concentration (above 6.0mg/ml) in both varieties of extracts their radical inhibition ability started to decrease. This was happened due to absorbing their complexes colors started once they reached their highest capacity in removing free radicals as illustrated in the table 4.31.

For this investigation, the positive control or standard used to determine the DPPH free radical inhibition capacity for both varieties of extracts was ascorbic acid. The percentage inhibition activities of the varieties of extracts were compared to the percentage of radical scavenging activity of the positive control or standard ascorbic acid, to investigate that the extracts had the capacity to remove free radicals or prevent the formation of radicals. As established in the table 4.31 or in the figure 4.13, the maximum percentage inhibition capacity of standard ascorbic acid was (94.94%) but the maximum radical scavenging activities of varieties, Washington and Valencia extracts were (92.8%) and (93.40%). No extremely significant difference existed as compared with standard and this was evidence that both extracts had radical inhibition activity with respect to standard. The other parameter used to compare potent of antioxidant activities of Washington and Valencia extract with standard ascorbic acid was $IC_{50,}$ the concentration of samples (ascorbic acid, Washington and Valencia) in which the samples had the capacity to remove 50% of free radicals. IC_{50} was calculated using linear regression. As illustrated in the table 4.31, ascorbic acid had the capacity to scavenge 50% of free radicals at the concentration

(3.1816mg/ml), similarly. Washington and Valencia extract had the capacities of removing 50% free radicals at the concentration of (1.1269mg/ml) and (0.6731mg/ml) respectively. Low IC_{50} indicates more potent in antioxidant activity. Hence, both variety extracts were more potent in antioxidant activity than positive control, ascorbic acid. Valencia extract was the most potent in antioxidant activity over both ascorbic acid and Washington extract.

The present study was compared with the past investigation in terms of IC_{50}. According to the investigation of Park *et al*., (2014), 50% antioxidant activity was achieved at the concentration (IC_{50}) of 1.1379 mg/ml for variety of Auranthium orange peel extract. IC_{50} value of Auranthium orange peel extract according to park *et al*., was higher than both variety extracts of this study indicates lower in antioxidant activity than both variety extracts. According to the study of Ebrahimzadeh *et al*., (2009), four different varieties of orange peel extract was studied and IC_{50} for each variety was investigated. Under Ebrahimzadeh *et al*., (2009), IC_{50} values for Citrus sinensis var. Washington Navel, Citrus sinensis var. Valencia, Citrus sinensis Sungin and Citrus reticulate var. clementine orange peel extracts were 1.1, 2.1, 1.7 and 1.7mg/ml. IC_{50} values of current study was compared with past studies listed before. A lower IC_{50} value indicates more potent antioxidant activity or radical removing activity. From the comparison, Valencia extract in the present study had lowest IC_{50} value and the value indicates that Valencia extract had more potent in antioxidant activity. Washington extract of this study had lower value of IC_{50} next to Valencia extract and Washington extract had better antioxidant activity than past studies except Citrus sinensis var, Washington Navel extract which had low value of IC_{50} (1.1mg/ml) Ebrahimzadeh *et al*., (2009).

Similarly, this study was also compared with other investigations in terms of percentage of total radical scavenging activity of the extract with other studies. According to Singh (2014), the total free radical scavenging activity of ethanolic orange peel extract was 71.4% and according to study Ibrahium (2012), the maximum antioxidant activity of ethanolic extract of Baladi orange peel was 78.14%. As compared, free radical scavenging activity of ethanolic extract of both varieties of orange peels in the present study were significantly higher than the both results reported by Ibrahium (2012) and Singh (2014). There was an excessive difference observed in percentage of total radical inhibition activity as compared with present research, 92.8% for Washington extract and 93.40% for Valencia extract.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In current study, antioxidants were extracted from orange peels of Washington and Valencia varieties. Maximum extraction yield was found in Washington orange peel. It can be concluded from this study that Washington orange peels had large amount of extracts than Valencia orange peels.

In both varieties, extraction yield was significantly depending on the extraction conditions. Generally, as extraction conditions (temperature, concentration and time) increased from low to center level, the extraction yield was increasing. But was slightly increasing at some interval, remaining unchanged at another interval and tended to decrease in extraction yield at final interval as extraction conditions increased from center to high level individually. Similarly, interactive extraction conditions had also effect on extraction yield, increasing interaction of extraction conditions from low to center level was increasing extraction yield but further increasing extraction conditions to high level, extraction yield was slightly increasing at some interval, remained constant at another interval and started to decrease at as approaching to high level.

According to the model regression equation developed, extraction yield was positively affected by linear effects for both varieties extracts (Washington and Valencia). Extraction yield was negatively affected by all quadratic (pure and interaction quadratic) effects for Washington orange peel. All pure quadratic effects and all interaction quadratic effects except interaction between temperature and concentration had negative effect on extraction yield for Valencia orange peel.

