JIMMA UNIVERSITY
JIMMA INSTITUTE OF TECHNOLOGY
SCHOOL OF CHEMICAL ENGINEERING
PROCESS ENGINEERING STREAM

EXTRACTION AND CHARACTERIZATION OF ANTIOXIDANTS FROM BANANA PEELS AND EVALUATION OF ITS FOOD PRESERVATIVE EFFECTS

A Thesis submitted to School of Graduate Studies, Jimma University, Jimma Institute of Technology, School of Chemical Engineering in Partial Fulfillment of the Requirements for the Degree of Master of Science in Process Engineering

By

Tsion Wolde

January 2021
Jimma, Ethiopia

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DECLARATION

I declare that this research entitled **"**Extraction and Characterization of Antioxidants from Banana peels and evaluation of its food Preservative effects" is my own original work, and has not been submitted as a requirement for the award of any degree in Jimma University or elsewhere.

Tsion wolde	_____	_____
NAME	SIGNATURE	DATE

As research Adviser, I hereby certify that I have read and evaluated this thesis paper prepared under my guidance, by TSION WOLDE entitled "Extraction and Characterization of Antioxidants from Banana peels and Evaluation of its Food Preservative Effects" and recommend and would be accepted as a fulfilling requirement for the Degree Master of Science in Process Engineering.

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JIMMA UNIVERSITY

JIMMA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL ENGINEERING

This is to certify that the thesis prepared by **Tsion Wolde Neri** entitled "**Extraction and Characterization of Antioxidants from Banana Peels and Evaluate its Preservative Effect**" and submitted in partial fulfillment of the requirements for the award of the Degree of Master of Science in Chemical Engineering (Process Engineering) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Approved By the Examining Board

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Abstract

Banana (Musa Cavendish) peels extracts high in phenolic compounds are important antioxidants because of its phenolic hydroxyl groups. Thus, the goal of this research is extraction and characterization of antioxidant from banana peel and evaluation of its preservative effects. The influence of different process parameters, like solvent ratio, extraction temperature and time on the extraction yield examined. The results presented the minimum and maximum extract yields was 13% and 24.6% for acetone BP extract, and 21.4% and 32.4% for methanol BP extract, respectively. The parameters taken up for the study significantly affect the extraction yield. Time had the highest positive effect on yield of both solvents from linear effects, and A^2 had the highest negative effect from quadratic effects. Characterization of antioxidants was carried through the evaluation of total phenolic content (TPC), total flavonoid content (TFC), and scavenging capacity against DPPH radical scavenging activity performed using spectrophotometer. Results were obtained under optimum conditions: total phenolic content 2.476-77.524 mg GAE/g acetone extract and 3.230-77.739 mg GAE/g for methanol extract; total flavonoid content 16.436-47.327 mg CE/g for acetone extract and 5.743-57.030 mg CE/g methanol extract and DDPH radical inhibition activity 7.18%-74.06% for acetone and 1.92%-88.37% for methanol BP extracts. The IC50 values for both BP extracts were 5.80 mg/ml and 4.35 mg/ml for acetone and methanol, respectively. The antioxidant activity of BP methanol extract is higher than that of BP acetone extract. Additionally, food applications of the extract obtained to inhibit oil oxidation have been carried out according to the Shaal Oven Test compared with butylated hydroxytoluene (BHT) by measuring per oxide value (PV). In the study, all samples were treated with 0, 0.1% and 0.2% levels of BP extract and analysis were performed in every 2 days. Based on the results, soybean oil oxidation showed that the extract was less than BHT, but the oxidative stability of oil improved with 0.2% BP extract sample. Therefore, banana peel has potential antioxidant activity also can be effective used as a preservative.

Key words; *Banana (Musa Cavendish) peels, yield, total phenolic Content, total flavonoid content, DPPH radical scavenging activity*

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ABBREVIATIONS

AA	Antioxidant activity
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
BP	Banana peel
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyl toluene
CAR	Carotene
CCD	Central composite design
CE	Catechin eqivalent
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
FCCCD	Face centered central composite design
FRAP	Ferric reducing ability of plasma
FTIR	Fourier transformation infrared spectroscopy
GAE	Gallic acid equivalent
IA	Inhibition activity
IT	Induction time
PRESS	Predicted residual sum of squares
OS	Oxidative stress
RSM	Response surface methodology
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RSA	Radical scavenging activity
S.D	Standard deviation
TBHQ	Tertiary butyl hydroquinone
TFC	Total flavonoid content
TPC	Total phenol content
PV	Peroxide value

CHAPTER ONE

INTRODUCTION

1.1 Background

Antioxidants are chemicals that inhibit the activation or progression of oxidative chain reactions, thus slowing or inhibiting the oxidation of lipids or other molecules (Velioglu *et al.*, 1998). Phenolic elements such as flavonoids, phenolic acids, and phenolic diterpenes are mainly responsible for the antioxidant effect. The redox properties of phenolic compounds contribute to their antioxidant activity, as they can absorb and neutralize free radicals, quench singlet and triplet oxygen, and decompose peroxides (Choe, 2009). Most of these phytochemicals have potent antioxidant properties that have been linked to lower cancer incidence and mortality rates in a variety of human populations.

Antioxidants are classified into two categories: synthetic and natural. Synthetic antioxidants are phenolic compounds that capture free radicals and inhibit chain reactions. They are synthetic chemicals that are applied to raw or pre-packaged foods to avoid rancidity and browning. Butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), and tertiary butyl hydroquinone (TBHQ) are the most widely used synthetic antioxidants, phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), and carotenoids, as well as ascorbic acid, are examples of natural antioxidants(Vu, Scarlett and Vuong, 2018).

Antioxidants commonly used as a food additive to improve health and prevent diseases such as cancer and cardiovascular disease. Furthermore, they frequently used in foods as preservatives. This occurred in the mid-twentieth century. It all started with scientists' efforts to prolong the shelf life of foods. The experiments were able to avoid the start of rancidity by mixing antioxidants with foods rich in unsaturated fat (Atta, Nawal H Mohamed and Abdelgawad, 2017). Many foods consist of various organic compounds and can quickly be oxidized. In addition, lipids (like fats, oils, plus waxes) has the highest potential to loss electrons. Food lipid auto-oxidation caused thru exposure to sunlight, ionizing radiation, heat, metal ions can have a degrading effect on the color, flavor, texture, consistency and protection of food. Food lipids chemically made up of triglycerides, and degradation occurs at the unsaturated sites of the triglyceride, resulting in rancidity. Therefore, antioxidants frequently applied to food as a protection against lipid oxidation or

auto oxidation. Food preserved usually by storing it in the dark, wrapping it in tubes, or even waxing it. These antioxidants are a particularly valuable group of preservatives because of bacterial or fungal spoilage. Oxidation reactions also happen very quickly in frozen or refrigerated products ,triggering spoilage (Genitha Immanuel, 2014).

In recent years, the attention of consumers when natural antioxidants are used has increased because they can be more beneficial to someone's health than synthetic antioxidants. This situation has contributed to the quest for natural compounds that have antioxidant properties and, therefore, a source of antioxidants; various natural products have been examined. Wastes, such as peels and seeds, have gained particular consideration. Plant-isolated phenolic compounds are thought to be the most promising family of molecules for preventing and inhibiting oxidation.

Currently, the usage of natural antioxidants in food and preventive medicine is becoming very common because of the arguments that they are safer and have characteristics that inhibit illness and improve health. Plants are widely recognized as the common source of bioactive phytochemicals and antioxidant nutrients (Elless *et al.*, 2000).Polyphenolic compounds such as phenolic acids as well as flavonoids are essential phytochemicals for their antioxidant properties, inactivation of lipid free radical chains, chelation of redox-active metal ions, and inhibition of hydro peroxide conversion to reactive oxyradicals. The phenolic and flavonoid composition of a commodity is used as a preliminary screen for every product that is meant to be used as a natural potent antioxidant in foods products (Viuda-Martos *et al.*, 2011).

Bananas are one of the most common fruits, rich in phytochemical compounds, which have a potential to extract antioxidants. The fruits eaten either raw, fried or converted into different items. As a result, every year several tones of banana peel are produced and this is a possible material for further use. However, the peel of the fruit, which accounts for about 30% of the weight of the fruit, is commonly regarded as waste, resulting in an environmental crisis because of banana cultivation and processing (González-Montelongo et al., 2010). Extraction process is the most significant stage in the production of bioactive compounds from plant materials and by-products. Therefore, extracting the antioxidant compounds from banana peels and studying their food preservative potential not only make it useful but it also serves as a waste management strategy.

1.2 Statement of the problem

Oxidation reactions are among the most common source of deterioration of food and food components. Due to reduced food protection and nutritional consistency, oxidative degradation of fats and oils decreases the shelf life of lipid-containing foods (Jacobsen, 2016). As a result, the issues associated to it can be addressed by developing food storage mechanisms that maintain oxidative stability of fats and oils while also extending the shelf life of foods by the use of antioxidants. Synthetic antioxidants are the ones in great use in the area of food processing industries as most food and pharmaceutical products contain them. These synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), can have harmful health consequences and presumed unsafe to be used. Therefore, the use of natural antioxidants in food and medicine is becoming very common because of they are safer and have features that prevent illness and improve health.

Phytochemicals such as polyphenols, flavonoids, and vitamins, which has been related to improved human health, can be found in fruits and vegetables. It is widely known that people who eat a lot of fruits and vegetables have a lower chance of heart disease, obesity, cancer, and other diseases. Higher demand for these goods, on the other hand, increases the number of residues like peel, bagasse, and nuts, resulting in environmental and economic costs. Bananas are among the most well-known fruits; the peel is the primary by-product, accounting for roughly 30% of the total fruit (González-Montelongo et al., 2010), phenolic compounds (gallocatechin), carotenoids (α-carotenoids, β-carotenoid, and xanthophylls), anthocyanin (delphinidin and cyanidin), sterols, catecholamines, and triterpenes are among the phytochemical compounds with strong antioxidant ability (Someya et al., 2002). This indicates that one of the natural sources of antioxidants may be banana peels. Antioxidant extraction from banana peels, which is a perfect way for reduce waste and environmental protection because banana peel is the primary by-product from banana. Its peel covers one third of fruit, also investigating their potential antioxidant activity of banana peel may help to inhibit lipid oxidation, enhance food consistency and nutritional value, and replace synthetic antioxidants.

1.3 Objectives of the study

1.3.1 General Objective

The general objective of this research is to extract and characterize antioxidants from banana peels and evaluation of their food preservative effect.

1.3.2 Specific Objectives

- ✓ To determine the yields of antioxidant extracts from banana peels using two organic solvents (acetone, methanol) with water.
- ✓ To analyze the proximate composition of banana peels.
- ✓ To optimize the extraction conditions which give the maximum yield of antioxidants.
- ✓ To characterize and evaluate the potent antioxidant activities of the extracts by determining total phenolic content, total flavonoid content and percentage free radical scavenging activity of the extract.
- ✓ To evaluate the food preservative effect of banana peels extracts.

1.4 Significance of the study

Oxidation of lipids is a significant source of degradation of food quality; it has been a struggle for food scientists and manufacturers. In food systems, lipid oxidation can result in the development of off-flavors and the degradation of essential amino acids, fat-soluble vitamins, and other bioactives. Synthetic antioxidants have been commonly used to retard lipid oxidation in different food systems to avoid having a loss of consistency due to lipid oxidation. However, questions about potential toxicity and side effects have been posed by the use of synthetic antioxidants. Thus, the usage of natural antioxidants has increasingly accepted as an effective method to prevent rancidity. Various phenolic-rich plant extracts had effectively established the delaying effect on lipid oxidation due to their good antioxidant capacity. It is well known that the presence of phenolic compounds is responsible for the antioxidant properties of fruit extracts. However, research into the effectiveness of fruit/fruit peel extracts in preventing oxidative rancidity is still lacking. So extracting phenolic compounds from fruit/fruit peel is very important.

Bananas are an important food crop, it peel contains potent antioxidant compounds. The most common antioxidants in banana peels are phenolic compounds, carotenoids, and minerals. The antioxidant capacity (AC) of banana peels has been found to be greater than that of banana pulp. These peels are biodegradable and because of their amount of nitrogen and phosphorus, they can cause environmental concerns. Therefore, extracting the banana peel will be the best resolution in order to protect human being and creating considerable economic benefit to food industry. Besides, banana fruit is easy obtains because it is not a seasonal fruit. These ensure that, banana can continuously act as the natural antioxidant

source, to prolong the shelf life of a food by avoiding rancidity, lipid peroxidation, and oxidative harm.

CHAPTER TWO

LITERATURE REVIEW

2.1 Banana (Musa Cavendish)

Banana on the world market is a very common fruit and is eaten in many countries as simple food. It is cultivated internationally and, in terms of world trade, it is the fifth most important agricultural food crop in the world. It has been categorized as sweet bananas or dessert and bananas or plantains for cooking. In different food items, it is consumed either fresh or cooked, plus as a useful ingredient. The peel of banana is a typical waste after consumption. There is a variety of bioactive substances in addition to banana peel, phenolics, carotenoids, biogenic amines, and phytosterols are some examples of these compounds, that are particularly beneficial in the diet because they have several favorable health and well-being benefits. Most of these substances are important in defending the body from different oxidative stresses and have antioxidant activities. The banana peel may also be one of the natural sources of antioxidants. Bananas have been successfully used for treatment of multiple diseases in the past, plus minimize the risk of certain progressive degenerative disorders (Singh *et al.*, 2016).

2.2 Banana Bioactive Compounds

Bioactive compounds obtained from plant secondary metabolism have a strong ability to contribute to the activity of antioxidants. The major phytochemicals contained in fruits and vegetables linked to human health are phenolics and carotenoids (Singh *et al.*, 2015). Bananas have a distinct variety of bioactive substances, including other essential fruits. Phenolics, cartonoids, flavonoids and biogenic amines are the ones that have gained special interest in the ripened and unripen banana. Some phytosterols in banana pulp have also been identify at low levels. Bananas has a greater antioxidant ability compared to berries, herbs and vegetables because of these bioactive substances, and this ability grows as the fruit ripens.

2.2.1 Phenolic and flavonoid compounds

Gallic acid, catechin, epicatechin, tannins, and anthocyanin are among the phenolic compounds contained in banana pulp and peel. Bananas contain significant numbers of phenolic compounds and flavonols in general. This compound give the unripe banana a bitter flavor. Various earlier studies have revealed phenolic compounds extracted from

banana measured ranges from 11.8 to 90.4 mg of GAE/g DW using the Folin-Ciocalteu colorimetric method. The discovery of certain free phenolic compounds in bananas from Tenerife and Ecuador was investigated by Del Mar Verde Méndez et al. (2003). High levels of free gallic acid and catechin (cianidanol) were observe in two banana varieties, with recovery percentages of 49.61.5 percent and 84.32.2 percent for catechin and gallic acid, respectively, using the proposed procedure. Study by Sulaiman et al. (2011) reported that the chloroform extract of the pulps of Awak cultivar presented the highest total phenolic content (23.42 $\pm$ 1.22 mg GAE/g d.w.) followed via the similar extract of Nangka cultivar (20.47 $\pm$ 0.49 mg GAE/g d.w.). Different extract concentrations and various techniques followed for TPC analysis are likely to provide different outcomes, including using gallic acid as a guide. In addition to reduced tannins and flavonoids, banana pulp produces a number of non-extractable phenolic compounds in the soluble cell wall fractions of the fruit pulp (catechin, gallocatechin, and epicatechin). The existence of anthocyanidin delphinidin was documented on the walls of the cells. Dwarf Cavendish bananas have been found to have epicatechin, epigallocatechin, and gallocatechin.

Banana is a fruit that can be eaten and has a variety of medicinal properties. It is cook as a vegetable in the southern parts of India and consumed alongside rice. Flavonols, like quercetin, myricetin, kaempferol and cyanidin, are the primary groups of flavonoids found in bananas. Many experts know the health advantages of flavonoids found in bananas. As defensive scavengers against free radicals results from oxygen and reactive oxygen species (ROS) responsible for ageing and numerous diseases, flavonoids function. This fruit is also high in phenolic acids, which are compounds with a phenolic ring and an organic carboxylic acid group attached to other plant components including polysaccharides and lignin in cell walls. The amount of phenolic compounds and flavonoid contents in different genotypes varied greatly, and many studies have reported superior genotypes with high amounts of these bioactive compounds (Sulaiman *et al.*, 2011). The study by Aboul-enein et al., (2016) shows different solvent systems significantly changes in total flavonoid contents and the values were also influenced by the polarity of the solvent, according to the findings the overall flavonoids in banana peel had the highest content (21.04 CEmg/g DW) in methanol extract followed by ethanol (18.52 CEmg/g DW), acetone (16.15 CEmg/g DW) then aqueous extract (8.56 CEmg/g DW). A study by Fatemeh *et al.*, (2012) using quercetin as a reference, records were made in two varieties (Cavendish and Dream) and two levels of ripeness (ripe and green). Cavendish ripe was 225.91 mg QE/100g of dry matter, and

Cavendish green was 389.33 mg QE/100g of dry matter, while Dream ripe was 72.46 mg QE/100g of dry matter.

2.2.2 Carotenoids

Carotenoids are beneficial to someone's health because of their specific physiological roles, such as their role as pro vitamins and antioxidants, particularly in scavenging singlet oxygen. This has been extensively researched for the role in lowering the risk of illnesses, especially certain cancers and eye diseases that have become global issues. Pure hydrocarbons (carotenes) and hydrocarbon oxygen derivatives are two of the most important classes of plant pigments (xanthophylls). This are members of the isoprenoid biosynthetic pathway, with key roles for light harvesting in plants as antioxidants and accessory pigments. Carotenoids can be present in excess in orange and yellow fruits, such as bananas. Lutein, α-carotene, β-carotene, violaxanthin, auroxanthin, neoxanthin, isolutein, beta-cryptoxanthin, and alpha-cryptoxanthin were known as carotenoids (Jaswir *et al.*, 2011). Banana cultivars with high levels of carotenoids were established using raw and cooked samples of bananas, giant swamp taro, and breadfruit cultivars. A comprehensive review of banana varieties has shown that there are actual high levels of pro-vitamin also maximum carotenoids in different genotypes. Banana cultivars rich in carotenoids and culturally acceptable were discovered by Englberger et al. (2003). They tested these cultivars for α-carotene and β-carotene and recommended that they be used to treat vitamin A deficiency using these culturally appropriate banana cultivars. The dietary and behavioural changes towards processed refined foods are linked to vitamin A deficiency and chronic diseases. According to research, banana cultivars with yellow or orange flesh have the highest levels of β-carotene, one of the most essential pro vitamin A carotenoids. These banana cultivars can be considered for promotion in vulnerable target populations with vitamin A deficiency and other chronic diseases. The main bioactive compounds and broader profiles of micronutrients in 16 Karat also other Micronesian banana cultivars were studied by Englberger et al., (2006). In Karat banana cultivars, they find high amounts of carotenoids and riboflavin and this rate were extraordinarily high for carotenoids relative to Micronesian banana cultivars. Usage of carotenoids-rich foods enhances safety and lowers the risk of diseases like cancer, diabetes, and heart disease.

Several carotenoid-rich banana cultivars were grown by breeding methods with the aim of providing targeted populations with special nutritional needs in regular diets. Davey *et al.*,

(2007) have studied Musa varieties grown under uniform field conditions, Orange-fleshed plantain (AAB) varieties were found to have higher levels of pro vitamin A carotenoids than dessert bananas (AAA). Arora *et al.*, (2008) described that the content of beta-carotene in selected Indian banana varieties was determined, and the karpooravalli banana cultivar was found to have a high level of beta-carotene. The amount of carotenoids in bananas was greater than those present in maize, which the main compounds were lutein and zeaxanthin and only 10-20 percent are provitamin A carotenoids. Multiple alleles decide the content of pro-vitamin A carotenoids and other carotenoids, and their content has been shown to vary between genotypes based on the environmental conditions of the developing regions.

Banana genotypes that have a great pro-vitamin A carotenoid content are possibly valuable foods for vitamin A-deficient populations. In small-income countries by significant health issues with vitamin A deficiency, certain genotypes can be grown and eaten. Provitamin A carotenoids are readily consumed and transformed to vitamin A in the human body, thus assisting in the treatment of vitamin A deficiency (Fungo and Pillay, 2015).

2.2.3 Biogenic amines

Decarboxylation of amino acids or amination and transamination of aldehydes or ketones produce biogenic amines (BA), which are essential nitrogenous compounds with a low molecular weight. (Biji *et al.*, 2016). Banana peel plus pulp have been presented to contain biogenic amines, like dopamine, serotonin and norepinephrine. Serotonin leads to the feelings of health and pleasure. Banana has significant quantities of dopamine also norepinephrine. Studies indicate that the formation of 3-hydroxytyramine, in the peel of ripening bananas, by C14 categorized tyrosine, phenylalanine, tyramine and L-DOPA 3,4-dihydroxyphenylalanine. They presented secondary suggestion for biosynthesis of dopamine, via tyramine trail with tyrosine decarboxylation, When demonstrating that peel skin alters to C_{14}-tyrosine to 3-hydroxytyramine thru ripening. Separated, processed, and classified the enzyme that catalyzes the conversion of tyramine to dopamine from banana fruit pulp (M. sapientum). The synthesis of dopamine in bananas was thought to be caused by tyramine hydroxylase(Deacon and Marsh, 1970).

Dopamine is a catecholamine generated by removing the carboxyl group from L-DOPA. In the human brain and body, it plays a significant function as a neurotransmitter with a major effect on our mood, capacity to focus and emotional health. Many experiments have

also detected large levels of catecholamines, including dopamine and its precursor L-DOPA, in bananas.

Bapat *et al.*, (2011) discovered that the concentrations of L-DOPA and tyramine in banana fruits rose as they ripened. Dopamine levels rise in both peels and pulp during the transition from unripe to ripening (climacteric) stages, then fall in the post-climacteric period. The decrease in dopamine levels associated with aging and senescence could indicate that it is oxidized into quinones, which then polymerize to melanin. González-Montelongo et al., (2010) characterized a variety of bioactive compounds (including catecholamines from banana peels) and recorded high levels of dopamine and L-DOPA in the peel area Dopamine content improved dramatically as the extraction time using methanol extracts was increased in their analysis.

2.3 Antioxidants

The term "antioxidant" refers to a substance that protects the body from oxidation. Antioxidants act to prevent radicals from oxidizing lipids. Antioxidants are efficient since they are prepared to give free radicals their own electrons. If a free radical obtains an antioxidant from the electron, the cell no longer needs to be targeted and the oxidation chain reaction is broken. By default, after contributing a electron, a free radical is generated when an antioxidant is destroyed. Antioxidants are not dangerous in this state and without being reactive; they have the ability to accept the transition in electrons. There is an elaborate antioxidant defense mechanism in the human body. Antioxidants are produced inside the body and can also be derived from foods such as bananas, vegetables, beans, nuts, meats, and oil that are eaten by humans(Atta et al., 2017).

Also, by neutralizing free radicals, antioxidants block the oxidation mechanism. As a result, the antioxidants themselves become oxidized. They will use one of two approaches: chain-breaking or prevention. As a free radical releases or abstracts an electron, a second radical is formed, which is known as chain-breaking. This molecule then does the same thing to a third molecule, creating more unstable products in the process. The mechanism proceeds before either the radical is stabilized by a chain-breaking antioxidant like beta-carotene or vitamins C and E, or it decays into a harmless substance (Atta et al., 2017). The other is Prevention; Antioxidants resist oxidation by slowing down the chain initiation process. By scavenging the initiating radicals, such antioxidants will prevent an oxidation chain from

ever beginning. They can also prevent oxidation by stabilizing altered metal radicals like copper and iron.

Antioxidants have been described in foods as substances capable of stopping or greatly slowing the oxidation of readily oxidizable compounds such as fats in limited amounts, therefore, Antioxidants are commonly compared with chain-breaking lipid peroxidation inhibitors in food chemistry, however not completely so. Antioxidants have been researched widely and can be present in a wide range of foods, including drinks. Antioxidants are compounds that can be related to the protection of macromolecules from oxidation in foods and beverages (Atta et al., 2017).

2.3.1 Types of Antioxidants

2.3.1.1 Natural antioxidant

Natural antioxidants be present in different elements with diverse chemical properties, which are commonly exist in plants. Antioxidants are compounds which prevent or delay the oxidation of additional molecules by preventing the start or spread of oxidizing chain reactions(Velioglu *et al.*, 1998). Therefore, natural antioxidants may protect all the biologically important cellular components and foods against oxidative processes triggered by reactive oxygen species. Recently, food companies are pleased to use natural antioxidants in different goods to slow oxidative degradation of lipids, increase consistency and nutritional value of foods, and substitute synthetic antioxidants (Velasco and Williams, 2011). The majority of natural antioxidants are found in common foods and have long been included in the diet in the form of fresh, cooked or processed products. Natural antioxidants offer electrons from two main electron-rich bases: hydroxyl groups plus paired bonds. Natural antioxidants go through extra chemical reactions after donating electrons to promote their degradation. Several antioxidants use their hydroxyl (-OH) groups to give electrons to other molecules, and is the first major tool they use to resist oxidation.

2.3.1.2 Synthetic antioxidants

Synthetic antioxidants are compounds that are synthesized chemically since they may not occur in nature and are used as preservatives in food to prevent lipid oxidation. To preserve fats and oils, some synthetic antioxidants have been used. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were developed to avoid oxidative gumming in petroleum. However, such compounds began to be used as antioxidants in human foods in

1954, and they are still the most popular antioxidants in such foods. BHT and BHA share the same name, as well as similar structures and antioxidants, and are present together in fats and oils.

Regardless of the information that both BHT and BHA are includes in the category of substances that are generally accepted as safe. BHT and BHA have been linked to a wide variety of health issues, including swollen livers, elevated liver microsomal enzyme production, and the conversion of certain ingested materials into poisonous and carcinogenic compounds, according to studies (Ballard et al., 2008). So that these common synthetic has increasingly become seen as a threat to human health because of synthetic antioxidant will not produce the same health benefits like natural antioxidants.

2.4 The chemistry of antioxidants

It incorporates the antioxidant's mechanism. Antioxidants are thought to work by two different pathways. The first is a chain-breaking process by which electrons are donated by primary antioxidants to free radicals in the process, example lipid radicals. The second mechanism includes elimination of ROS (reactive oxygen species) and RNS (reactive nitrogen species) initiator by quenching chain initiator catalyst.

Chain reactions of free radicals

Initiation stage

(1) $RH \longrightarrow R^{\bullet} + H^{\bullet}$

(2) $R^{\bullet} \longrightarrow R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$

(3) $2ROOH \longrightarrow ROO^{\bullet} + RO^{\bullet} + H_2O$

Propagation stage

(1) $R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$

(2) $ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$

(3) $RO^{\bullet} + RH \longrightarrow ROH + R^{\bullet}$

Termination stage

(1) $R^{\cdot} + R^{\cdot} \longrightarrow R-R$

(2) $R^{\cdot} + ROO^{\cdot} \longrightarrow ROOR$

(3) $ROO^{\cdot} + ROO^{\cdot} \longrightarrow ROOR + O_2$

(4) Antioxidants + O_2 $\longrightarrow$ Oxidized antioxidants

The initiation stage – involves an additional factor creating a free organic radical or an unpaired electron as part of the substrate (oxidizing agent).

The propagation stage - requires the reaction of the free radical (highly reactive species) free during initiation stage through oxygen to peroxide radical. Radical peroxide is another reactive product that could react with the substrate or other materials in the substrate surface that could contribute to further decomposition.

The termination stage – involves the formation of a stable organic compound from the radical species produced during the initial and propagation stages of oxidation, followed by the removal of free radicals from the substrate. The termination stage will be active in attenuating or stopping the oxidation step if no further radicals are produced during the initiation stage.

Additional, as fats come in interact with oxygen in free radical chain reactions, it forms unsaturated fatty acids that result in free radicals. Hydroperoxide, which occurs until the oxidation reaction in trace amounts, commonly breaks down into radicals, which separate a hydrogen atom from some other molecule to form a hydroperoxide, which releases more radicals. The antioxidants would neutralize the free radicals by sacrificing one of their own electrons, thereby stopping the reactions. These can be seen all over the body.

2.5 Antioxidant Mechanisms in the Oxidation of Foods

Antioxidants work by scavenging free radicals, chelating metals, quenching singlet oxygen and photosensitizers, and inactivating lipoxygenase to slow down the oxidation rate of foods.

2.5.1 Free radical scavenging

By donating hydrogen to them, antioxidants scavenge free food radicals and generate reasonably stable antioxidant radicals with a low standard reduction capacity, less than 500

mV. Rates of hydrogen abstraction from antioxidants are higher than that of lipids. The greater stability of antioxidant radicals than that of food radicals is due to resonance delocalization across the phenolic ring structure. The efficacy of antioxidants in scavenging food free radicals depends on the energy of the bond dissociation between oxygen and phenolic hydrogen, the constant pH related to acid dissociation, and the capacity for antioxidant radicals to be decreased and delocalized. When the bond dissociation energy for O-H in the antioxidants is low, hydrogen transfer from antioxidants to the peroxy or alkyl radicals of foods is thermodynamically more desirable. Bond dissociation energy for O-H of phenolic antioxidants decreases in the order of δ>γ>β>α-tocopherol (Choe and Min, 2015).Bond dissociation energy for O-H of phenolic antioxidants is influenced by means of nearby solvents; it is greater in polar solvents like acetonitrile plus tertbutyl alcohol than nonpolar benzene. Therefore, because of the intermolecular hydrogen bonding between oxygen or nitrogen in the polar solvent group and OH in phenolic antioxidants, polar solvents reduce the radical scavenging activity of antioxidants.

The phenolic antioxidants' bond dissociation energy for O-H also projects the stabilization of antioxidant radicals. For the O-H group of antioxidants, the lower the bond dissociation energy, the more stable the antioxidant radical is. With antioxidants.Thus, low bond dissociation energies are more effective donors of hydrogen and stronger antioxidants. Hydrogen replacement in a benzene ring changes the O-H bond frequency of phenolic antioxidants. The antioxidant activity of the phenolic antioxidants depends on the equilibrium between the substituents' electron donation effect and the steric crowding around the phenolic OH groups that is connected to the site of the substituents. The O-H bond strength is diminished by some substituent that destabilizes phenolic antioxidants in the ground state and stabilizes the phenoxy radical type of the antioxidants. Substituents like an alkyl group or a 2nd hydroxyl group promote antioxidant radical stability and improve 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, 2015). In the 6-hydroxy group, tocopherol donates hydrogen to the alkyl peroxy radical on a chromanol ring, and alkyl hydroperoxide plus tocopherol radical are formed. Due to a resonance structure, tocopherol radical is comparatively stable. To generate tocopherol semiquinone without vitamin E action, tocopherol radical may react with lipid peroxy radical, or react through each other to create

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 and Sutherland, 1982).

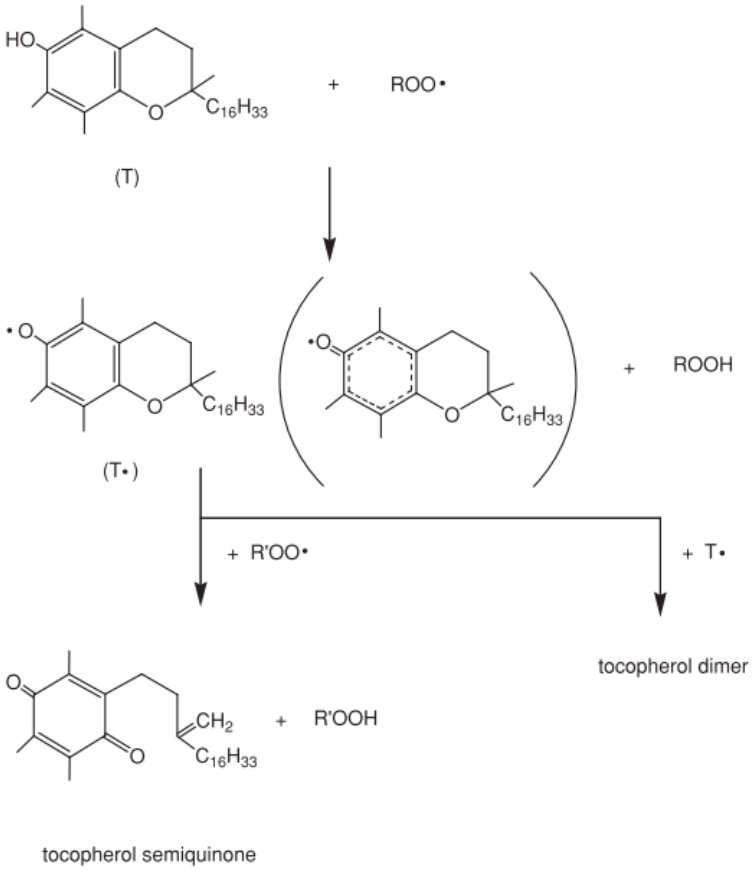


Figure 2.1 Reaction of α-tocopherol with lipid peroxy radical

Flavonoids should have special structural features for scavenging free radicals: the orthodihydroxy 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 best effective 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, 2006). Phenoxy radicals undergo disproportionation and produce phenolic quinone and a dihydroxy phenolic compound (Shahidi et al., 1992).

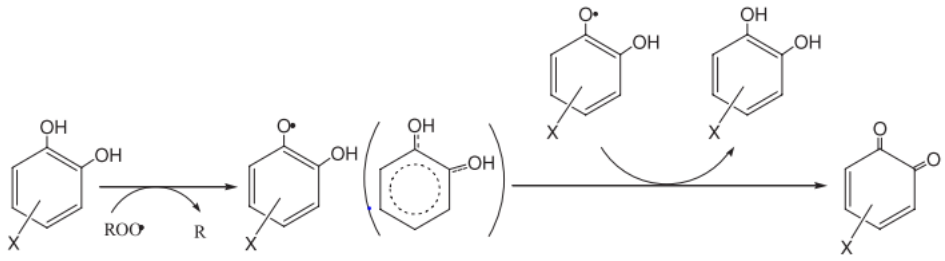


Figure 2.2 Reactions of catechol structured flavonoid with lipid peroxy radicals

Carotenoids can provide electrons and so hydrogen can be donated. With 2 reduction potentials, E1 and E2, two electrons instead of one are moved per carotenoid. The ease of carotenoid electron donation depends on the existence of the carotenoid substituents. In canthaxanthin and astaxanthin, the reduction potential for sequential movement of 2 electrons varies, generally E1 < E2, while lycopene, β-carotene, and zeaxanthin have similar E1 and E2 values. 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 end-group electron-acceptance power decreases, ΔE (E1 – E2) decreases or cation radical can be reduced to carotenoid radical with an E3 reduction potential that is generally much lower than E1(Jeevarajan and Kispert, 1996).

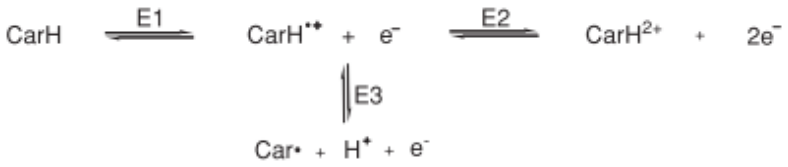


Figure 2.3 Hydrogen release from carotenoids (CarH) via electron donation.

β-Carotene may donate hydrogen to lipid peroxy radical with some limitations and produce carotene radical. Due to the delocalization of unpaired electrons in its conjugated polyene, Carotene radical is a fairly stable species and has sufficient lifetime for a reaction with low oxygen concentration lipid peroxy radicals 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 create carotene peroxy radical (ROO–Car•), specifically at oxygen pressure greater than 150 mm Hg(Choe and Min, 2009).

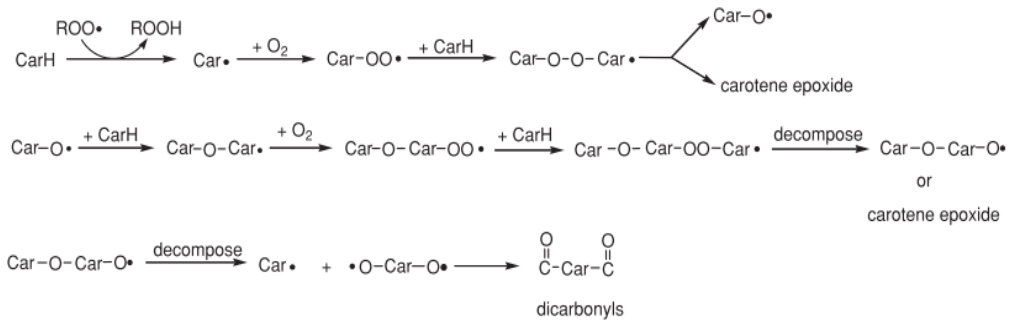


Figure 2.4 Reaction of β-carotene and lipid peroxy radicals.

β-Carotene peroxy radical reaction of triplet oxygen to create 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 chain reaction of lipid oxidation, thus β-carotene acts as a prooxidant:

$$\mathrm{Car} + \mathrm{ROO}\bullet \rightarrow \mathrm{ROO} - \mathrm{Car}\bullet$$

$$\mathrm{ROO} - \mathrm{Car}\bullet + {}^{3}\mathrm{O}_2 \rightarrow \mathrm{ROO} - \mathrm{Car} - \mathrm{OO}\bullet$$

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

By donating hydrogen to food radicals, Ascorbic acid and glutathione scavenge free radicals, creating more stable ascorbic acid and glutathione radicals than food radicals. Ascorbic acid radicals become dehydroascorbic acid with proton depletion. Free radicals are also inactivated by amino acids having sulfhydryl or hydroxy groups, such as cysteine, tyrosine, phenylalanine, and proline. The inactivation of food radicals by protein compounds may be a result of rivalry for high-energy food radicals between protein compounds and lipids, rather than an actual chain breaker.

2.5.2 Metal chelating

Metals decrease the activation energy of the oxidation, particularly in the initiation step, to speed up oil oxidation. Metals catalyze food radical formation by abstracting hydrogen. They also produce hydroxy radicals using catalyzing decay of hydrogen peroxide or hydro peroxides. Ferric ions reduce the oxidative stability of olive oil by decaying phenolic antioxidants such as caffeic acid. Crude oil contains transition metals for instance iron or copper, often existing in chelated form rather than in a free form. Oil refining losses metal

contents. Edible oils produced minus refining, as if extra virgin olive oil and roasted sesame oil contain comparatively high amounts of transition metals (Decker et al., 2005).

Metal chelators reduced oxidation by inhibiting metal redox cycling, creating insoluble metal complexes, or providing steric obstruction between metals and food components or intermediates for their oxidation. In food items, the most common metal chelators are EDTA and citric acid. Most chelators are water-soluble, but citric acid can be dissolved in oils in the oil system with some restriction on chelate metals. Phospholipids are also acting as metal chelators(Chen and Xu, 2018). The metal ions can also bind the flavonoids 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 (Hudson and Lewis 1983,Ferrali et al. 1997). Metals can also chelate lignans, polyphenols, ascorbic acid, and amino acids such as carnosine and histidine (Decker et al., 2005).

2.5.3 Singlet oxygen quenching

Singlet oxygen reacts with lipids at a higher rate than triplet oxygen with high energy of 93.6 kJ above the ground state triplet oxygen. Tocopherols, carotenoids, curcumin, phenolics, urate, plus ascorbate could quench singlet oxygen(Reviews and Science, 2005). Physical quenching results in the deactivation by energy transfer or charge transfer of singlet oxygen to the ground-state triplet oxygen. There is neither the absorption of oxygen nor the formation of materials. When the energy level of a quencher (Q) is very near or below that of singlet oxygen, singlet oxygen quenching by energy transfer occurs:

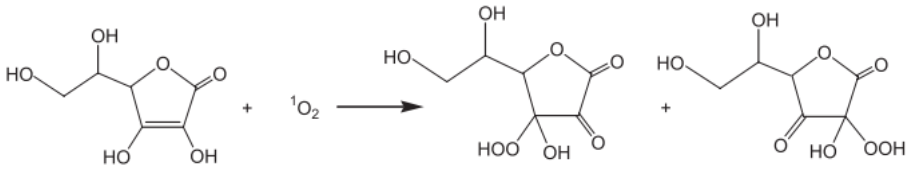


Figure 2.5 Formation of ascorbic acid hydro peroxides by singlet oxygen

By power conversion, carotenoids with 9 or additional conjugated double bonds are strong singlet oxygen quenchers. Singlet oxygen-quenching function of carotenoids depends on the amount 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, 2009b). 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. When the quencher has a high reduction potential and low triplet capacity, the singlet oxygen is extinguished by a charging transmission system.

These forms of quenchers, which all have several electrons, are amines, phenols (including tocopherols), sulfides, iodides, and azides. In order to form a singlet state charge transfer complex, the quencher can donate the electron to singlet oxygen and then shift the complex to triplet by intersystem crossing. Finally, the triplet conversion 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 related to the oxidation of the quencher relative to the quencher resulting in a degradation or oxidation of the quencher. β-Carotene, tocopherols, ascorbic acid, amino acids (such as tryptophan, cysteine, and methionine), peptides, and phenolic are oxidized with singlet oxygen, and they are all chemical quenchers of singlet oxygen. Reaction of ascorbic acid using singlet oxygen produces an unstable hydro peroxide of ascorbic acid. Tocopherol reacts irreversibly with singlet oxygen and produces tocopherol hydroperoxydienone, tocopherylquinone, and quinone epoxide.

2.5.4 Photosensitizer inactivation

Foods are containing chlorophyll and riboflavin sensitizers, which remain activated by light. Photo activated sensitizers transfer the energy to triplet atmospheric oxygen towards form singlet oxygen, or transfers an electron to the triplet oxygen towards 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 superior by carotenoids with 9 or more conjugated double bonds(Virtanen *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. 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(Liang *et al.*, 1999).

2.5.5 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℃ for 2 min or 116℃ under 44.5N for 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 (Engeseth et al., 1987).

2.6 Main Influential Factors of Antioxidant properties

The increased use of antioxidants research has helped establish strategies to assess the antioxidant activity of these compounds. The antioxidant potential of plant extracts can be determined by various laboratory methods, with the outcome depending on the principle of the method and the chemical structure of the sample (Brewer,2011). However, spectrophotometry, where the solution changes color after the oxidation agent has decreased, is the tool most widely used to measure a certain compound or phenolic class.

Factors that can influence the extraction and solubility of antioxidants include the nature of the compound to be determined, the extraction method, sample storage conditions, extraction time, temperature of the extraction, solvent polarity, particle size, the degree of polymerizing compounds, and interactions with other constituents of the sample. Moreover, destruction of the antioxidants can occur if adaptations are not made for the specific compound of interest or if the above mentioned parameters are not controlled. In particular, the extraction process requires the ultimate care because antioxidants are highly sensitive to light, oxygen, and heat(Moreira-Araújo et al., 2017). The antioxidant content of vegetal material depends on the chemical composition of the plant species, which varies with genetics, the plant variety, its stage of maturation, environmental conditions (specifically temperature and relative humidity), cultivation area, ground conditions, season, and time of collection. These factors may influence the reproducibility of the analytical method (Fank-de-Carvalho et al., 2015).

Solvent is one of the influences that can directly influence the process due to its chemical nature. Thus the solvent used for the extraction can preferentially extract specific bioactive compounds based on their solubility, although this can vary due to the possible interactions

of the bioactive compounds with other constituents. Furthermore, the solvent affects the tendency of the substrate to pass the free radical (for example, OH) of the hydrogen atom, which is continued by stabling reactive species to a stable compound.

The extraction time must take between 1 min and 24 h; however, depending on the substrate type, very long extractions may cause oxidation of the phenolics. Furthermore, the temperature range used in the extraction may be underestimated regarding the decomposition, chemical and enzymatic degradation, and the volatilization of compounds (Andreo and Jorge, 2006). When selecting a particular antioxidant or antioxidants, thought should be given to their efficiency at low concentrations, the characteristics of the extract including its compatibility with the food in which it is to be used (i.e., it must have no negative effect on the color, odor, and flavor of the final product and provide stability during processing and storage), its toxicity (Lucera et al., 2012).