Optimal values of extraction conditions which gave maximum yield were selected using numerical optimization of design expert for both varieties. 28.2356% response yield was obtained at operation conditions were 41.57 ºC, 73.79% and 44.15h for Washington variety, similarly, 25.8555% was yield was found at 40.77 ºC, 64.78% and 49.46 h for Valencia orange peel. These values were selected from 10 alternative optimal solutions set by design expert,

based on consider and compromise of yield, energy, economy and antioxidant activity during extraction.

According to spectrophotometer analysis for the identification and quantification of total phenol contents, total flavonoid contents and percentage of inhibition DPPH activity, Valencia peel extract was the most leading in phenolic compounds (phenol and flavonoid contents). There were differences observed in total phenol and total flavonoids content between Washington and Valencia orange peel extracts. Valencia Orange peel extracts treated with ethanol had higher phenolic content than Washington. Valencia orange peel extract had higher DPPH radical scavenging activity than Washington peel extract.

The antioxidant activities of the extracts of the Washington and Valencia orange peel revealed the radical scavenging activity, which is powerfully associated with the presence of flavonoids, phenolic acids, and their derivatives, thereby supporting the relevance of sweet orange peel as important dietary source of antioxidant compounds. Valencia orange peel was exploited as natural antioxidant in food application as well as for health supplements or functional food, to alleviate oxidative stress than Washington orange peel. It can be concluded that Valencia orange peels due to its high antioxidant activity, phenolic content and flavonoid content may prove to be a better substitute in place of synthetic antioxidants in extending the shelf life of food product by preventing the peroxide formation in the product containing fat and oil. In addition natural antioxidants are safe and impart health benefit to the consumer

5.2 Recommendations

During the experimental session, both fresh orange peel and final extract were dried in the oven with temperature 30 and 50 ºC respectively. But antioxidants are sensitive in temperature, heat metal and oxygen. For better potent of antioxidant activity, future studies should focus on freeze drying, with taking a care that they should be dried away from oxidation facilitating factors like heat, presence of oxygen and metal ions.

Food oxidation constitutes both lipid phase oxidation and aqueous phase oxidation (protein oxidation). The present study has been focused on anti-oxidative protection of lipid oxidation in food products. Antioxidants that protect lipid oxidation do not necessarily protect the protein oxidation. Future researches should pay attention in protection of food oxidation from holistic approach to the chemistry behind browning reactions, protein oxidation and lipid oxidation involving kinetic modeling.

Most of food preparation in our country is traditional and foods are easily exposed to the food oxidation enhancing factors like temperature, heat, oxygen and metals. Especially preparation of lipid containing food products like oil, chips, bread, cookies, biscuits and dairy products were not tight from oxygen and not keep away from light and heat. These conditions easily accelerate oxidation and results deterioration and rancidity of foods. Therefore, concerning bodies should give awareness to the people about natural antioxidants which are safe, effective, easy to use and renewable.

Food processing industries should emphasize their direction towards functional, health promoting natural foods and food additives like natural antioxidants, use natural antioxidant applied packaging polymers and dietary recommendation for health maintenance and wellbeing throughout life.

Natural antioxidants continue to be held in high regard because many consumers do not want to purchase products containing synthetic antioxidants. Developing a cost effective natural extract with efficacy similar to or better than current orange peel extracts could draw a substantial market share.

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APPENDICES

Appendix A: Comparison of present study with other past investigations

Table B: Comparison of present study other past investigation on antioxidant extraction from orange peel

Study	Variety	Yield (%)	TPC (mg/g)	TFC (mg/g)	IA (%)	IC_{50} (mg/ml)
Present study	Washington	18-29	29.24-135.22	22.12	47.07 - 92.80	1.1269
	Valencia	14.4-25.9	61.94-182.58	25.456	55.91 -93.40	0.6731
Singh (2014)	-	23.9	169.56	0.3	71.4	-
Ibrahium (2012)	Baladi	27.96	169.56	0.02975	78.14	-
Fidrianny et al (2014)	Kintamani	-	100.8	12.2	-	-
	Banyuwangi	-	88.5	9.3	-	-
	Jember	-	95.4	15	-	-
Ebrahimzadeh et al (2009)	Washington	-	160.3	23.2	-	1.1
	Valencia	-	132.9	7.2	-	2.1
	Sungin	-	153.8	7.2	-	1.7
	Clementine	-	161.7	2.1	-	1.7
Asad et al (2013)	Washington	-	148.5	25.7	-	-
	Valencia	-	128.5	2.5	-	-
	Sungin	-	145.9	6.9	-	-
	Page	-	99.4	0.2	-	-
Park et al. (2014)	Auranthium	-	-	-	-	1.1379

Declaration

I, undersigned, declare that the thesis for the M.Sc. degree in Process Engineering at the University of Addis Ababa, hereby submitted by me, is my original work and has not previously been submitted for degree at this or any other university, and that all resources of materials used for this thesis have been duly acknowledged.

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This thesis has been submitted for examination with my approval as a university advisor.

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