2.7 Characterization and Evaluation of Antioxidant Properties

Emerging attention to trends related to the bioactive recovery processes lead to the exploration of accurate techniques to assess their antioxidant properties. Therefore to isolated, identify, and quantify these compounds, several examinations are typically performed, which are created on the transfer of electrons and hydrogen atoms. Three analytical techniques most widely used are the Folin–Ciocalteu assay, Ferric-reducing ability of plasma (FRAP) assay, and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay(Berker *et al.*, 2013)

2.7.1 Folin–Ciocalteu assay

The spectrophotometric quantification of antioxidant efficacy using Folin–Ciocalteu reagent is one of the methods used to measure the reducing capacity of samples, which allows inference of its phenolic compound content (Kiren et al., 2014). It's an electron transfer-based test that tests the oxidant's declining capacity. The colour of the reaction solution varies with the quality of reduction compounds. The Folin–Ciocalteu reagent is a mixture of phosphomolybdic and phosphotungstic acids, where molybdenum and tungsten stay in the oxidation state, initially producing a yellow solution. In the existence of phenolic compounds, a blue molybdenum–tungsten complex is formed, allowing absorption at 760 nm. Measurement of the absorbance at this wavelength can thus be used to conclude the concentration of the reducing compounds present (Georgé et al., 2005). The reaction occurs in a basic medium by oxidation of the phenolic compounds, producing phenolate anions

and reducing the molybdenum ions in the Folin– Ciocalteu reagent, which changes its color, as mentioned above. According to the standard reagent, the results can be described as gallic acid equivalents (GAE), catechin, quercetin, tannic acid, chlorogenic acid, caffeic acid, hydroxytyrosol, or tyrosol equivalents. Most literature suggests that the results are most commonly expressed as GAE. From the results obtained, it can be inferred that the higher the reducing power of the sample, the higher its antioxidant power (Paula et al., 2018).

2.7.2 Ferric-reducing ability of plasma (FRAP) assay

The ferric reducing potential is determined using the FRAP test for the overall antioxidant activity of a sample. The FRAP is made by combining the 2.4.6-tripyridyl-s-triazine and ferric chloride solution sodium acetate buffer, (pH 3.6). In the acidic medium, orange ferric-tripyridyltriazine in the existence of compounds decreased by donation or electron transfer is reduced to a ferrous form. This helps the development of a deep blue color by using absorption maximum at 593 nm. Test conditions favor the reduction of the complex, hence the formation of the blue complex is due to the reducing ability of the sample and the complex performs the function of the agent (Becker et al., 2004). At 593 nm the absorbance is aligned to the level of the antioxidant-containing sample. Using a calibration curve prepared from different specifications mentioned in the literature, the final results are usually presented as μM (μmol/L).These standards are obtainable for ascorbic acid, bilirubin, uric acid, albumin, and α-tocopherol ; Trolox ; and catechin and vitamin E (Gbor et al., 2005).

2.7.3 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) assay

The DPPH assay is based on evaluating the antiradical activity of antioxidants. The DPPH radical initially presents with a purple color and after a chemical reaction is reduced to 2,2-diphenylpicryl hydrazine, resulting in the discoloration of the solution. The radical may be reduced by an antioxidant donating an H atom or through reaction with a free radical to form a stable compound (Krishnaiah et al, 2010).The DPPH antioxidant activity is evaluated by competition kinetics in the decay curve, with lower absorbance values of the reaction mixture at various concentrations of the test sample indicating higher free radical-scavenging activity. The absorption in a spectrophotometer is measured at 515 nm and the results expressed as IC50 or EC50 values. Hence the percentage of antioxidant activity or

radical-scavenging activity is related with the amount of DPPH consumed during the reaction(de Oliveira et al., 2009, Koşar et al., 2005).

2.8 Oxidation of food

The degradation of rancid oils and fats plays a significant part in the oxidation process. Due to the production of a bad flavor and odor and changes of color, viscosity, density and solubility, the most typical changes which are becoming visible during oxidation. The oxidation process primary stages produces hydro peroxides. As these hydro peroxides degrade, compounds are formed which are considered to have a certain toxic potential in higher concentrations. Oxidation products are likely to react with other ingredients, contributing to texture or color changes. Therefore, oxidation is very important in terms of the tastiness, toxicity and nutritional value. Many foods consist of many organic compounds and can be readily oxidized. The greatest potential is for lipids (for example fats, oils, and waxes) to lose electrons. Auto oxidation of lipids in food caused by exposure to sunlight, high temperature, ionizing radiation, metal ions or metallo-protein catalysts can have a deteriorating effect on the food colour, flavour, texture, quality, and safety. Fats contained in food are chemically composed of triglycerides and oxidation leading to the rancidity of foods occurs at the unsaturated sites of the triglycerides (Atta et al., 2017).The synthetic antioxidants addition to these foods is one of the most efficient ways to reduce rancidity, reduce the production of toxic oxidation molecules and to prolong the food shelf life. The major source of degradation is the oxidative reactions of food. The reductions in nutritional value and scent, flavor and texture are due to them. Furthermore, biological oxidation products can distort homeostasis and cytotoxically contribute to various conditions including tumors, heart disease, cataract, brain dysthma through the association of essential organism function molecules. The deteriorative process of oxidation occurs naturally in all foods, not just those with high fat content (McGhee et al, 2015). Oxidation of foods could be reduced by removing prooxidants for instance free fatty acids, metals, and oxidized compounds, and by keeping foods from light, by decreasing pressure or adding oxygen scavengers can also reduce oxidation. Then it is very hard to totally eliminate all the pro-oxidants and air, antioxidants are now increasingly added to foods to slow down the process of oxidation.

2.8.1 Oxidation Mechanisms of Fats and Oils

Various chemical mechanisms are accountable for the oxidation of fats and oils throughout processing, storing, and cooking. Two kinds of oxygen might react with fats and oils, atmospheric triplet oxygen and singlet oxygen. The radical character of triplet oxygen interacts with radicals and induces autoxidation. The non-radical electrophil singlet oxygen does not need the reaction of radicals, it reacts directly with the dual interactions of high electron density, unsaturated fats and oils called photosensitized oxidation Type II.

2.8.1.1 Autoxidation

Fats and oils can be in radical forms to react with triplet oxygen in autoxidation. Lipids are usually in non-radical singlet state and heat, metals, or light increase their radical formation. Hydrogen attached to the carbon between two double bonds, is easily removed due to low bond dissociation energy(Choe and Min, 2006). The formation of hydrogen removing lipid radicals and the double bond adjacent to linoleic and linolenic acid radical carbon transitions to the more stable next carbon, contributing to the creation of conjugated diene structures. The evolving double bond generally takes the more stable transition thermodynamically. The radical lipid reacts very easily with three-fold oxygen at normal pressure of oxygen and forms radical lipid peroxide. The lipid peroxide radical absorbs hydrogen to form lipid hydroperoxide and another lipid radical from other lipid molecules. The radicals catalyze the reaction spontaneously and the autoxidation is called free radical reaction in the chain. If radicals react with each other, the reaction is interrupted by non-radical species.

2.8.1.2 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. 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, 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(Choe and Min, 2005). The resulting hydro peroxides by singlet oxygen are both

conjugated and non-conjugated. Production of non-conjugated hydro peroxides does not occur in autoxidation.

2.8.1.3 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, and the unstable primary oxidation products, hydro peroxides, are decomposed rapidly into secondary oxidation products such as aldehydes and ketones (Choe and Min, 2007). Thermal oxidation of oil produces many volatiles and non-volatiles. Volatiles such as aldehydes, ketones, short-chain hydrocarbons, lactones, alcohols, and esters are produced from decomposition of hydro peroxides by the same mechanisms as the autoxidation. Through radical reactions, several nonvolatile polar compounds plus triacylglycerol dimers and polymers are formed in thermally oxidized oil. Dimerization and polymerization in the thermal oxidation of oil are major reactions.

2.8.1.4 Enzymatic oxidation

Lipid oxidation is catalyzed by lipoxygenase in a non-radical mechanism. 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 (Engeseth et al., 1987), and oils containing linoleic, linolenic, and arachidonic acids are favored substrates (Hsieh and Kinsella, 1989). Eicosapentaenoic acid (EPA) and DHA can also be oxidized by lipoxygenase (Wang *et al.*, 2012). Lipoxygenase with ferric-state iron (LOX-Fe3+) forms a stereospecific complex with a 1, 4-pentadienyl system (RH) of unsaturated fatty acid and it abstracts hydrogens from interrupted fatty acid methylene. It binds to a pentadienyl radical rearranged in a conjugated diene scheme, followed by an oxygen reaction to create lipid peroxy radicals (ROO). The iron in the enzyme is lowered (LOX-Fe2+) to the ferrous state. 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 (Grosch, 2013).

2.9 The Use of Antioxidants in Foods

Antioxidants are compounds that function to protect biological systems against the harmful effects of processes or reactions that are triggered by ROS. Different study reports that antioxidants had positive effects on food quality, using methods that determined lipid and

protein oxidation and tests based on electron transfer and the capacity to donate hydrogen atoms.

Synthetic antioxidants commonly used by the food industry to maintain the quality of foods due to their ability to minimize the occurrence of oxidative reactions. However, due to their potential health risks and the increase in consumer concern about food safety, the food industry aims to decrease the use of these synthetic compounds (Shahidi and Zhong, 2010). Thus many studies have been performed that replaced synthetic additives with natural alternatives with the aim of increasing the shelf life of foods and decreasing the incidence of chronic and degenerative diseases, such as cardiovascular disease, diabetes, obesity, osteoporosis, and cancer (Brewer, 2011). Plants and vegetal material have important bioactive constituents, studies ensure that using plant-derived antioxidant compounds, which have underlined their importance in the inhibition of oxidative reactions in foods rich in oils and fats, mostly due to their high level of phenolic compounds. More than 8000 naturally derived phenolic compounds are known (Demirata, 2007). As natural antioxidants, phenolic compounds can prevent oxidative processes, eliminate free radicals, and act as metal chelators(Teets and Were, 2008), thus delaying or preventing oxidative damage and the formation of undesirable flavors and odors in the substrates (Brewer, 2011).

Many foods are rich in unsaturated fatty acids and are therefore highly sensitive to deterioration caused by oxidation (Brewer, 2011). This is a particularly common problem in meat and meat-derived products. Hence controlling oxidation in these products typically involves the addition of antioxidants alongside the use of appropriate storage conditions (Van Boekel et al., 2010). There is currently a great demand for convenience (i.e., fast and easy to prepare) products combined with a major trend in the use of natural ingredients. Thus products prepared using plant material or their extracts will benefit from the presence of antioxidant compounds contained in these natural sources by maintaining the original flavor and quality of the product during its shelf life (Brewer, 2011). The intake of functional products is greatly encouraged and thus functional constituents are extensively studied as the preference for minimal synthetic compounds in foods is a reality. The addition of plants (or their extracts) that display antioxidant properties to processed products is a great opportunity to promote the consumption of functional ingredients without causing drastic changes in eating habits of the population(Karabagias et al., 2011). Furthermore, numerous studies have reported satisfactory results when assessing the use of

natural extracts to decrease food deterioration, And requires synthetic compounds to be substituted. As a result, the use of plant-based antioxidants is becoming increasingly important due to their strong consumer acceptability, low cost, high availability and, in particular, their ability to capture free radicals, which would otherwise encourage improvements in nucleic acids, proteins, lipids and cells. In addition, they are able to keep the value of food by preventing or delaying oxidation-induced adverse impacts in texture, flavor, and nutritional value, and thus prolong the shelf life of products. All of these functions mean that natural antioxidants have properties that can decrease the occurrence of chronic diseases and progressive diseases, such as cardiovascular and circulatory diseases, cancer, atherosclerosis, arthritis, and inflammation (Balunas et al., 2006).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw materials and Equipment

Fresh banana (*Musa Cavendish*) variety was collected from fruit market in Jimma city and its by-product was chosen as a feedstock for extraction. The experiment was done in the school of chemical and Bio-Engineering and material science laboratories in JIT and Chemistry Department Laboratory, Jimma University.

The apparatus used for the experiment was Drying oven, Crucible, Desiccator, Electric oven, Condenser, Volumetric Flask, Filter Paper, Beaker, Glass Dish, Muffle Furnace, Erlenmeyer Flask, Funnel, Measuring Cylinder, Spatula, Soxhlet Apparatus, Thimble, pH Meter, Computerized UV Visible Spectrometer(Model SPECORD 200/PLUS, analytiKjena, Germany), Fourier Transform Spectroscopy(Model Perkin Elmer Spectrum Two), Conical Flask, Electrical Grinder, pestle and mortal, Balance, Knife, Kjeldahl Flask, Micro Pipette, Test tube, Vacuum Rotary Evaporator (Model Laborers 4000),Refrigerator, Sonicator, Cuvettes, Laboratory thermo meter, Sieve.

3.1.2 Chemicals

The chemicals used during the experiment are Sulphuric acid (H_2SO_4), Boric acid, Hydrochloric acid (HCl), Potassium Sulphate (K_2SO_4), Copper sulphate ($CuSO_4$), Sodium hydroxide (NaOH), Methyl red indicator, Petroleum ether, Ethanol, Acetone, Methanol, Folin-ciocalteu reagent, Gallic acid, Sodium carbonate(Na_2CO_3), Catechin, Sodium nitrate($NaNO_2$), Aluminum chloride($AlCl_3$), 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) reagent, Ascorbic acid, Potassium Bormide(KBr), Butylated hydroxytoluene(BHT), Glacial acetic acid, Chloroform, Potassium iodide(KI), Sodium thiosulfate, Starch indicator, Distilled water.

3.2 Experimental frame work

The research framework was conducted as shown below in the following general diagram (Figure 3.1) which shows all activities performed during the work.

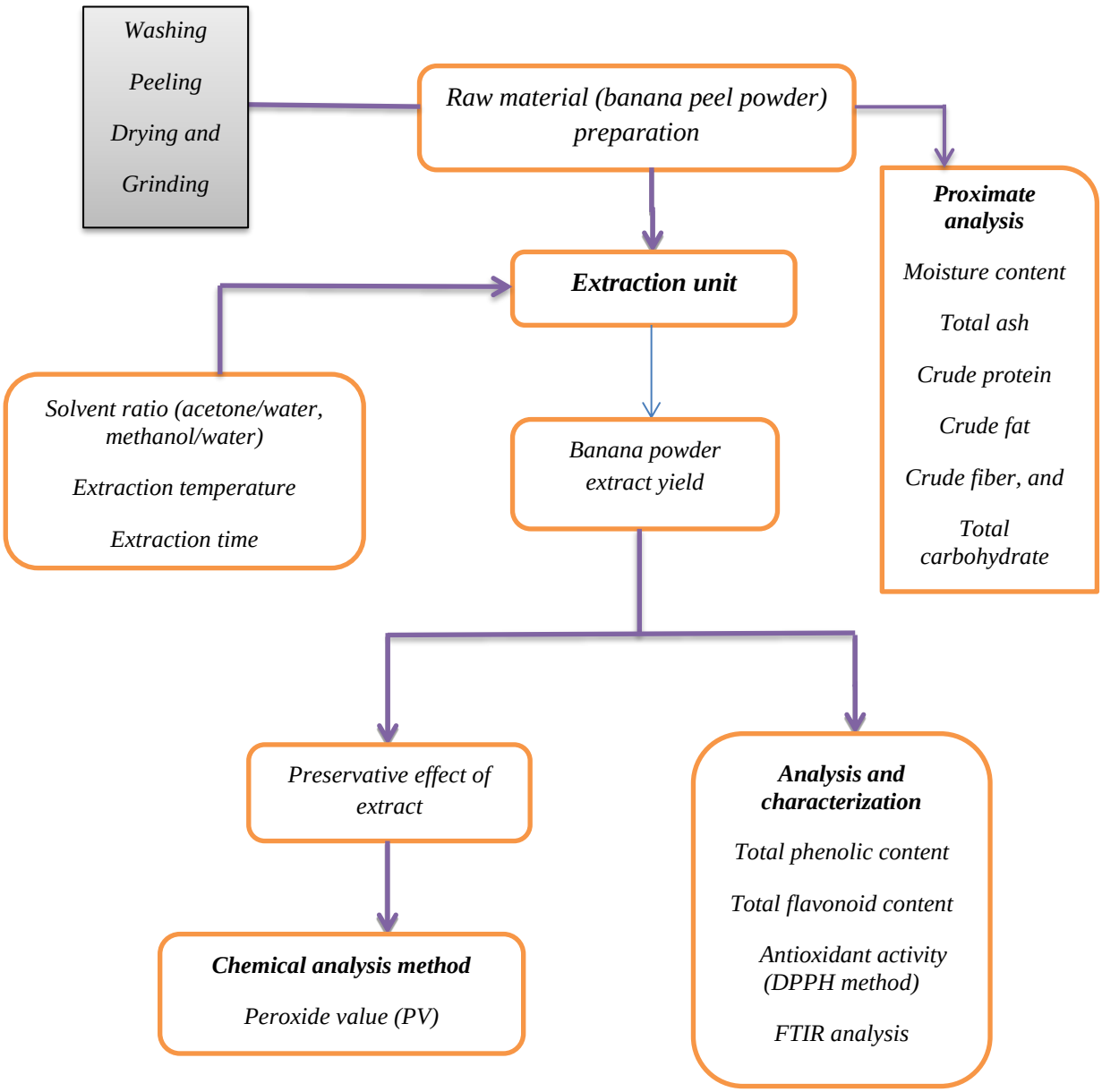


Figure 3.1 Experimental framework of the thesis

3.3 Methods

3.3.1 Preparation of banana peels

The banana fruit purchased was taken to the JIT chemical engineering laboratory. To remove impurities, debris and dust particles, and banana were washed using water. Then the peels were separated from the fruit. Once the peels are separated, it was cut in to small piece using a knife. To remove the moisture content, the banana peels were oven dried at 40–45°C for 24 h(Peschel et al., 2006). and then ground into fine powder by using electrical

grinder and place through using 355µm sieve, then placed into plastic containers and stored until the extractions is carried out.

3.3.2 Proximate analysis

The proximate evaluation was conducted using the method of (AOAC, 2003) and the parameters determined was moisture content, total ash, crude protein, crude fat, crude fiber, and carbohydrate. The experiment was conducted in duplicate.

Moisture content determination: Moisture was measured by the process of oven drying method. 2 g of mixed well sample was correctly weighed in clean, dried crucible (W_1). The crucible was allowed in an oven at 100-105°C for 6-12 h until a constant weight was obtained. Then the crucible was placed in the desiccator for 30 min to cool. After cooling it was weighed again (W_2). The percent moisture was calculated Eqn 3.1:

$$\% \text{ Moisture content} = \frac{(W_1 - W_2)}{Wt.of\ the\ sample} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.1)$$

Where W_1 = Initial weight of crucible + Sample, W_2= Final weight of crucible + Sample

Crude protein determination: Protein in the sample determined using Kjeldahl method. The sample was digested through heating with concentrated sulphuric acid (H_2SO_4) in the form of digestion mixture. Thus, ammonium sulphate formed, produced ammonia that was collected in a 2% solution of boric acid and titrated against standard HCl. Total protein was calculated by using the amount of nitrogen with appropriate factor (6.25) then the amount of protein was calculate.

Procedure: Protein in the sample was determined using Kjeldahl method. 0.5-1.0 g of dried samples was brought into digestion flask. Then add 10-15 ml of concentrated H_2SO_4 plus 8 g of digestion mixture i.e. K_2SO_4: Cu_2SO_4 (8: 1). The flask was whirled in order to blend the contents thoroughly after this placed on heater to start digestion until the mixture become clear (blue green in color). It will 2 h to complete. The digest was cooled then moved to 100 ml volumetric flask and the volume was made up by the addition of distilled water to mark. Distillation of the digest was then performed. In the distillation tube, 10 ml of digest was introduced, and then 10 ml of 0.5N NaOH was gradually added in the same way. Distillation was continuous at least for 10 min and NH_3 produced was obtained as NH_4OH in a conical flask containing 20 ml of 2% boric acid solution with some drops of

modified methyl red indicator. Throughout distillation yellowish color appears due to NH_4OH. The distillate was then titrated against regular 0.1 N HCl solutions until the presence of pink color. Blank samples also run through all steps as above. Percent crude protein content of the sample was calculated using Eqn 3.2 & 3.3:

$$\% \text{ Crude Protein} = 6.25^* \times \%N \text{ (*. Correction factor)} \ldots\ldots\ldots\ldots\ldots\ldots \quad (3.2)$$

$$\% \text{ Nitrogen } = \frac{(S-B)*N*0.014*D}{\text{Wt.of the sample}*V} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3.3)$$

Where S = Sample titration reading, B = Blank titration reading, N = Normality of HCl, D=Dilution of sample after digestion, V = Volume taken for distillation, 0.014 = Milli equivalent weight of Nitrogen

Crude fat of determination: Crude fat determined using dry extraction method. It requires collecting dry samples of any organic solvent, when all fat products are collected together, e.g. fats, phospholipids, sterols, fatty acids, carotenoids, pigments, chlorophyll, etc., thus the findings are also referred to as crude fat. By intermittent soxhlet extraction equipment, fats were determined. Crude fat was measured using ether extract method using Soxhlet apparatus. Approximately 1 g of the moisture-free sample was covered in filter paper, put in a thimble free of fat, and then placed in the extraction tube. Measured, washed and dried receiving beaker was filled with petroleum ether then fitted into the apparatus. To begin extraction, turn on the water and heater. After siphoning 4-6h, before last siphoning, ether will evaporate and disconnect the beaker. The extract was moved to a clean glass dish and the ether evaporated in a water bath. Then the dish was put in a 105 °C oven for 2 h and cooled in a desiccator. Using Eqn 3.4, the percent of crude fat was determined:

$$\% \text{ Crude protein} = \frac{Wt\ .of\ ether\ extract}{Wt.of\ the\ sample} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3.4)$$

Total ash determination: To determine the ash content, clean empty crucible was placed in an oven at 110℃ for an hour, cooled in desiccator and then weight of empty crucible was taken (W_1). In the crucible, 2g of the dry material is taken, then the weight of the crucible and the sample was taken (W2). The crucible was then put in the muffle furnace for 2-4 h at 550 °C. The appearances of gray white ash indicate complete oxidation of all organic matter in the sample. Then the crucible was cooled and weighed (W_3). Eqn 3.5 was used to determine percent ash:

$$\% \text{ Ash} = \frac{\text{differance in wt.of ash}}{\text{wt.of sample}} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{ (3.5)}$$

Difference in wt. of Ash= $W_2 - W_3$

Crude fiber determination: For determination of crude fiber About 3.0g defatted samples (from crude fat determination above) were transferred (W_1) into a 750 ml Erlenmeyer flasks and 200ml of boiling 1.25% H_2SO_4 was added and the flask was immediately set on a hot plate electric oven at 130℃ and condenser connected to it. The content was brought to boil within 1 minute then digest for 30 min. The flask was withdrawn at the end of 30 min and the content was filtered in a funnel through a linen cloth and then cleaned with hot water until the washing was no longer acidic. The samples were washed back into the flask with 200ml boiling 1.25% NaOH solution. The condenser was again connected to the flask and the content of the flask was boiled for 30 minutes. Then it was filtered through the linen fabric and cleaned vigorously with hot water until it was no longer alkaline. The residue was moved with a spatula to a new crucible and the remaining particles were washed into the crucible with 15ml of ethanol. The crucible with its contents was then dried overnight at 105°C in an oven and cooled in a desiccator and weighed (W2). The crucible and its contents were then ignited for 2 h in a furnace at 550°C, cooled and re-weighed (W_3). The weight loss gave the content of crude fiber and was expressed as a percentage of the initial weight of the sample using Eqn 3.6. The total crude fiber was expressed in percentage as:

$$\% \text{ Crude fiber} = \frac{W_2 - W_3}{W_1} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{ (3.6)}$$

Carbohydrate determination: After analysis of all the other item approaches in the proximate analysis, carbohydrate was determined as shown in Eqn 3.7.

% Carbohydrate = (100-% moisture + % crude protein + % crude fat + % crude fiber + % ash)..... (3.7)

3.4 Extraction of Antioxidants from banana peel

Experimental Procedure: The antioxidants was extracted by taking the banana peel powder into soxhlet apparatus using two organic solvents. The solvents were poured in to round bottom flask at different solvent ratio and sample (banana peel) placed in the thimble and inserted in the center of extractor. The soxhlet was heated at different temperature using

electrical oven and when the solvent boilies, the vapor rises through the vertical tube into the condenser at the top. The liquid condensates rip in to thimble in the center which contains the solid sample to be extracted. The extract product mixed with solvent was heated by the end of the extraction to recover the solvent from the antioxidant. The solvent free antioxidant will be then obtained for further use. The extracts was processed as described by (Javier David Vega et al., 2017).

The three factors: extraction solvent ratio (60-90 v/v), extraction temperature (40-60°C) and extraction time (1-4h) was selected as independent variables, because of their influence on antioxidant properties of phenolic extracts in plant materials (Wettasinghe and Shahidi, 1999). In this study, the particle size was controlled as constant by passing through on 355μm sieve openings. The 355-500μm sieve size is optimal for extraction, while smaller particles may become slimy during extraction and create difficulty.

3.4.1 Characterization of the extract

3.4.1.1 Determination of Extract Yield

The extraction yield is a calculation of the efficiency of the solvents to extract specific components from the original material and the amount of extract recovered in mass relative to the initial quantity of peel is calculated. It is given in percentage (%) using Eqn 3.8, for each factor, total yield is calculated as:

$$\text{Extraction yield (\%)} = \left(\frac{\text{weight of the extract}}{\text{total dried weight of peel powder}}\right) * 100 \quad \text{..... (3.8)}$$

3.4.1.2 Determination of Specific Density of Extract

The exact density of the extract determined by the weight ratio of 1 ml of solid extract to 1 ml of water or by the density ratio of 1 ml of extract to the density of 1 ml of water at room temperature.

Procedure

The extract is prepared by dissolving 100 mg of extract in 2 ml of solvent to get 50 mg/ml stock solution. Then 1 ml of stock solution was taken and weighed and the mass was known. The weight of solvent (acetone, methanol) in the stock solution was determined by subtracting the weight of pure extract found in the 1ml of stock solution from the total weight of 1 ml stock solution. Once the mass of the solvent (acetone, methanol) in the stock solution was known, 1 ml of pure solvent (acetone, or methanol) was weighed and the mass

of pure 1ml of solvent was known and it is used to calculate the volume of the solvent. After the volume of the solvent in 1ml stock solution was known, it was assumed that the rest in 1ml of stock solution was the volume occupied by the extract. The extract density was calculated by dividing the extract mass into the extract volume (1.0 g/ml). Then 1ml of water was taken and its mass was weighed. The density of water was determined (1g/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) (Eqn 3.9)(Genitha Immanuel, 2014)

$$\text{Specific density} = \frac{\text{density of the extract}}{\text{density of water}} \quad \ldots\ldots (3.9)$$

3.4.1.3 Determination of pH value of extract

The extract's pH value was directly measured at room temperature by a pH meter. The stock solution (50 mg/ml) was prepared through 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.

3.4.1.4 Total phenolic contents of extracts

The amount of phenolic compounds in the extracts was determined using the Folin–Ciocalteu method described as in Vuong et al.(2013) with some modifications. Briefly, 2 ml of 10% v/v Folin–Ciocalteu reagent in water was added to 1 ml of the diluted extract or Gallic acid (a standard solution). The mixture was settled for 3-5 min at room temperature. The blue color complexes was formed due to redox reaction and lead to increase in absorbance of the extracts, then 2 ml of 7.5% (w/v) Na_2CO_3 was added, adjusted the solution to with 10 ml distil water, mixed well and incubated in the dark for 1h at room temperature. Absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Model SPECORD 200/PLUS, analytiKjena, Germany). Gallic acid was used to make a standard curve. The results were described as mg Gallic acid equivalent (GAE)/g DM. The total phenol content was calculated using Eqn 3.10:

$$\text{TPC} = \frac{C * V}{W} \quad \ldots\ldots (3.10)$$

Where: TPC = total phenol content (mg of GAE/g dry extract), V= volume of stock solution of extract (ml), W = weight of extract found in the stock solution (g), C = concentration of Gallic acid obtained from the calibration curve (mg/ml).

Dilution factor was incorporated in Eqn 3.10 as shown in Eqn 3.11 if dilution of the solution was used for proper concentration to achieve the necessary absorbance.

$$\mathrm{TPC} = \frac{\mathrm{C*DF*V}}{\mathrm{W}} \quad \ldots\ldots\ldots\ldots (3.11)$$

Where DF = dilution factor

Procedures

Reagent preparation

The solution for sodium carbonate reagents was prepared by weighing 18.75g of $NaCO_3$ & dissolving in methanol using 250 ml flask to get 7.5% (w/v) Na_2CO_3 solution. Solution of Folin-Ciocalteu's reagent was prepared by taking 1ml of Folin-Ciocalteu's into 9ml of distil water.

Blank solution Preparation

2 ml Folin-Ciocalteu reagent solution was applied to a test tube with 1 ml methanol mixed well, and incubated for 3-5 min. Then, a 2 ml saturated Na_2CO_3 solution was added, and the solution was adjusted to 10 ml with distil water, diluted thoroughly, and incubated at room temperature in the dark for 1 h.

Preparation of standard solution

Gallic acid was used as a positive control or standard to determine the 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 dissolve in 50 ml Volumetric Measuring flask using methanol. Aliquots of 20, 40, 80, 120,160, 200, and 240µl of standard Gallic acid were withdrawn from the stock solution and mixed with 980, 960, 880, 840, 800, and 760µl of methanol solvent to get Gallic acid 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 solution of 2 ml of Folin-Ciocalteus reagent was added, mixed well and incubated for 3-5 min, then solution of 2ml of saturated Na_2CO_3 was added and adjusted the solution to 10 ml with distil water, mixed well and incubated in the dark at room temperature for 1h. After 1hr incubation, the absorbance was determined against the blank (the same mixture without the Gallic acid) at 765nm using a UV-Visible Spectrophotometer (Model SPECORD 200/PLUS, analytiKjena, Germany). The study was conducted in triplicates.

Preparation of sample solution

Sample stock solution of banana peel extract (50 mg/ml) was prepared by dissolving 1g of extract in to 20 ml of solvent. 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 solution of 2 ml of Folin-Ciocalteus reagent was added, mixed well and incubated for 3-5 min. then solution of 2 ml of saturated Na_2CO_3 was added and adjusted the solution to 10 ml with distil water, mixed well and incubated in the dark for 1h at room temperature. After 1hr incubation, the absorbance was determined against the blank (the same mixture without the extract) at 765 nm using a UV-Visible Spectrophotometer (Model SPECORD 200/PLUS, analytiKjena, Germany). The study was conducted in triplicates. The total phenol content was determined and described as milligram of Gallic acid equivalents (mg of GAL/g dry extract) using the Gallic acid calibration curve and the curve used to calculate the corresponding Gallic acid concentration of the samples.

3.4.1.5 Total Flavonoid contents in the extracts

The total flavonoid content was measured according to aluminum chloride colorimetric method by modification of Singh *et al.*, (2015). Briefly, 1ml of the extract or catechin standard solution was 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 has been added and allowed to stand for another 5 min until adding 0.5 ml of 1 M NaOH. The mixture was taken to 2.5 ml with distilled water and mixed well. The absorbance was measured instantly against the blank (the same mixture without the sample) at 510 nm using a UV- Visible Spectrophotometer (Model SPECORD 200/PLUS, analytiKjena, Germany). The results were calculated then expressed as milligrams of catechin equivalents (mg of CAE/g dry extract) using the calibration curve of catechin.

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 using Eqn 3.12:

$$\mathrm{TFC} = \frac{\mathrm{C}*\mathrm{V}}{\mathrm{W}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.12)$$

Where: TFC = total flavonoid content expressed as (mg of CE/g dry extract), V = volume of stock solution of extract (ml), W = weight of extract found in the stock solution (g), C = catechin equivalent concentration obtained from the calibration curve (mg/ml)

Procedures

Preparation Reagents

5 g of $NaNO_2$ dissolve in to 100 ml of distill water to prepare 5%$NaNO_2$ and 10 mg $AlCl_3$ dissolve in to 100 ml of distill water to prepare 10%$AlCl_3.6H_2O$. Also, 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 (50 mg/ml) of catechin was prepared by dissolving 1 g of catechin in to 20 ml of methanol. 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, 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 by using a UV-Visible Spectrophotometer (Model SPECORD 200/PLUS, analytiKjena, Germany). The study was conducted in triplicates. The standard catechin calibration curve was developed by plotting linear regression curve of absorbance versus catechin concentration (mg/ml).

Preparation of sample solution

A stock solution (50 mg/ml) of banana extract was prepared by dissolving 1 g of banana extract in to 20ml of acetone and methanol. 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. A 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 by using a UV-Visible Spectrophotometer (Model SPECORD 200/PLUS, analytiKjena, Germany). The study was conducted in triplicates.

3.4.1.6 Determination of free radical scavenging activity (DPPH Method)

Radical Scavenging Activity was determined according a modified version of the DPPH method by Singh *et al.*, (2015). The extract stock solution was prepared 0.004% DPPH solution was prepared by dissolving 0.01 g DPPH 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 min. The absorbance of the resulting solution was measured using a UV-Visible Spectrophotometer (Model SPECORD 200/PLUS, analytiKjena, Germany) at 517 nm by monitoring the decrease in absorbance. Ascorbic acid was used as standard. The scavenging activity of the extract was calculated using Eqn 3.13.

$$\% \text{ Inhibition} = [(A_B - A_A]/A_B] * 100 \ldots\ldots\ldots\ldots \quad (3.13)$$

Where: A_B–absorption of blank sample (t= 0 min), A_A–absorption of extract sample (t= 30 min)

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.

Preparation of blank solution

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 (50 mg/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 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 (Model SPECORD 200/PLUS, analytiKjena, Germany). The study was conducted in triplicates.

Preparation of sample solution

Sample stock solution of extract (50 mg/ml) was prepared by dissolving 1g of extract in to 20 ml of acetone and methanol. 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 (Model SPECORD 200/PLUS, analytiKjena, Germany). The study was conducted in triplicates.

3.4.1.7 Fourier Transformation Infrared (FTIR) Spectroscopy

Fourier Transform Infrared Spectroscopy, also known as FTIR Analysis or FTIR Spectroscopy, is an analytical method used to detect organic, polymeric, and, in some cases, inorganic materials. The FTIR analysis system uses infrared light to scan test samples and observe chemical properties.

The functional group of the antioxidant extracted from banana peel was tested by FTIR and the spectra were recorded for each sample range from 4000 cm^{-1} to 450 cm^{-1} using the

conventional KBr technique. The functional group was described based on the reading of the infrared spectrum frequencies. The following procedure was followed to perform the analysis: First, the sample for the analysis was ground in a ceramic pestle and mortal to powder. The powder was then mixed with KBr particles to make it stable to infrared analysis. Then the mixture pressed to a small thickness, slightly below 1mm, required for FTIR analysis. Then the power of the instrument was turned on and the initialization started. Then the sample holders was cleaned by acetone with wipes and carefully remove the pressed sample from die and place in the FTIR sample holder and attach with scotch tape. Perform a spectrum analysis and the obtained data was graphed by using origin lab software.

3.5 Preservative effect of the extract

3.5.1 Preservative Effect of the Extract on Soybean Oil

The preservative effect of the extract tested on oil sample by Schaal oven test method at 60°C. The oxidative stability of oil was tested at 60°C for 8 days at a daily period of 2 days according to the AOCS Official Methods by peroxide value (PV, Method Cd 8-53). Synthetic antioxidants Butylated hydroxytoluene (BHT) was mixed in soybean oil for a comparative study and control samples without antioxidant were placed under identical conditions. All oil samples of each treatment were prepared in duplicate. Oil samples were withdrawn every 2 days to assess PV measurement. In a Schaal oven test, 40g samples oil sample treated with 0.1% and 0.2% extract and placed in a drying oven at 60°C(Gebrehana Ashine, 2012). In contrast, the same conditions were prepared and saved for positive (BHT 0.05%) and negative (without extract) treatments.

3.5.1.1 Determine by Peroxide value

The peroxide value was evaluated according to AOCS Official Method Cd 8-53 as described by Rybak-Chmielewska, (2003). Five gram oil samples were weighed into a conical flask and 30 ml of solvent mixture of glacial acetic acid-chloroform in the ratio of 3:2, respectively, were added to the oil samples. Half ml saturated potassium iodide (KI) solution was added to the solution and allowed to stand for 1 min thereafter, 30 ml of distilled water were added and titrated with 0.01 N sodium thiosulfate solution using starch indicator until the yellow color was discharged. A blank was prepared alongside the oil samples. Peroxide value was calculated as:

$$PV = \frac{10*(V_1 - V_2)}{m} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.14)$$

Where: V1 =Volume of $Na_2S_2O_3$ for determination of test samples in ml, V2 =Volume of $Na_2S_2O_3$ for determination of blank solution in ml and m = mass of test portion in g

3.6 Experimental design and statistical Analysis

In this study, Face Centered Central Composite Design (FCCCD) under the Response Surface Methodology (RSM) was used to define the influence of extraction solvent ratio, extraction temperature and extraction time on the total yield of antioxidants extracts from banana peel and to identify the optimum levels. Response Surface Methodology (RSM) contains a group of mathematical also statistical methods that can be used to describe the relations between the response and the independent variables. Also describes the influence of the independent variables, alone or in combination, in the processes. In addition to examine the effects of the independent variables, this experimental methodology also generates a mathematical model. For FCCCD, 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 previous evidence about the site of the optimum. Therefore, designs provide equal precision in all directions is not preferred in this case. The response variable was the extraction yield.

A three-factor and three level face center central composite design consisting of 20 runs for total yield were including six replicates at the center point (Table 3.1). Experimental design was analyzed and done by Design-Expert 7.0.0 software. The level of fit of the regression model expressed as the coefficients of determination (R^2), the statistical significance determined by ANOVA the response surface and the contour plots were all study to estimate the models as well as to decide the optimum levels.

Table 3.1 Level of independet variables for extraction process

Independent variable	**Factors**	**Coded level**		
		-1	0	1
Solvent ratio(v:v)	A	60/40	75/25	90/10
Temperature (°C)	B	40	50	60
Time (h)	C	1	2½	4

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Proximate analysis of banana peel powder

The major components identified in the proximate study were moisture content, crude protein, ash content, crude fat, crude fiber and carbohydrates. Results from respective analyses were used to characterize banana peel powder as presented in Table 4.1.

Table 4.1 Proximate analysis of banana peel powder

Proximate analysis	**(%)**
Moisture content	32 ± 0.2
Ash content	9.8 ± 0.54
Crude protein	10.5 ± 1.00
Crude fat	3.8 ± 0.14
Crude fiber	14.4 ± 0.1
Carbohydrate	29.5 ± 0.3

4.2. Effects of Extraction Parameter on the yield

The yield of extracts obtained from the extract at each of the 20 run is given in Table 4.2. The experiments conducted for each solvent were used for extraction by varying the selected parameters that affects the yield. The factors selected in this study were solvent ratio (acetone and methanol), extraction temperature and extraction time. All of these variables individually and interactively affected the extraction yield of polyphenol compounds. The levels of solvent ratio 60/40, 75/25 and 90/10 (v/v), extraction temperature 40, 50 and 60 ºC, extraction time 1, 2 and 4 h were used.

Table 4.2 Extracts yield at different extraction parameter for acetone and methanol BP extract

Run	Extraction parameters			Yields(%)	
	Solvent ratio(v/v)	Temperature (°C)	Time (h.)	Acetone extract	Methanol extract
1	60/40	40	1	13	21.4
2	75/25	50	2½	21.3	29.7
3	60/40	60	4	22.5	30.9
4	60/40	50	2½	20.3	28.4
5	60/40	60	1	19.6	27.5
6	90/10	60	4	24.6	32.4
7	75/25	50	1	19.3	27.7
8	75/25	50	2½	22.5	31.9
9	75/25	50	2½	23.2	32.1
10	75/25	50	4	23.8	31.6
11	90/10	60	1	21.5	29.5
12	60/40	40	4	18.9	27.3
13	75/25	50	2½	23.1	31.6
14	90/10	50	2½	23.6	32
15	75/25	60	2½	22.7	30.5
16	90/10	40	1	18	26.4
17	90/10	40	4	23.2	31.6
18	75/25	50	2½	23.1	30.9
19	75/25	50	2½	23.5	31.4
20	75/25	40	2½	20	28

The extraction yields obtained were ranged 13-24.6% and 21.4-32.4% for acetone and methanol extract, respectively. The maximum extraction yield was obtained on the experiment run number six (6) at higher levels of solvent ratio of 90/10 v/v, 4h extraction time and extraction temperature of 60℃ extraction parameters. On other hand, the minimum extraction yield was experimental run numbers one (1) at lower solvent ratio of 60/40, 2½-extraction time and 40℃ extraction temperature. The present yield from banana

peel extract is compatible with the finding by González-Montelongo, Gloria Lobo and González, (2010) that obtained a yield of extract ranged 3% to 54% with methanol: water and acetone: water extraction.

Increasing the solvent ratio from lower to higher level maximizes the yields. As seen in table 4.2, when solvent ratio increases from 60/40 into 90/10, the yield also increases. The study by Toh et al.,(2016) reports that, the highest yield is obtained when the aqueous acetone and methanol extraction done at 90%,as the aqueous content decrease the extract yield increases. In addition, when solvent ratio increases, the rate diffusion of polyphenols and flavonoids towards the solvent was higher due to the amount of polar soluble phytochemical compounds. Increasing in temperature from lower to higher-level affects the yields obtain. González-Montelongo et al., (2010) reported that the increase in temperature of the extracted phenolic compound improved the performance of the extraction of phenolic compounds by raising the temperature. Vuong et al.(2013) also reported that the extracts yield increased when temperature of extraction increased to 70 °C and subsequently decreased when the extraction temperature exceeded 90 °C.

The extraction time has also an influence from lower to higher level. The extract yield increase in both solvents with time. A longer extraction time generally led to a higher percentage yield. Different research reports that by keeping the temperature at a minimum level for a maximum extraction period achieved the highest yields. Hence, a sustained exposure of the sample in the solvent provided adequate time for the desired compounds to migrate into the solvent. The longer extraction time values allow for the longer amount of time the solute and solvent were in contact, so it make the systems have been successfully mass transfer.

The maximum yield was obtained at higher level of solvent ratio, extraction time and temperature. Based on the results, the highest extract yield obtained was by methanol (32.4%) than acetone solvent (24.6%). Banana peel phenolic compounds are highly soluble in methanol rather than acetone. This is because methanol is more polar than acetone. Alcoholic solvents have been frequently used to extract phenolic constituents from natural sources: they offer high yield of total extract. Particularly, mixtures of alcohol and water have shown that they are more successful in extracting phenolic constituents from the corresponding solvent system with mono components (Yilmaz and Toledo, 2006).

4.2.1 Experimental Design Analysis

The experimental design selected for ANOVA (Table 4.3 and Table 4.4). Under response surface methodology, FCCCD was selected. The response of the analysis was extraction yield. The response yield for acetone and methanol range from 13-24.6% and 21.4-32.4%, respectively. The ratio of maximum to minimum yield for acetone and methanol was 1.8923 and 1.5140, respectively. A ratio greater than 10 usually indicates a transformation is required. For a ratio less than 3, the power transformation has a little effect. The aim of the model fit summary was maximizing the adjusted R-Squared and predicted R-Squared. Model significances was checked for both model and model factor, linear model factor solvent ratio (A), temperature (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 4.3 Analyses Of Variance (ANOVA) for yield of acetone extract Banana Peel

Source	**Sum of square**	**df**	**Mean square**	**F value**	**p-value Prob > F**	
Model	136.48	9	15.16	38.59	< 0.0001	**significant**
A	27.56	1	27.56	70.13	< 0.0001	
B	31.68	1	31.68	80.63	< 0.0001	
C	46.66	1	46.66	118.73	< 0.0001	
AB	3.51	1	3.51	8.94	0.0136	
AC	0.031	1	0.031	0.080	0.7837	
BC	3.25	1	3.25	8.27	0.0165	
A^2	0.71	1	0.71	1.81	0.2078	
B^2	3.38	1	3.38	8.61	0.0149	
C^2	2.27	1	2.27	5.78	0.0370	
Residual	3.93	10	0.39			
Lack of Fit	0.90	5	0.18	0.30	0.8965	**not significant**
Pure Error	3.03	5	0.61			
Cor Total	140.41	19				

The model was statistically significant as seen from the very low p-value (<0.0001) shown in Table 4.3. The model F-value for acetone extract represent 38.59, which implies the model, is significant. There is only a 0.01% chance that a "Model F- Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case acetone extract, A, B, C, AB, BC, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Model reduction will improve the model if there are several insignificant terms. This shows that the solvent ratio, extraction temperature, extraction time, interaction between solvent ratio and extraction temperature, interaction between extraction time and extraction temperature, square of temperature and square of time affect the yield of banana peel extract significantly. The "Lack of Fit F-value" of 0.30 for acetone extract implies the Lack of Fit is not significant relative to the pure error. There is 89.65% for acetone chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good because we need the model to fit.

Table 4.4 Analyses Of Variance (ANOVA) for yield of methanol extract Banana Peel

Source	**Sum of square**	**df**	**Mean square**	**F value**	**p-value Prob > F**	
Model	135.52	9	15.06	36.00	< 0.0001	**significant**
A	26.90	1	26.90	64.30	< 0.0001	
B	24.65	1	24.65	58.93	< 0.0001	
C	45.37	1	45.37	108.46	< 0.0001	
AB	4.20	1	4.20	10.05	0.0100	
AC	0.18	1	0.18	0.43	0.5266	
BC	2.88	1	2.88	6.88	0.0254	
A^2	0.78	1	0.78	1.86	0.2026	
B^2	4.52	1	4.52	10.80	0.0082	
C^2	3.22	1	3.22	7.69	0.0197	
Residual	4.18	10	0.42			
Lack of Fit	2.53	5	0.51	1.53	0.3267	**not significant**
Pure Error	1.66	5	0.33			
Cor Total	139.71	19				

The model was statistically significant as seen from the very low p-value (<0.0001) shown in Table 4.4. The model F-value for methanol extract represent 36.00, which implies the model, is significant. There is only a 0.01% chance that a "Model F- Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case methanol extract, A, B, C, AB, BC, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Model reduction will improve the model if there are several insignificant terms. This shows that the solvent ratio, temperature, time, interaction between solvent ratio and temperature, interaction between time and temperature, square of temperature and square of time affect the banana peel extract yield significantly. The "Lack of Fit F-value" of 1.53 for methanol extract implies the Lack of Fit is not significant relative to the pure error. There is 32.67% methanol extract chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good because we want the model to fit. The model fit summary statistics were listed in Table 4.5 and 4.6.

Table 4. 5 Analysis of variance (ANOVA) for the Response of acetone yield of Banana Peel

Std. Dev.	0.63	**R-Squared**	0.9720
Mean	21.38	**Adj R-Squared**	0.9468
C.V.%	2.93	**Pred R-Squared**	0.9194
PRESS	11.32	**Adeq Precision**	25.268

The "Pred R-Squared" of 0.9194 is in reasonable agreement with the "Adj R-Squared" of 0.9468. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case the ratio is 25.268 indicates an adequate signal. This model can be used to navigate the design space.

Table 4.6 Analysis of variance (ANOVA) for the Response of methanol yield of Banana Peel

Std. Dev.	0.65	**R-Squared**	0.9701
Mean	29.71	**Adj R-Squared**	0.9431
C.V.%	2.18	**Pred R-Squared**	0.8874
PRESS	15.72	**Adeq Precision**	23.656

The "Pred R-Squared" of 0.8874 is in reasonable agreement with the "Adj R-Squared" of 0.9431."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case ratio of 23.656 indicates an adequate signal.

4.2.2 Development of Model equation

An empirical relation among the response and the independent variables is given by the RSM application. The mathematical relation between the response and the independent variables such as solvent ratio(A), temperature(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 variables of extraction process was given by quadratic model as equation 4.1 and 4.3 based on coded factors and equation 4.2 and 4.4 based on actual factors, respectively.

Final Equation in Terms of Coded Factors:

Acetone yield = +22.64 + 1.66* A + 1.78 * B + 2.16 * C - 0.66 * A *B - 0.062 *A *C - 0.64* B *C - 0.51* A^2 -1.11 * B^2 - 0.91 * C^2 ……..……. (4.1)

Where A=solvent ratio

B=temperature

C=time

Final Equation in Terms of Actual Factors:

Acetone yield =+22.64364 + 1.66000 * concentration + 1.78000 * temperature +2.16000* time -0.66250* concentration * temperature -0.062500 * concentration * time - 0.63750* temperature * time -0.50909 * $concentration^2$ -1.10909 * $temperture^2$ -0.90909* $time^2$……. (4.2)

From the regression model equation developed in terms of coded factors, the response yield was affected by linear terms solvent ratio(A) , temperature (B) and time(C) and, quadratic terms, pure quadratic terms (A^2 , B^2 , C^2) and interaction quadratic terms (AB, BC, AC). On the basis of the coefficient in the equation , it was evident that the response yield increases with an increase in the solvent ratio (A), temperature(B), and time (C) which have positive linear effect on the yield of extraction but time has a more substantial linear effect on yield compare to solvent ratio and temperature. Pure quadratic terms (A^2, B^2, C^2) have negative effects on the response yield but the pure quadratic term (A^2) has a substantial

effect than the other quadratic terms. Interaction quadratic term of interaction of solvent ratio and temperature (AB), interaction of temperature and time (BC) and interaction of solvent ratio and time has a negative quadratic effect on yield.

Final Equation in Terms of Coded Factors:

Methanol yield =+31.16 +1.64*A +1.57*B +2.13*C -0.72* A * B -0.15 *A* C -0.60*B* C -0.53 * A^2 -1.28* B^2 -1.08* C^2…… (4.3)

Where A=solvent ratio

B=temperature

C=time

Final Equation in Terms of Actual Factors:

Methanol yield = +31.16273 +1.64000* concentration +1.57000* temperature +2.13000 * time -0.72500 * concentration * temperature -0.15000 * concentration * time -0.60000*temperature * time -0.53182* concentration^2 -1.28182* temperture^2 -1.08182* time^2…… (4.4)

From the regression model equation developed in terms of coded factors, the response yield was affected by linear terms solvent ratio (A) , temperature (B) and time(C) and, quadratic terms, pure quadratic terms (A^2 , B^2, C^2) and interaction quadratic terms (AB, BC, AC). On the basis of the coefficient in the equation , it was evident that the response yield increases with an increase in the solvent ratio (A), temperature(B), and time (C) which have positive linear effect on the yield of extraction but time has a more linear effect on yield compare to solvent ratio and temperature. Pure quadratic terms (A^2 , B^2, C^2) have negative effects on the response yield but the pure quadratic term (A^2) has a substantial effect than the other quadratic terms. Interaction quadratic term of interaction of solvent ratio and temperature (AB), interaction of temperature and time (BC) and interaction of solvent ratio and time has a negative quadratic effect on yield.

4.2.2.1 Model adequacy check

The model was tested for adequacy by analysis of variance. The regression model has been found to be highly significant with the correlation coefficients of determination of R -Squared, adjusted R-Squared and predicted R-Squared values of acetone and methanol extract from BP having a value of 0.9720, 0.9468, 0.9194 and 0.9701, 0.9431, 0.8874

respectively. The reliability of the model developed could be evaluated from their coefficients of correlation. The value of R-squared for the developed correlation for acetone is 0.9720. It means that 97.20% of the overall variation in the percentage of conversion is attributed to the experimental variables studied. In the other word, only 2.8% of the overall variations were not describe by the model, and for methanol the value of R-squared for the developed correlation is 0.9701. It means that 97.01% of the overall variation in the percentage of conversion is attributed to the experimental variables studied. In the other word, only 2.99% of the overall variation was not describe by the model.

The normal probability plot was shown in figure 4.1(a) and (b) for acetone and methanol extract form BP respectively. The normal probability plot indicates whether the residuals follow a normal distribution, in which case the points will follow a straight line. However, some moderate scatter even with normal data. Thus, the data can be normally distributed in the responses of certain models.

The result in Figure 4.2(a) and (b) shows Residuals vs. Predicted plot. This is a plot of the residuals versus the predicted response values. It examines the assumption of constant variance. The plot must be a random scatter (constant range of residuals across the graph). The scale of the residual should be independent of its predicted value. This means that, the vertical distribution of the studentized residuals must be approximately the same through all levels of the predicted values. The plot looks all right in both situations.

The results in Figure 4.3(a) and (b) shows that the regression model equation provided a very accurate description of the experimental data, in which all the points are close to the line of perfect fit. This result indicates that it was successful in capturing the relationship between the three variables to the production of antioxidant extract from BP. The adequacy of the model was further checked with ANOVA, based on a 95% confidence level, F-value is a test for comparing model variance with residual (error) variance. If the variances are close to be the same, the ratio will be close to one and it is likely that any of the factors have a significant effect on the response with the P-value less than 0.05. It was calculated by model mean square divided by residual mean square. The effectiveness of the model could also be measured to assure its approximation to the true value. Thus, regression coefficient, R^2, could be used for checking its adequacy. The regression value is between 0 and 1, and as it approaches to 1 it fits well to the experimental data otherwise it indicates failure of approximation. In both case, R^2 obtained for acetone was 0.9720and methanol

0.9701, which was close to one and the value Adj-R^2 for acetone 0.9468 and methanol 0.943, both are in a reasonable agreement with R^2.

(a)

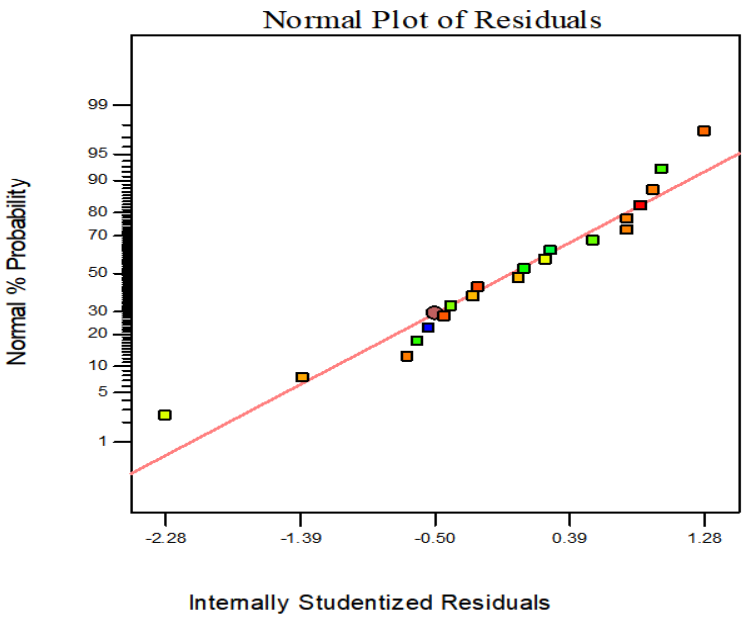


Figure 4.1 (a) Normal probability plot of residual of acetone extract

(b)

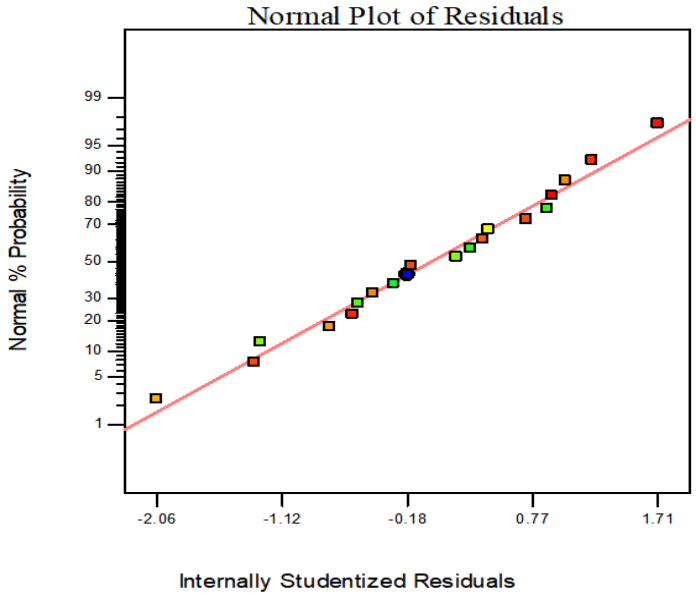


Figure 4.1 (b) Normal probability plot of residual of methanol extract

(a)

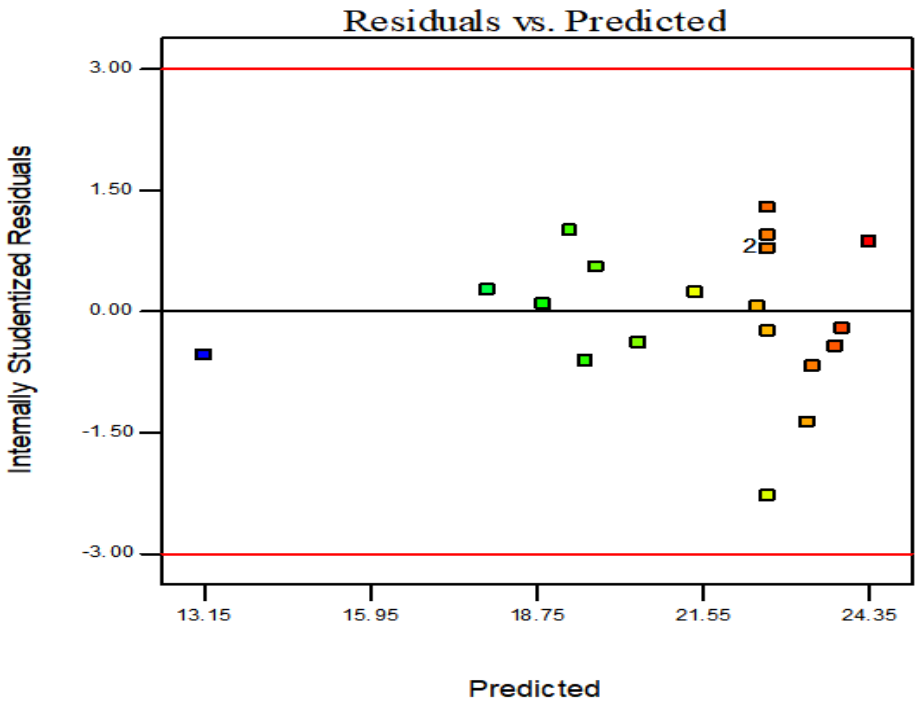


Figure 4.2 (a) Residual vs. Predicted plot for acetone extract

(b)

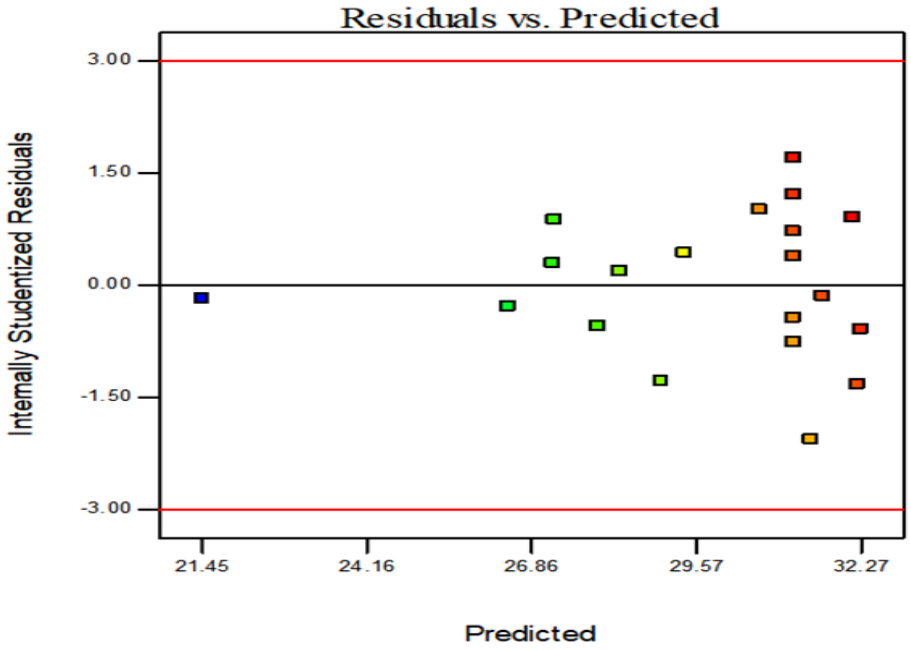


Figure 4.2 (b) Residual vs. Predicted plot for methanol extract

(a)

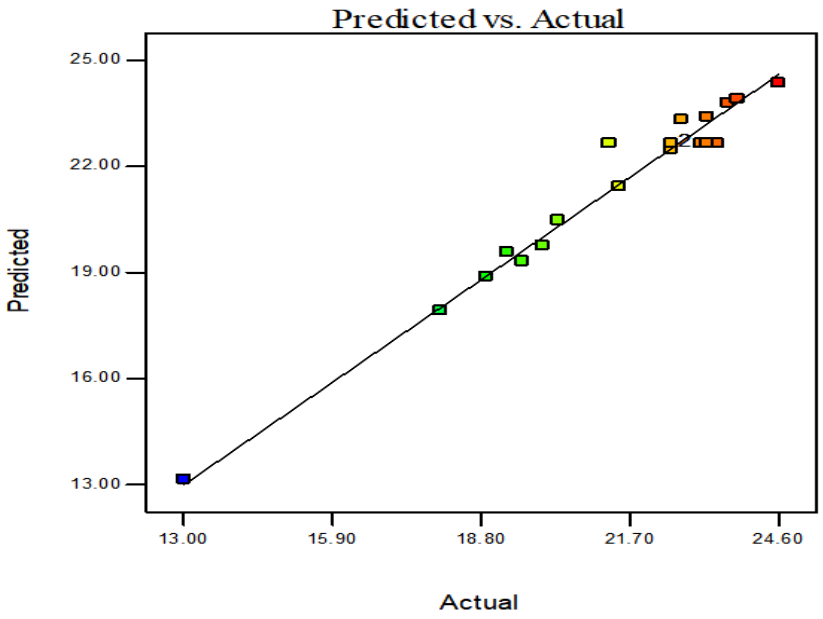


Figure 4.3 (a) Predicted vs. Actual plot for acetone extracts

(b)

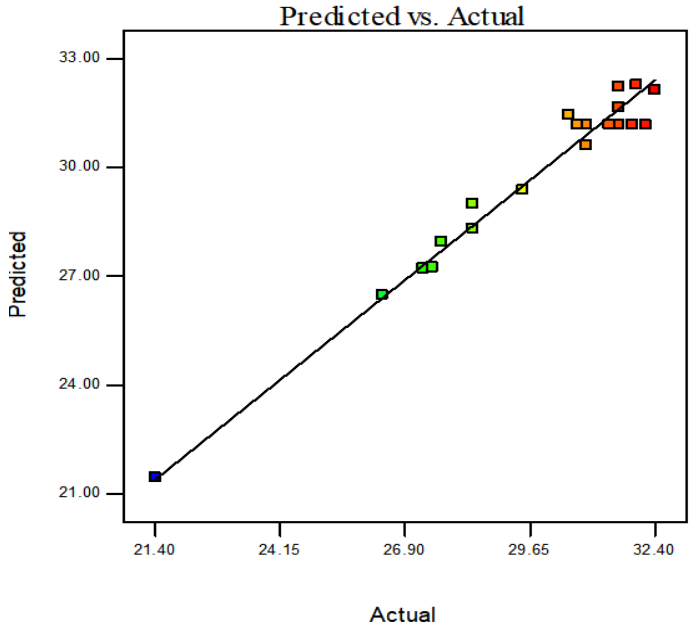


Figure 4.3 (b) Predicted vs. Actual plot for methanol extract

4.3 Effects of individual factor on yield

Based on variance analysis, the antioxidant yield was significantly influenced by different process variables. Important individual process variables influencing yield are solvent ratio,

extraction temperature and extraction time. These factors have an effect on the yield of the BP antioxidant extract.

4.3.1. Effect of solvent ratio on Yield

One of the significant factors influencing the yield of BP antioxidant extract is solvent ratio. The two solvents used, acetone and methanol combined with water at various extraction ratios. The solvent ratio has a major effect on the amount of BP antioxidant extract as seen in the Figure 4.4(a) and (b) The effect of both solvents ratio acetone and methanol concentration on the yield, the yield increases with increasing concentration from low level (-1) to high level (1) at fixed time and temperature at their center level (0). The maximum yield obtain when acetone/water and methanol/water solvent ratio were 90/10. The study by Toh et al.,(2016) indicated that the maximum yield is obtained when the aqueous acetone and methanol extraction done at 90%,as the aqueous content decrease the extract yield increases. Aqueous methanol has been commonly used as extraction medium. Chirinos et al., (2007) explained that 70% methanol was the best to extract antioxidants from plants, where it was able to inactivate polyphenol oxidases for enhancing the extraction of flavonoids. However, 90% methanol yielded a maximum polyphenol from the banana peel sample. (González-Montelongo et al, 2010) also reports that the acetone: water mixture is an effective solvent for extracting phenolic compounds from fruit by-products. However, each substance has a different molecular weight, different polarity and is bound to the matrix in different ways. Therefore, for the purpose of extraction, a specific solvent mixture, or the use of more than one solvent (sequential extraction) might be required. In addition, banana peels antioxidant activity is related to polar substances therefore extractions by polar solvents are more powerful relative to non-polar solvents. Besides organic solvents, water is also considered as an effective ‘green’ solvent in extracting phenolic compounds from banana peel (Someya et al, 2002).

(a)

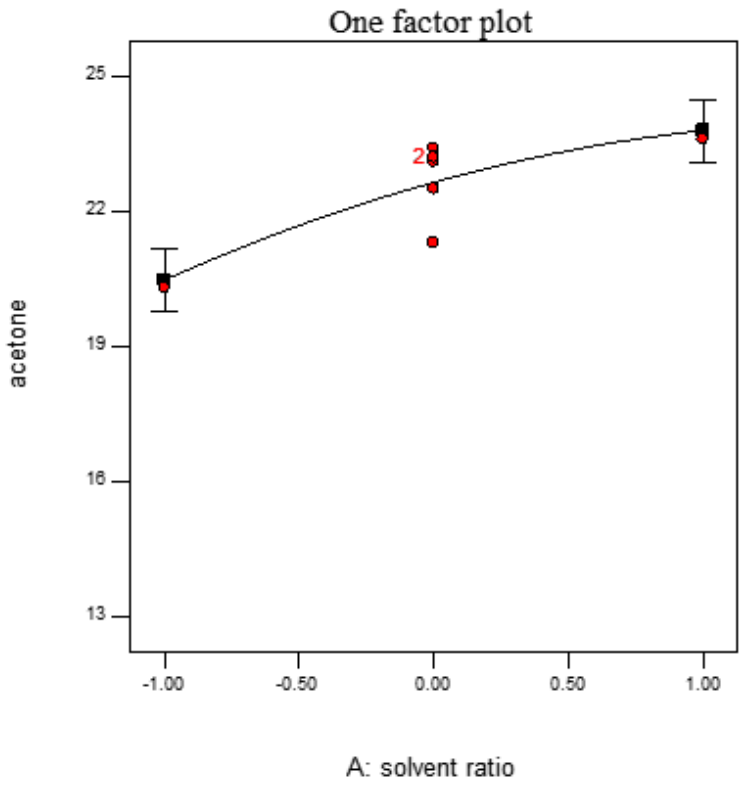


Figure 4.4 (a) Effect of acetone to water ratio on yield at fixed time and temperature

(b)

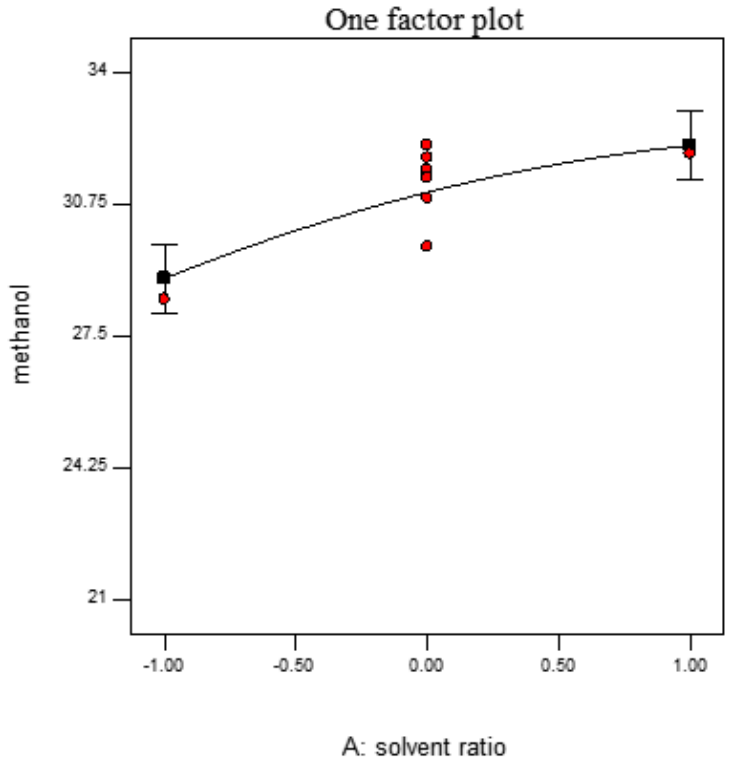


Figure 4.4 (b) Effect of methanol to water ratio on yield at fixed time and temperature

4.3.2 Effects of temperature on yield

The extraction temperature has an impact on the recovery of the maximum yield from BP was evaluated using temperatures ranging from 40 to 60°C. Results have shown that extraction temperature has a major impact on yield. From the results, it can be shown at Figure 4.5(a) and (b) that the yield increases over a wide range and starts to be stable at a higher level. Generally, the temperature has a significant influence on the extraction from the BP extract of phenolic compounds. The findings obtained also indicate a very strong effect of temperature on the polyphenols extraction of banana (Figure 4.5(a) and (b)). At 60°C, the yields of polyphenols extracted were higher compared to the yields obtained at 40°C. Ling et al.(2015) reported that at 60°C the extracted polyphenols yield were tripled comparing to the yields obtained at 20°C.The experiential positive effect of temperature could be described by the higher solubility of polyphenols in the solvent, the higher diffusivities of the extracted molecules and the improved mass transfer at higher temperatures. A rise in extraction temperature could also break the phenolic matrix bonds and influence the membrane structure of plant cells and therefore facilitate the extraction process (Ling et al., 2015).

(a)

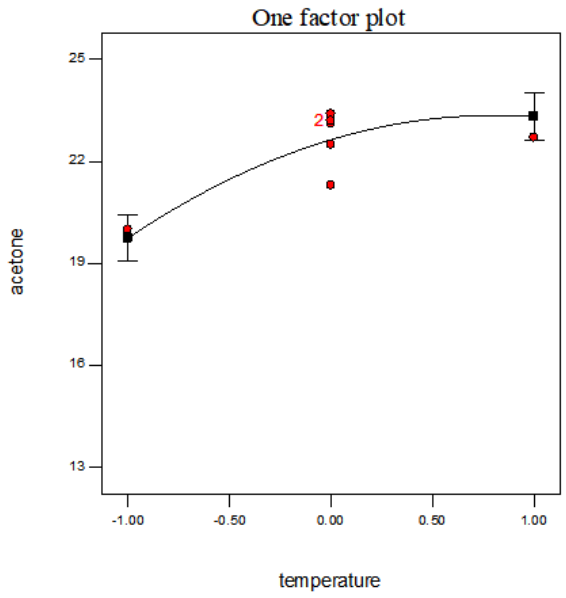


Figure 4.5 (a) Effect of temperature on yield of acetone extract at fixed solvent ratio and time

(b)

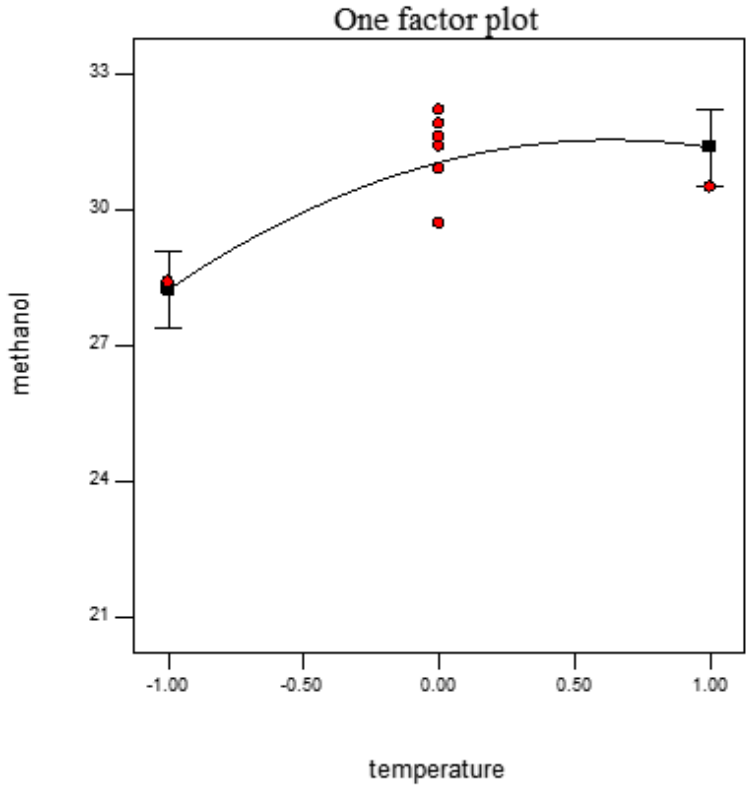


Figure 4.5 (b) Effect of temperature on yield of methanol extract at fixed solvent ratio and time

4.3.3 Effects of time on yield

The influence of the extraction time on the maximum yield recovery from BP was evaluated using time ranging from 1h to 4h. Results have shown that extraction time has a major impact on yield. From the results, it can be shown at Figure 4.6(a) and (b) that the yield increases when the time goes from lower level to higher level. The maximum yields were obtained when extraction is done for 4h at a fixed solvent ratio plus temperature. When extraction time increase the yield discover were higher because of the longer contact surface between the solvent and the fine solid particles favored the extraction of polyphenols. The longer extraction time values allow for the longer amount of time the solute and solvent were in contact, so it make the systems have been successfully mass transfer. As seen from figure 4.6 (a) and (b), the time of extraction went from low to high level, the contact time of solvent was increased and the total number of phytochemical compounds distributed to solvent increased, this leads to increasing the extract yields.

(a)

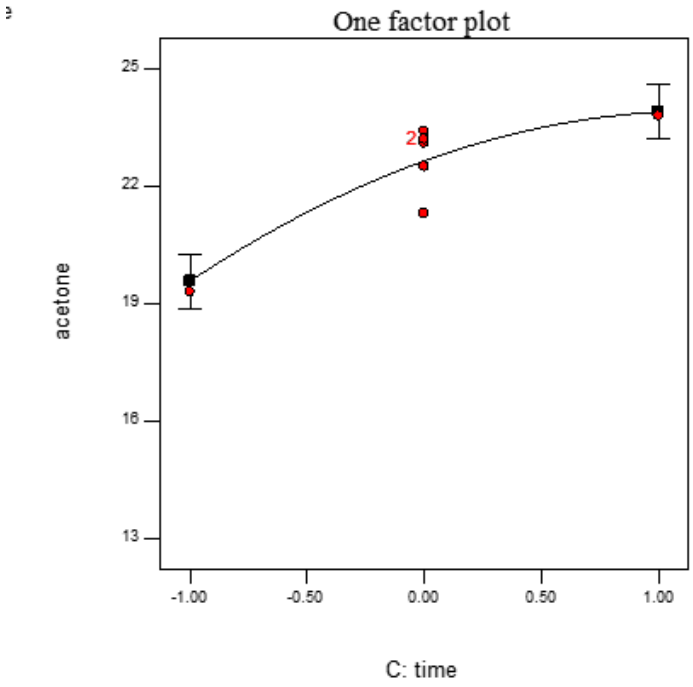


Figure 4.6 (a) Effect of time on yield of acetone extract at fixed solvent ratio and temperature

(b)

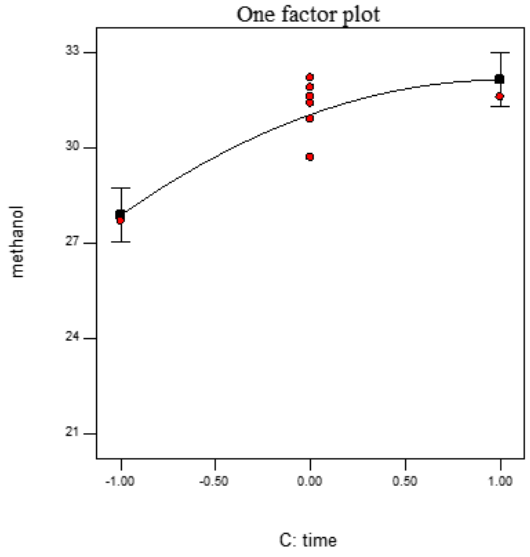


Figure 4.6 (b) Effect of time on yield of methanol extract at fixed solvent ratio and temperature

4.4 Effect of Interaction of Factors on Yield

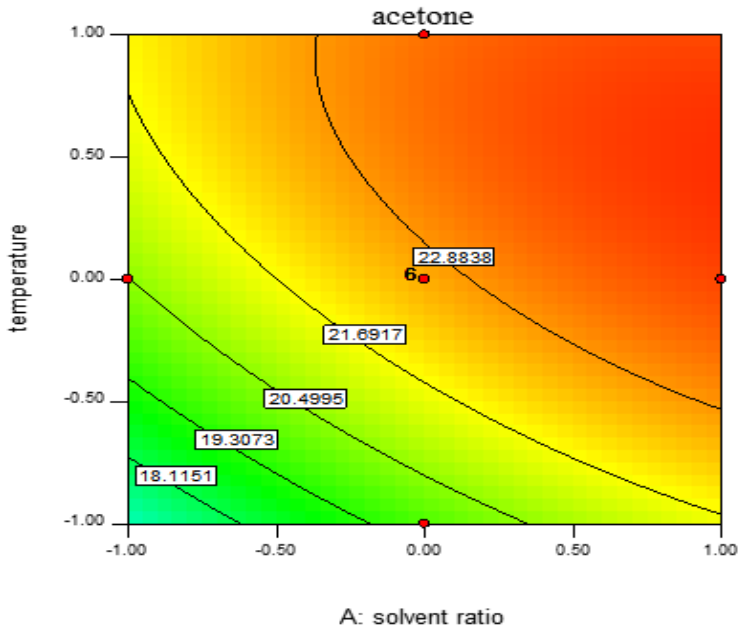
An interaction happens when the response was different based on the setting of two factors. The parts were created by holding one variable at the central of the experimental range, and varying the other two. Plots make it possible to interpret the correlation between two variables. Two non-parallel lines appear, indicating that the influence of one factor depends on the level of another factor. The interaction factor effects on the antioxidant yield can be understood easily by the coefficient of interaction factors from regression model equation. There were three interaction factors analyzed by the model equation. These are: solvent ratio and time, solvent ratio and temperature, and temperature and time. The sign of the coefficient of the interaction factor indicates the effect of interaction factor on antioxidant yield. Therefore, the interaction factors with positive sign have positive effect on antioxidant yield (as interaction factors increase antioxidant yield increase). Whereas, interaction factors with negative signs have a negative effect on antioxidant yield (as the interaction factors increase antioxidant yield decrease). The 3D response surface also contour plots below discussed the influence of the interaction of solvent ratio, extraction temperature and extraction time with the response of antioxidant yield.

4.4.1. Effect of solvent ratio and temperature on yield

Figure 4.7 shows the interaction effect of solvent ratio and temperature on antioxidant yield in the form of 3D contour and surface contour. For both acetone and methanol extracts, solvent ratio and temperature had an important effect on the response rate at a fixed center level of extraction time. Figure 4.7(a), (b), (c) and (d) indicates that antioxidant yield increase with solvent concentration and extraction temperature increases from low to center level and becomes stable and start to decline at high level slightly. This is for the reason that the lower water soluble phytochemical compounds happens at both lower solvent ratio and temperature, the rate of phytochemical compounds diffused into the solvent was minimal. Therefore, the extracted yield was low at a low solvent ratio and temperature. Increasing temperature can affect extraction by increasing the rate of solubility of phytochemicals in solvent-water binary mixture. In addition, soluble phytochemical compounds increased with solvent ratio increase. So that the outcome from extraction was influenced by both solvent ratio and temperature. Due to low solvent ratio and temperature the solubility of compounds becomes minimal so that amount of antioxidant obtain will be small. When solvent ratio and temperature became higher the amount of antioxidant recovery increase, as seen in the contour and 3D plots the yield increases with solvent ratio

and temperature increase. At a solvent ratio of 90:10 at 60°C at a fixed time, the highest yield was obtained.

(a)



(b)

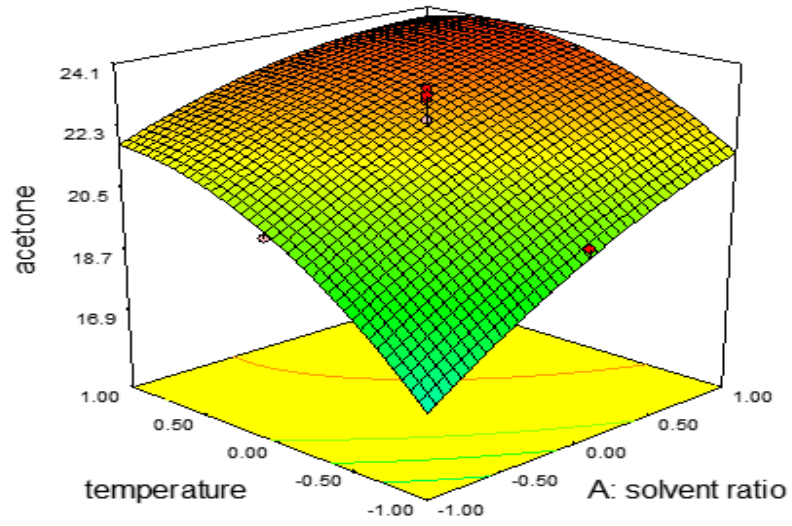
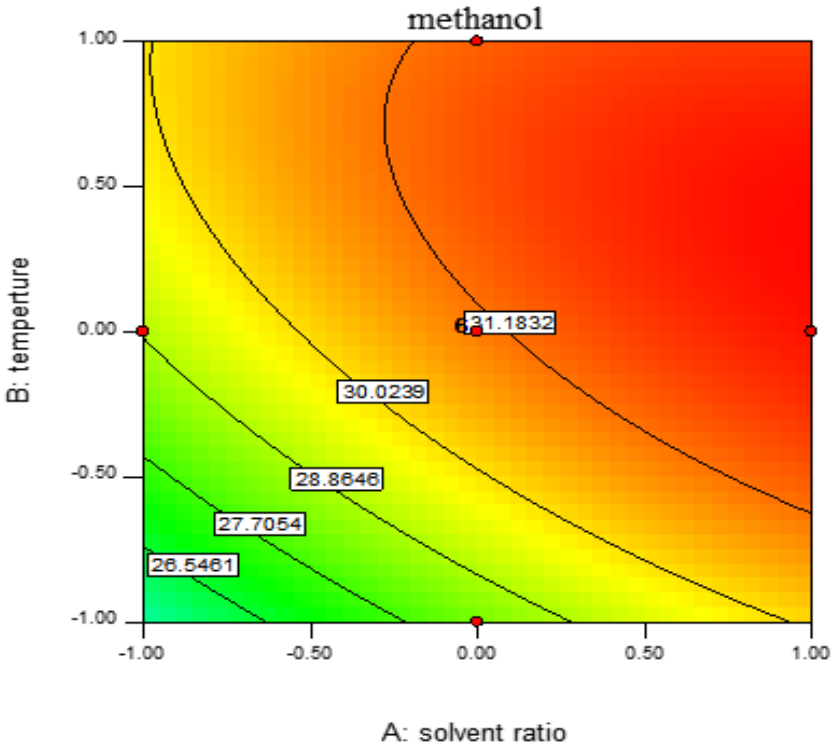


Figure 4.7 Contour plot (a) and 3D surface (b) showing effect of solvent ratio and temperature on yield at fixed time for BP acetone extract

(c)



(d)

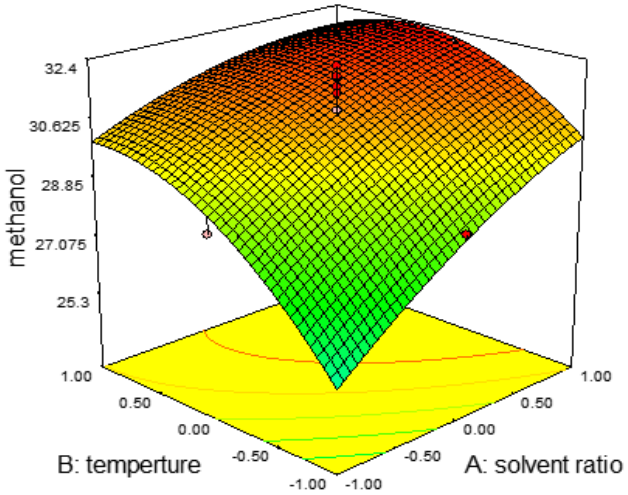


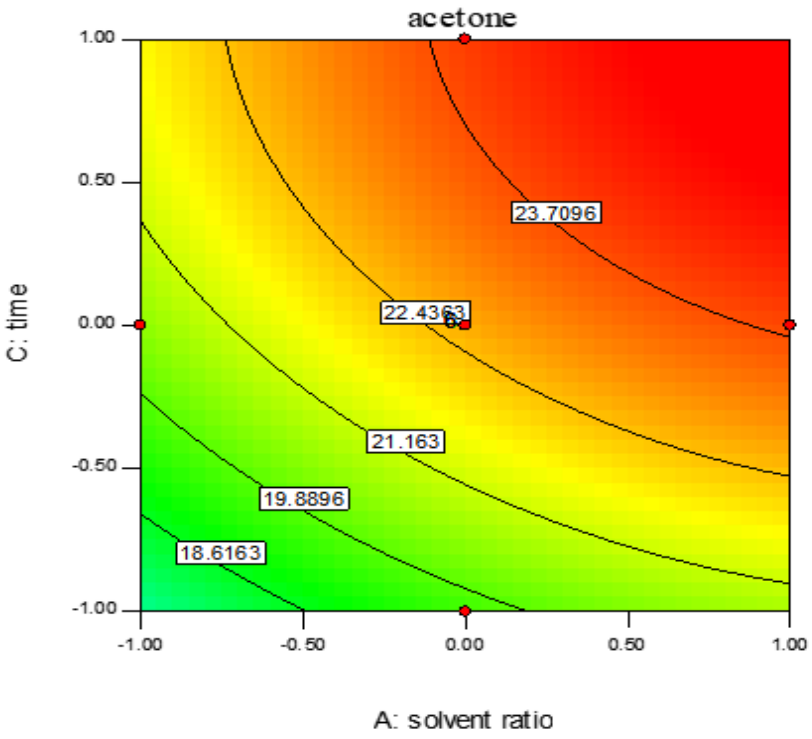
Figure 4.7 Contour plot (c) and 3D surface (d) showing effect of solvent ratio and temperature on yield at fixed time for BP methanol extract

4.4.2. Effects of solvent ratio and time on yield

Figure 4.8 shows the solvent ratio and extraction time interaction effect on the antioxidant yield in form of 3D contour and surface contour. For both acetone and methanol extracts,

solvent ratio and extraction time had an important effect on the response rate at a fixed center level of extraction. Figure 4.8(a), (b), (c) and (d) indicates that yield increase with solvent ratio and extraction time increases from low to high level. This is due the solvent and time effect. Shorter extraction time is insufficient to completely extract the bound phenolic compound; lower solvent ratio results minimal rate of phytochemical compounds diffused into the solvent. The extraction yield was therefore low at a low solvent ratio and extraction time. As presented in figure 4.8 the extraction temperature fixed a center level. The response yield increase with increasing solvent ratio (acetone/water and methanol/water) and extraction time.

(a)



(b)

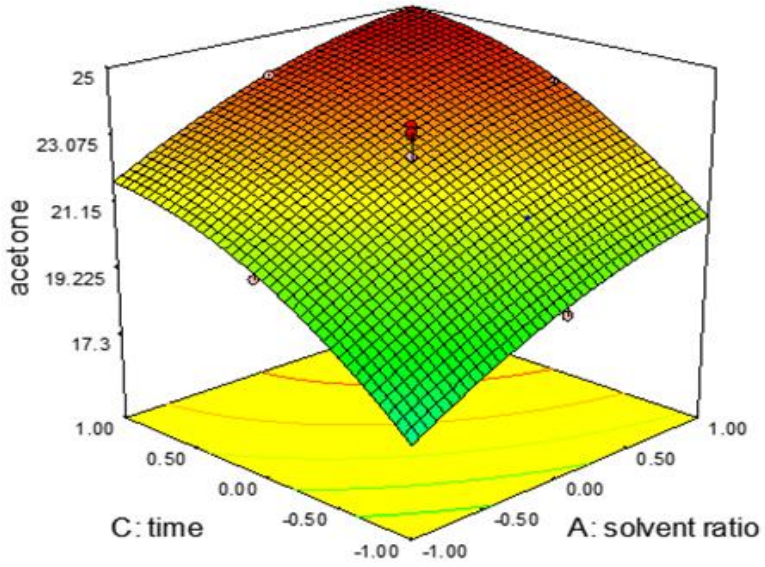
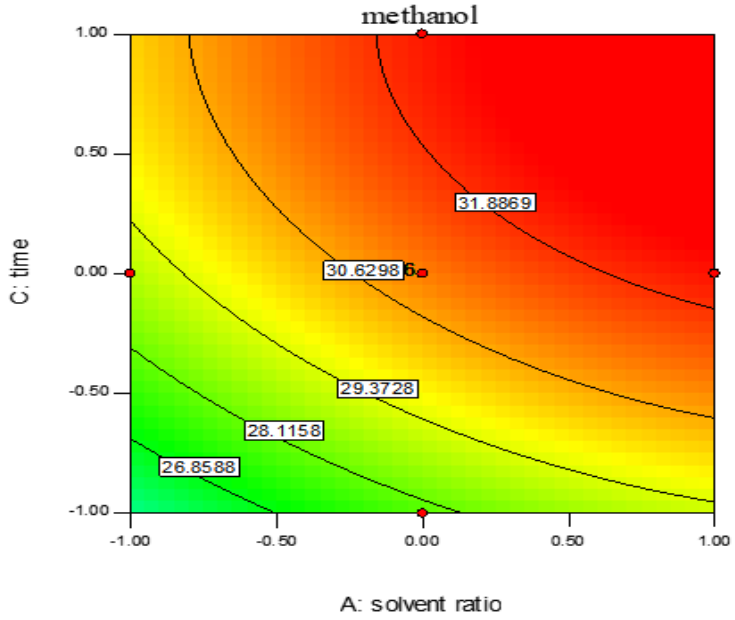


Figure 4.8 Contour plot (a) and 3D surface (b) showing effect of solvent ratio and time on Yield at fixed temperature for BP acetone extract

(c)



(d)

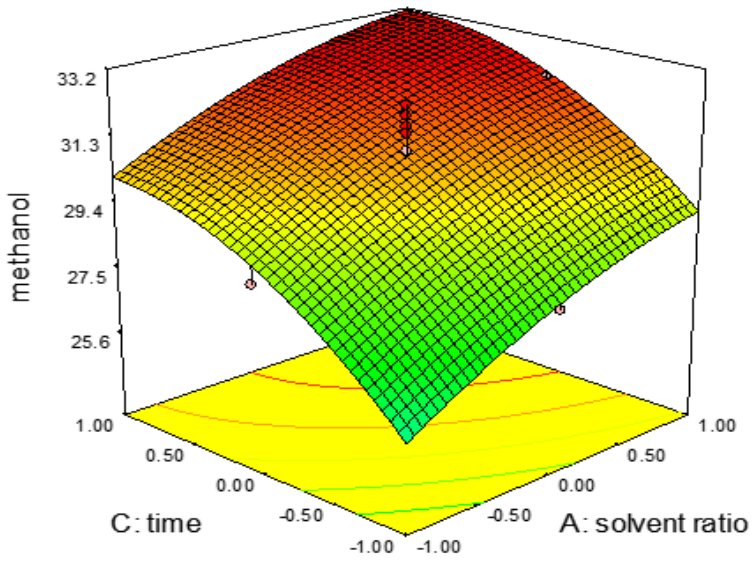
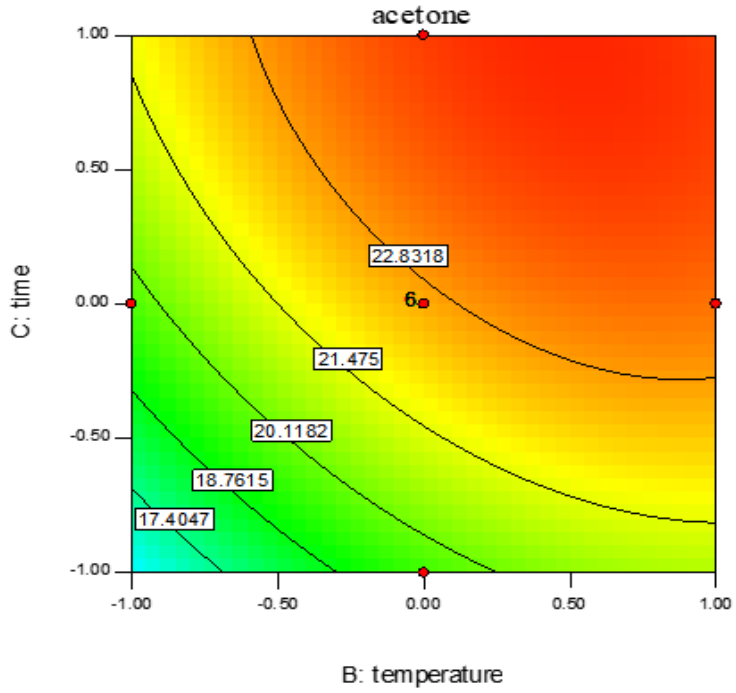


Figure 4.8 Contour plot (c) and 3D surface (d) showing effect of solvent ratio and time on Yield at fixed temperature for BP methanol extract

4.4.3. Effects of temperature and time on yield

Figure 4.9 shows the temperature and extraction time interaction effect on the antioxidant yield in form of 3D contour and surface contour. For both acetone and methanol extracts, temperature and extraction time had an important effect on the response rate at a fixed center level of solvent ratio. Figure 4.9(a), (b), (c) and (d) indicates that yield increase with temperature and extraction time increases from low to center level and at high level there is a slightly decreases. This is due the temperature and time effect. Shorter extraction time is insufficient to completely extract the bound phenolic compound; lower temperature results minimal rate of phytochemical compounds diffused into the solvent. And so at low temperature and extraction time the extracted yield was low.as shown in figure 4.9(a), (b), (c) and (d) the extraction solvent ratio fixed a center level. Study report that due to a prolonged contact of the sample in the solvent, the maximum extraction time and temperature formed the highest yield provided adequate time aimed at the desired compounds to migrate into the solvent and rise the temperature could break the phenolic matrix bonds and influence the membrane structure of plant cells and therefore facilitate the extraction process ((Ling et al., 2015, Che Sulaiman et al., 2017).

(a)



(b)

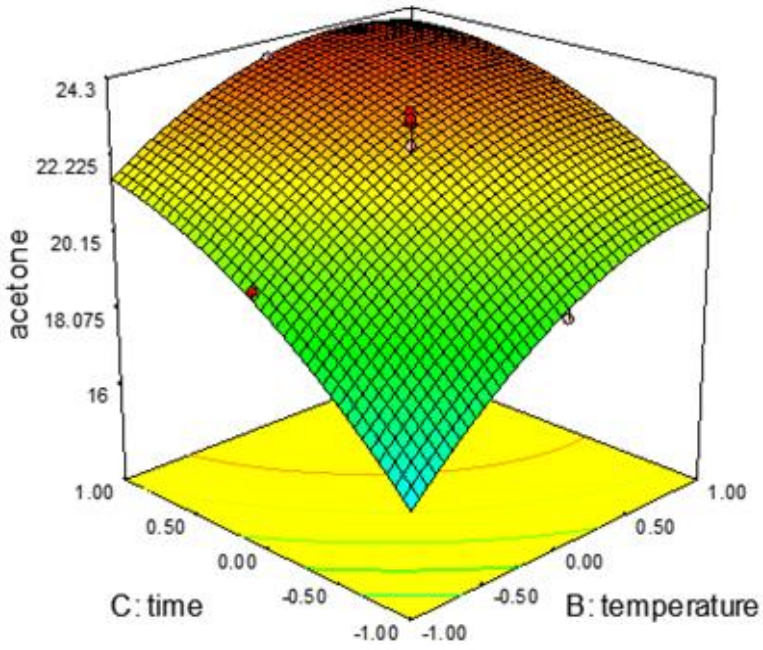
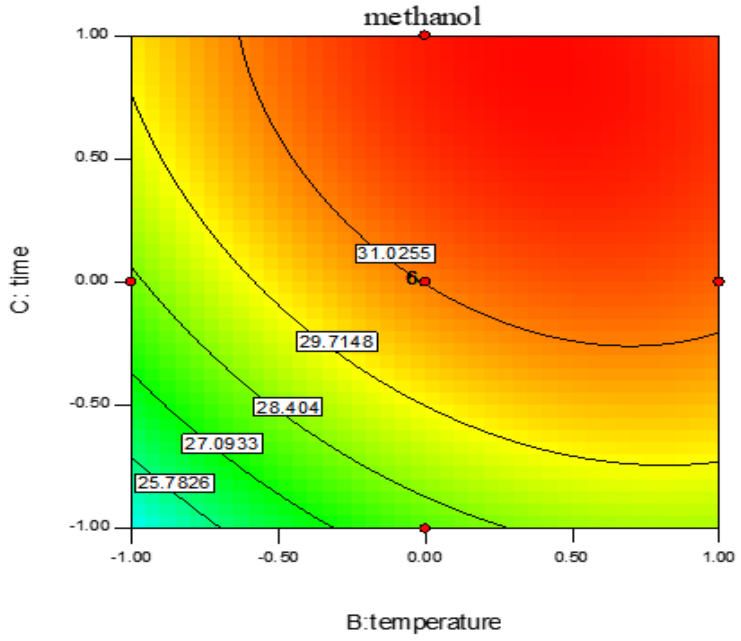


Figure 4.9 Contour plot (a) and 3D surface (b) showing effect of temperture and time on Yield at fixed solvent ratio for BP acetone extract

(c)



(d)

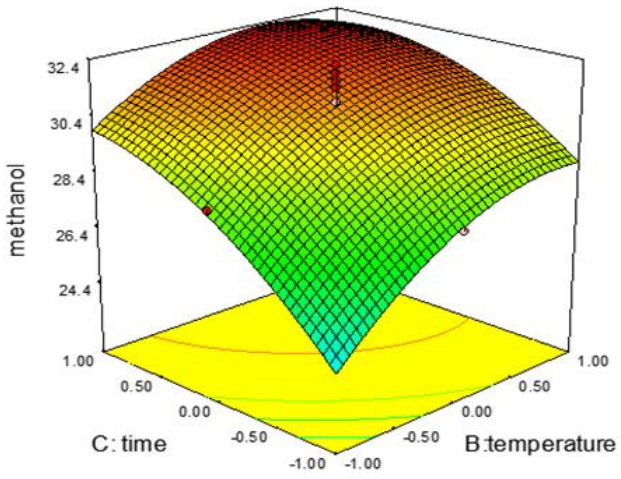


Figure 4.9 Contour plot (c) and 3D surface (d) showing effect of temperature and time on Yield at fixed solvent ratio for BP methanol extract

4.5. Optimization of the extraction parameters

One of the objectives of the present study was to find the optimal process variables for a better antioxidant yield. The process variables such as solvent ratio, extraction temperature

and extraction time were optimized. In optimizing the response yield, solvent ratio, extraction temperature and extraction time are a set of process variables held to be in range while the yield was the response that needs to be maximized. Table 4.7 and 4.8 showed the summary of factors, goals and the corresponding set of specific objectives that could enhance the process condition to have the highest responses. The expert design has three different optimization choices, namely, 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 predication optimization (enter desired operating conditions and discover predicted response values with confidence interval). In numerical optimization choice, depending on constraints selected different alternatives solution of optimization was given by expert design. For this study, numerical optimization was selected to obtain the better highest response of extract.

Table 4.7 Constraints for Optimization BP acetone extract

Name	**Goal**	**Lower limit**	**Upper limit**	**Lower weight**	**Upper weight**	**Importance**
Concentration	Is in range	60	90	1	1	3
Temperature	Is in range	40	60	1	1	3
Time	Is in range	1	4	1	1	3
acetone	maximize	13	24.6	1	1	3

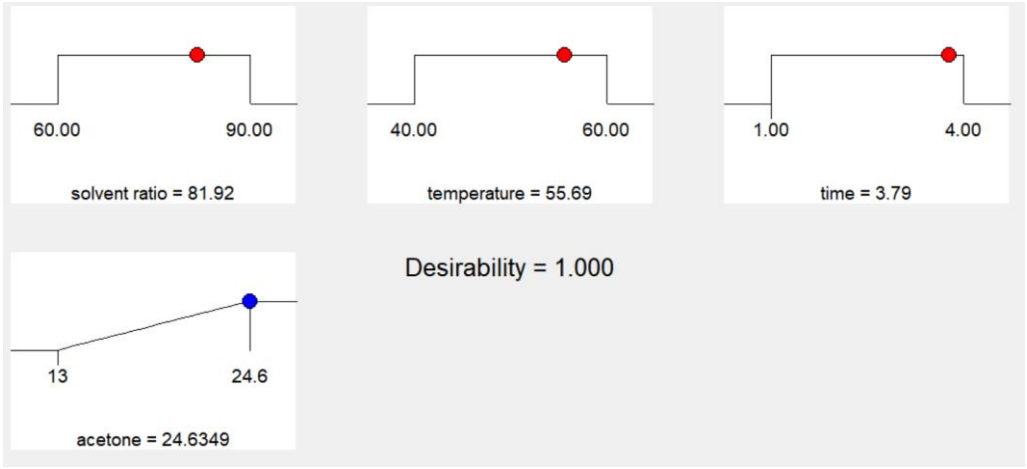
Table 4.8 Constraints for optimization BP methanol extract

Name	**Goal**	**Lower limit**	**Upper limit**	**Lower weight**	**Upper weight**	**Importance**
Concentration	Is in range	60	90	1	1	3
Temperature	Is in range	40	60	1	1	3
Time	Is in range	1	4	1	1	3
methanol	maximize	21.4	32.4	1	1	3

The design expert under numerical optimization gave 15 different optimization solutions. Depending on the parameters (solvent ratio, extraction time and temperature) by compromising yield, the best solution was selected among the alternatives. Undertaking the consideration of these constraints, for acetone solvent ratio (acetone/water) 81.92/18.08,extraction time 3.79 h, and temperature 55.69℃ were selected which gave

24.6349 % yield, for methanol solvent ratio (methanol/water) 88.56/11.44, extraction time 3.62 h, and temperature 53.35℃ were selected which gave 32.4629% yield among optimizing alternatives given by the design expert software. Desirability function has been used to identify the optimum level of factors and get a maximum desirable response. The optimized batch was selected with maximum combined yield desirability value i.e. 1.00 and the graph of the optimized solution for the yield of antioxidant from acetone and methanol extracts is presented in Figure 4.10(a)and(b).

(a)



(b)

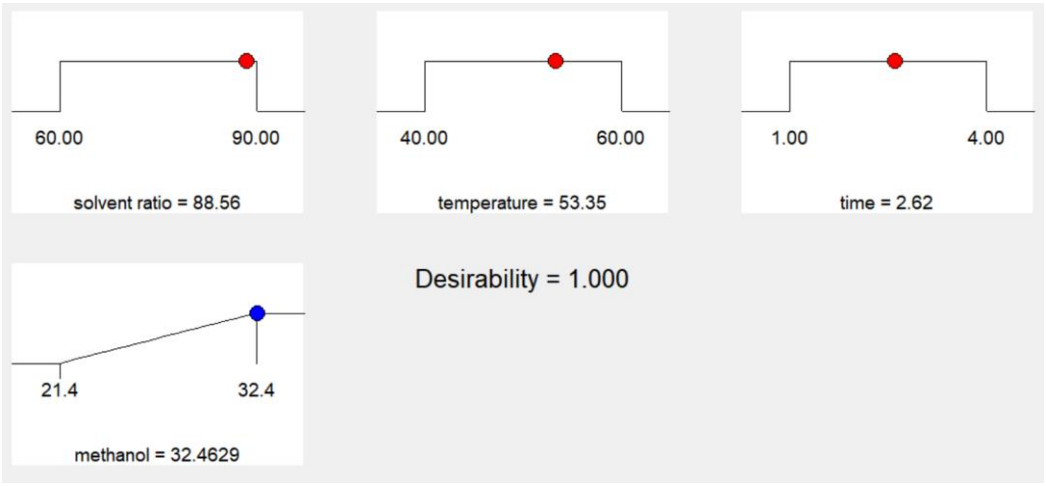


Figure 4.10: Ramp Plot of Optimization Solution for the Response, (a) acetone and (b) methanol BP extract response

The ramp plot of the optimization solution for the yield of antioxidant was shown in Figure 4.10. For easier interpretation, the ramp show combines the individual graphs and the dot on each ramp represent the factor setting or response prediction for that solution. The predicted highest screened yield was produced from a solution of optimization for acetone

at solvent ratio (81.92), extraction time (3.79h), temperature (55.69 ºC) and for methanol at solvent ration (88.56), extraction time (2.62h) and temperature (53.35 ºC) where the predicted yield was at 24.6349% and 32.4629% respectively. These are the optimal parameters to obtain maximum yield of antioxidant from BP in both solvents as seen in figure 4.10. So two parallel experiments for each were carried out under the optimal conditions. Comparing with the predicted value by Design Expert version 7.0.0, the results showed that the actual value of yield for acetone 24.5% and for methanol 32% which was very close to the predicted results. This indicated that the optimization achieved in the current study was reliable. The yields obtain from the optimization selected for further characterization the sample.

4.6 Characterization of the extracts

4.6.1 Specific density of the extract

The specific density of extract determined via 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 as mentioned in Eqn 3.9. The specific density of the acetone and methanol extracts was 1.00 for both solvents extracts.

4.6.2 pH value of extract

The pH value of methanol and acetone was 5.713 and 6.788 respectively. The lower pH values, the more acid of the extracts. Low- acid foods require pH values higher than 4.6 to 6.9. From the values, both the extract solutions were acidic and the methanol extract has low pH value than acetone extract that means it is high acidic and better in antioxidant activity than BP acetone extract.

4.6.3 Total Phenolic Content of the extracts

The phenolic content of banana peels extracts was determined by using the Folin-Ciocalteu method. Phenolic compounds are important fruit constituents because they exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydro peroxides into free radicals (Kuala *et al.*, 2012). The TPC of acetone and methanol extracts from banana peel shown in table 4.10 and 4.1, respectively. As shown in table 4.10 and 4.11, both solvents extracts contain significant amount of phenolic compounds in the extracts. The total phenol content calculated from the calibration curve of Gallic acid

(y=0.0743x+0.0095, R^2= 0.999), was expressed as Gallic acid equivalents/g (GAE/g) in dry base.

Table 4.9 Concentrations of Gallic acid standard solution and their corresponding absorbance

Gallic acid concentration(mg/ml)	Absorbance(nm)
0	0.0143
1	0.0791
2	0.1675
4	0.2992
6	0.4558
8	0.6025
10	0.7358
12	0.9176

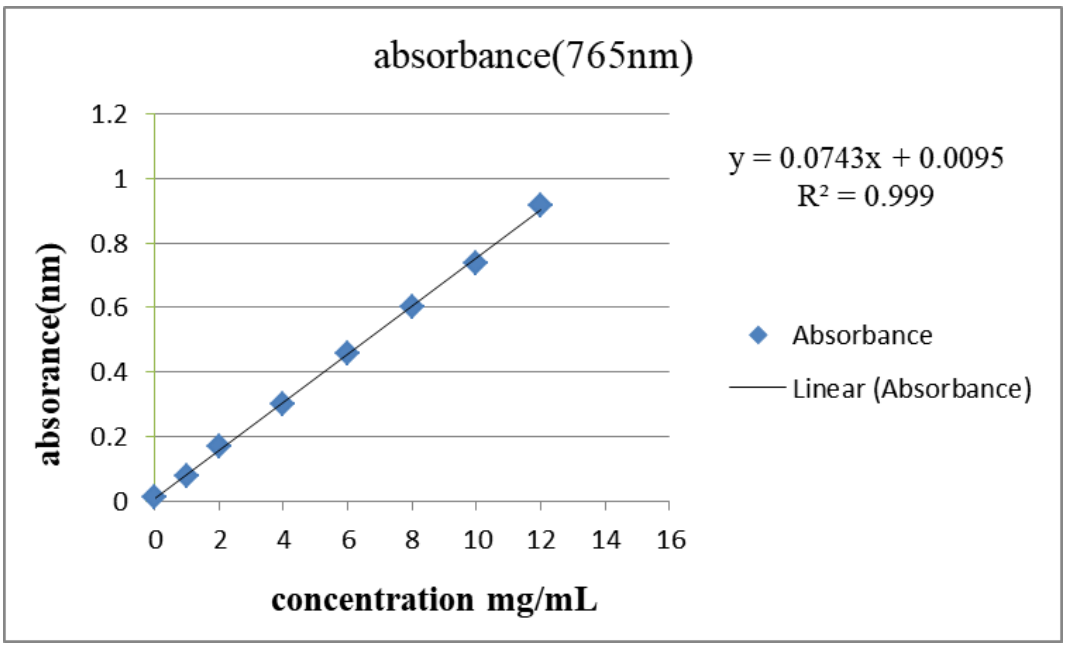


Figure 4.11 Gallic acid standard calibration curves

Table 4. 10 Amount of total phenol content from a gram of acetone extract from banana peel

Sample Concentration (mg/ml)	Acetone extract absorbance(nm)	Gallic acid equivalent Concentration (mg/ml)	Total phenol content (mg GAE/g extract)
1	0.0187	0.124	2.476
2	0.0493	0.536	10.713
4	0.119	1.474	29.475
6	0.1586	2.007	40.135
8	0.1947	2.493	49.852
10	0.2169	2.791	55.827
12	0.2975	3.876	77.524

The TPC of acetone and methanol extract from banana peel shown in table 4.10 and 4.11. The TPC in both extracts range from 2.476 to 77.739 mg GAE/g extract. In acetone extract, the highest TPC was 77.524 mg GAE/g dry weight basis, obtained at sample concentration 12.0 mg/ml. The other sample concentration 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/ml contains TPC 2.476, 10.713, 29.475, 40.135, 49.852 and 55.827 mg GAE/g dry weight basis. For methanol extract the highest TPC found was 77.739 mg GAE/g dry weight basis, obtained at sample concentration 12.0 mg/ml. The other sample concentration 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/ml give TPC 3.230, 9.609, 24.065, 35.828, 49.609 and 57.308 mg GAE/g dry weight basis. The TPC increases with concentration because of the absorbance has a direct relation with the concentration, as the concentration of the sample increase the absorbance also increase.

From the result obtained (Table 4.10 & 4.11), Methanol extract has a higher TPC than acetone extract. Methanol extract of banana peel had higher TPC in the previous study by Fatemeh et al., (2012) who studied the influence of variety Cavendish and Dream variety. The TPC found in methanol extract varied greatly from 75.01 to 685.57 mg GAE/ 100g from green and ripe banana peel and pulp. These results were lower than that obtained in the present results. Aboul-enein et al., (2016) also reveal that the total phenol in banana peel from *Musa paradaisica L*. variety recorded the highest content (17.89 mg GAE/g) in methanol extract followed by acetone (15.44 mg GAE/g),ethanol (15.21mg GAE/g) then aqueous extract (9.89 mg GAE/g), the methanol and acetone results close to the present

results obtained in low concentration. Liu et al. (2007) supported our findings that the TPC of Xylaria spices were the highest in methanol extract as compared to hexane extract. Methanol is often recognized as the best extraction solvent for fruit peels. In general terms, the methanol and acetone extracts exhibited higher phenolic contents than the other conventional solvent extracts (i.e., apple, strawberry, cucumber, chicory, golden rod and woad)(Peschel et al., 2006). Different extract concentrations and different procedures followed for TPC analysis, even using Gallic acid, as comparisons are likely to provide different results.

Table 4.11 Amount of total phenol content from a gram of methanol extract from banana peel

Sample Concentration (mg/ml)	Methanol extract absorbance(nm)	Gallic acid equivalent Concentration (mg/ml)	Total phenol content (mg GAE/g extract)
1	0.0215	0.162	3.230
2	0.0452	0.480	9.609
4	0.0989	1.203	24.065
6	0.1426	1.791	35.828
8	0.1938	2.480	49.609
10	0.2224	2.865	57.308
12	0.2983	3.887	77.739

4.6.4 Total Flavonoid Content of the Extracts

The recovery of flavonoid content using catechin as a standard was calculated according to the aluminum chloride colorimetric method. Flavonoids are found to be strong antioxidants because of the existence of phenolic hydroxyl groups, which enables them to effectively scavenge the reactive oxygen types. The TFC in the banana peel extract is expressed in terms of catechin equivalent. The value obtained for the total flavanoids is expressed as mg of catechin/g of the extract (standard curve equation: $y = 0.0101x + 0.0061$, $R^2 = 0.9675$). The TFC of acetone and methanol extract shown in table 4.13 and 4.14.

Table 4.12 Concentrations and Absorbance for Catechin standard calibration curve

Catechin concentration(mg/ml)	absorbance(nm)
0	0.0085
1	0.0092
2	0.0182
4	0.0624
6	0.0712
8	0.0834
10	0.1002
12	0.1289

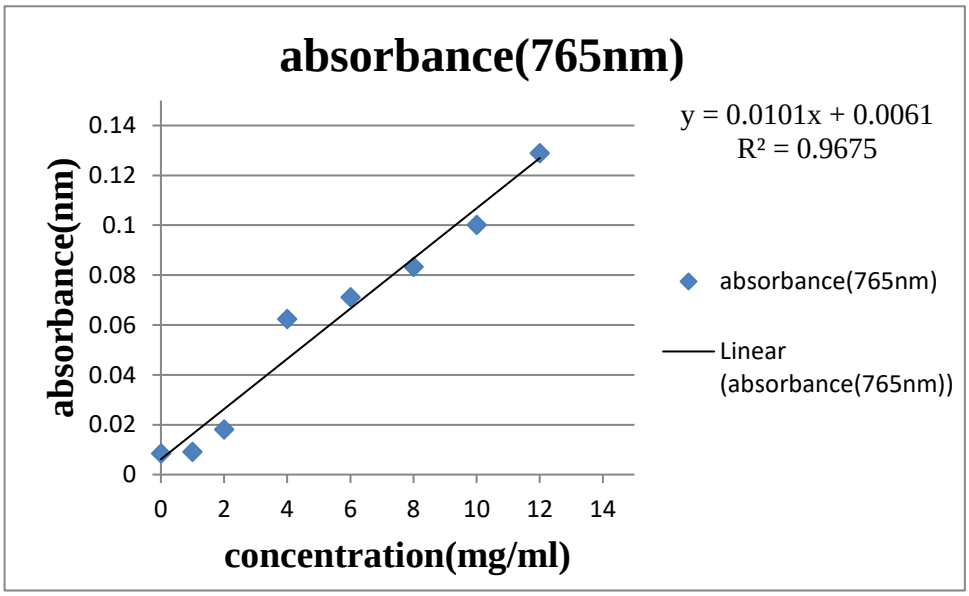


Figure 4.12 Catechin standard calibration curves

The TFC of acetone and methanol extract from banana peel are shown in table 4.13 and 4.14. The TFC in both extracts range from 5.743 to 57.030 mg CE/g dry weight basis. In acetone extract, the highest TFC was 47.327 mg CE/g dry weight basis, obtained at sample concentration 12.0 mg/ml. The sample concentration 4.0, 6.0, 8.0 and 10.0 mg/ml satisfy the catechin calibration curve, contains TFC 16.436, 22.772, 34.257, and 38.019 mg CE/g dry weight basis. For methanol extract the highest TFC found was 57.030 mg CE/g extract, obtained at sample concentration 12.0 mg/ml. The sample concentration 2.0, 4.0, 6.0, 8.0 and 10.0 mg/ml satisfy the calibration curve, which gives TFC 5.743, 26.337, 34.851, 37.228, and 41.980 CE/g dry weight basis. As observed from the results the absorbance has

a direct relation with the concentration, as the concentration increase of the sample increase the absorbance also increase.

Table 4.13 Amount of total flavonoid content from a gram of acetone extract from banana peel

Sample Concentration (mg/ml)	Acetone extract absorbance(nm)	Catechin equivalent Concentration (mg/ml)	Total flavonoid content (mg CE/g extract)
1	0.001	-	-
2	0.0028	-	-
4	0.0144	0.822	16.436
6	0.0176	1.139	22.772
8	0.0234	1.713	34.257
10	0.253	1.901	38.019
12	0.03	2.366	47.327

Table 4.14 Amount of total flavonoid content from a gram of methanol extract from banana peel

Sample Concentration (mg/ml)	Methanol extract absorbance(nm)	catechin equivalent Concentration (mg/ml)	Total flavonoid content (mg CE/g extract)
1	0.003	-	-
2	0.009	0.287	5.743
4	0.0194	1.317	26.337
6	0.0237	1.742	34.851
8	0.0249	1.861	37.228
10	0.0273	2.099	41.980
12	0.0349	2.851	57.030

The results reported by Aboul-enein,.(2016) reveal that the total flavonoids in banana peel recorded the highest content (21.04 CEmg/g DW) in methanol extract followed by ethanol (18.52 CEmg/g DW),acetone (16.15 CEmg/g DW) then aqueous extract (8.56 CEmg/g DW), Fatemeh et al.,(2012) reported that in two varieties (Cavendish and Dream) and two stages of ripeness (ripe and green) hence (Cavendish ripe was 225.91 and Cavendish green was 389.33 mg QE/100g of dry matter) while (Dream ripe was 72.46 and Dream green 96.92 mg QE/100g of dry matter). This is less those result obtained in the study.

The differences between the present results may be attributed to plant species, environmental condition and sample preparation. Also, the differences in flavonoid content could be related to the part of fruit used for making the extract and type of solvent. The data obtained indicate that phenolic, flavonoids of banana peels were extracted with methanol better than the other solvents tested.

4.6.5 Antioxidant activity of the extracts

Antioxidant activity was measured by DPPH method. This method measured the ability of plant extracts to donate hydrogen or electrons by changing the color of DPPH solution. Additionally, DPPH has the advantage of being unaffected by certain side reactions of polyphenols, such as metal ion chelation and enzyme inhibition(Babbar *et al.*, 2014). Table 4.15, 4.16 and 4.17 show that the absorbance of sample in comparison with ascorbic acid as standard at different concentration. Therefore, DPPH scavenging activity of every sample was reported as the percentage of DPPH inhibition, with a higher value is associated to a stronger antioxidant activity.

Free radical scavenging activity or inhibition activity of free radical DPPH percentage (IA %) of standard Ascorbic acid, BP acetone extract and BP methanol extract was calculated by using the relationship written in section three.

Table 4.15 absorbance of ascorbic acid and corresponding concentration

Ascorbic acid concentration (mg/ml)	**Absorbance (517nm)**	**%IA**	**IC_{50}**
0	0.5211		
1	0.4206	19.29	
2	0.3193	38.73	
4	0.120	77.16	2.59
6	0.0395	92.42	
8	0.0185	96.45	
10	0.0189	96.37	
12	0.0194	96.28	

As shown in Table 4.15, 4.16 and 4.17 the extract were capable of neutralizing the DPPH free radicals via hydrogen donating activity by 7.18, 21.36, 44.04, 60.74, 79.18, 78.93 and 74.06% for BP acetone extract and for BP methanol extract 1.92, 22.84, 64.46, 89.1, 89.81, 89.71 and 88.37% at concentrations of 1, 2, 4, 6, 8, 10, and 12 mg/ml, respectively. The tables shows IC_{50} value of ascorbic acid, BP acetone and methanol extract were determined from the regression line of concentration versus % of inhibition and IC_{50} value of ascorbic acid (2.59 mg/ml),BP acetone extract (5.08mg/ml) and for BP methanol extract (4.35 mg/ml) dry weight.

Table 4.16 DPPH absorbance of sample concentration for BP acetone extract

Sample concentration (mg/ml)	**Absorbance (517nm)**	**%IA**	**IC_{50}**
0	0.5211		
1	0.4837	7.18	
2	0.4098	21.36	
4	0.2916	44.04	5.80
6	0.2046	60.74	
8	0.1085	79.18	
10	0.1098	78.93	
12	0.1352	74.06	

The antioxidant activity of two different extracts from the BP is expressed in terms of percentage of inhibition (%) and the examination of antioxidant activities of plant extracts from BP acetone and methanol extract showed different values. The obtained values varied from 7.18% to 79.18% for BP acetone extract and for BP methanol extract it varied form 1.92% to 89.81%. The largest capacity to neutralize DPPH radicals was found for BP methanol extract, which scavenges 89.81% of free radicals at the concentration of 8.0 mg/mL. The extract from acetone were lower than methanol extract and the standard ascorbic acid, the BP acetone higher scavenging activity were higher at concentration 8mg/mL the free radical is 79.18%. Increased in concentration (above 8.0mg/ml) in both 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.

Table 4.17 DPPH absorbance of sample concentration for BP methanol extract

Sample concentration (mg/ml)	**Absorbance (517nm)**	**%IA**	**IC_{50}**
0	0.5211		
1	0.5111	1.92	
2	0.4021	22.84	
4	0.1852	64.46	4.35
6	0.0568	89.1	
8	0.0531	89.81	
10	0.0536	89.71	
12	0.0606	88.37	

The other parameter used to compare potent of antioxidant activities of BP acetone and methanol extract with standard ascorbic acid was IC50, the concentration of samples (ascorbic acid, BP acetone and methanol extract) in which the samples had the capacity to remove 50% of free radicals. IC50 was calculated using linear regression. As illustrated in the table 4.14, 4.15 and 4.16 ascorbic acid had the capacity to scavenge 50% of free radicals at the concentration (2.59mg/ml), similarly. Acetone and methanol extract had the capacities of removing 50% free radicals at the concentration of (5.80mg/ml) and (4.35mg/ml) respectively. Low IC50 indicates more potent in antioxidant activity. Hence, both BP extracts had low potent in antioxidant activity than positive control, ascorbic acid, so it requires much higher concentrations to reduce 50% of free-radical concentrations. But comparison between the two extracts methanol extract has more potent activity in antioxidant than the acetone extract as presented in figure 4.13.

The inhibition of DPPH radical of banana peel reported by Fatemeh et al.(2012) ranged from 26.55 to 52.66%.The acetone and methanol extracts however, showed greater DPPH scavenging activity than the ethanol and aqueous extracts, results were obtained for banana peel with acetone and methanol extract (46.63% and 45.76%) respectively followed by ethanol (40.45 %) and aqueous (37.85%) with IC50 values 55.45, 56.03 and 75.34 µg/ml

respectively compared to BHT (4.73 µg/ml). Aboul-enein *et al.*, (2016) the *Musa paradisiaca* banana that was extracted with methanol solvent, which showed IC50 values of 0.77 mg/ml(Schmidt *et al.*, 2015).much better free radical inhibitory than the present study. Lower IC50 value represents more potent free radical inhibitory activity. Thus, the study by Aboul-enein, *et al.*, (2016) results indicated that, compared to ethanol and aqueous extracts, acetone and methanol extracts have strong antioxidant activity. The strong antioxidant activity effects of banana extracts could be due to the existence of various antioxidant components. Reports show that the strong activity of DPPH radical scavenging activity may be attributed to the existence of higher levels of total phenolic and flavonoids as they play a key role as proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants (Choe and Min, 2015).

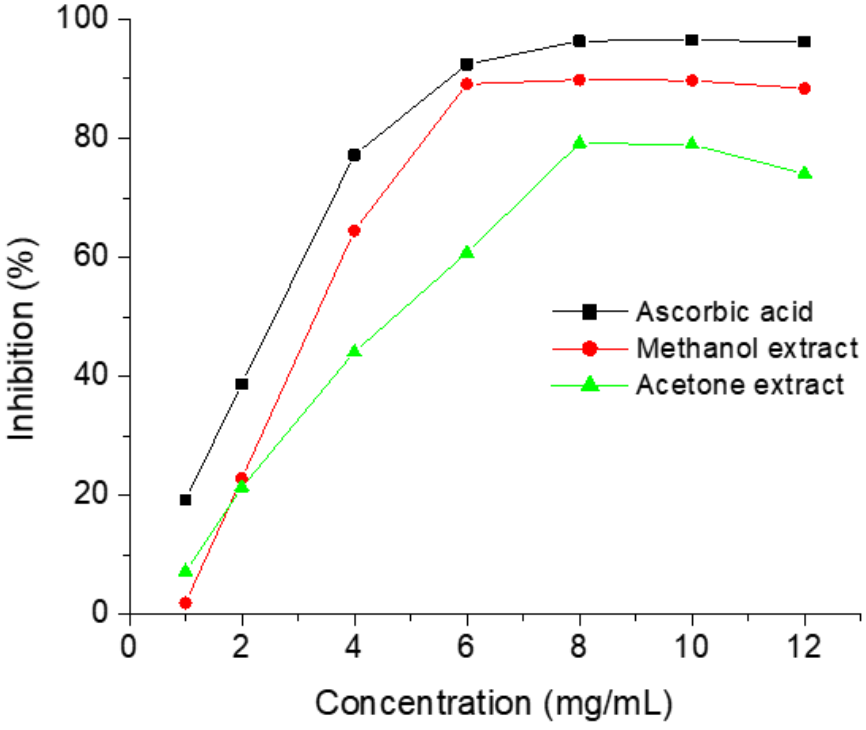


Figure 4.13 Comparison of percent DPPH free radical inhibition activities of ascorbic acid, acetone and methanol BP extracts

4.6.6. Fourier Transformation Infrared (FTIR) Analysis of Antioxidant Yield

Form FTIR analysis the result shows various functional groups in both acetone and methanol BP extracts. The result of acetone BP extract showed at figure 4.14. It can be observed that band at 3462cm^{-1}, related to hydrogen bond O-H stretch, that O-H stretch due

the alcohol group or phenols. The bands at 2932.5 cm^{-1} and 2857 cm^{-1} due to C-H stretch alkanes group. The band at 1737.25 cm^{-1} and 1725 cm^{-1} shows C=O stretch (general carbonyl, carboxylic acid, ketone, aldehydes). The band at 1647 cm^{-1} shows C=C stretching vibration of aromatic rings. The band at 1469 cm^{-1} shows a C-H bend alkanes group. The band at 1384 cm^{-1} shows CH_3C-H bend alkanes and alkyl group. The band at 1256.75 cm^{-1} shows C-N stretch aromatic amines and at 1181 cm^{-1},1082.75 cm^{-1} and 1003 cm^{-1} shows C-O stretch can be due to alcohol ,carboxylic acid group. The band at 948 cm^{-1}, 889.75 cm^{-1} 784 cm^{-1} and 724 cm^{-1} show the C-H bend due to the aromatic compound or alkenes groups or carboxylic acid.

Figure 4.15 shows the FTIR spectrum of BP methanol extract. It can be observed that the band at 3423.5 cm^{-1} indicates the O-H stretching of alcohol or phenol.The band at 2945cm^{1} shows the C-H starch due to alkanes. The band at 2105cm^{-1} show –C≡C– stretch due to alkynes. The band at 1652.25 cm^{-1} could be related to C=O of amides groups, C=C stretch of alkenes. The band at 1428.25 cm^{-1} is due to C-C stretch in aromatic rings. The band at 1263 cm^{-1} shows C-O stretch group also it could be C-N stretch aromatic rings. The 1069.25 cm^{-1} band C-O alcohols or C-N stretch aliphatic amines. The band at 915.25cm^{-1} shows C-H stretch or O-H bend carboxylic acid. And the band 867cm^{-1},824cm^{-1} and 783cm^{-1} shows C-H bend aromatic ring compounds.

The functional groups like O-H exist in all phenolic compounds also C-N is common to all alkaloids. The presence of O-H and C-N groups confirmed the presence of phenolic compounds and alkaloids. Therefore, both extracts could show remarkable antioxidant activities.

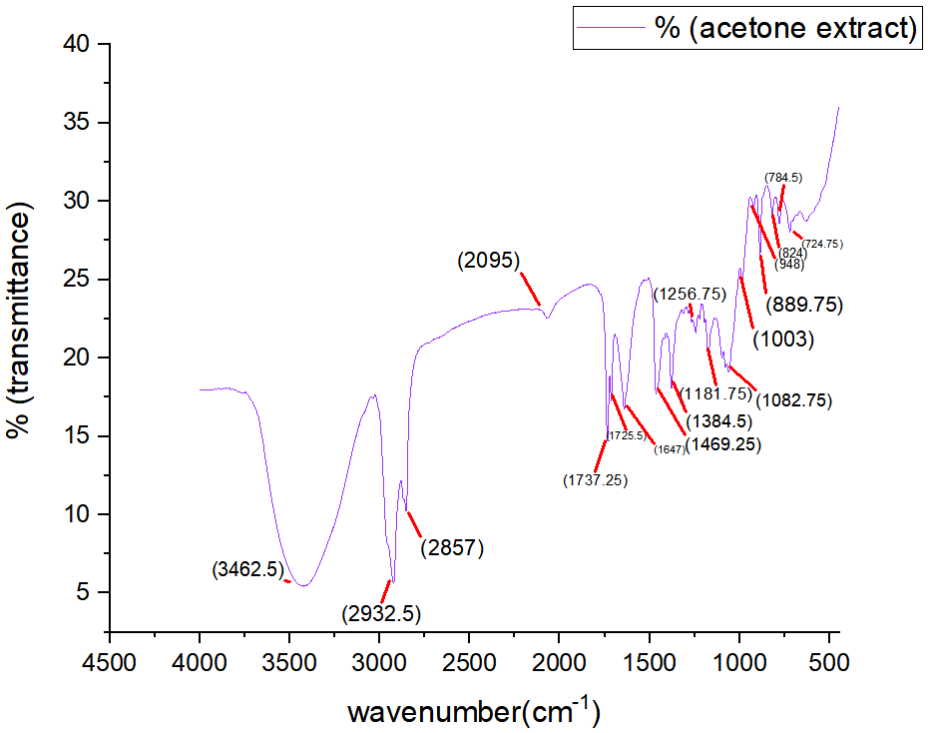


Figure 4.14 FTIR Spectrum of BP acetone extract

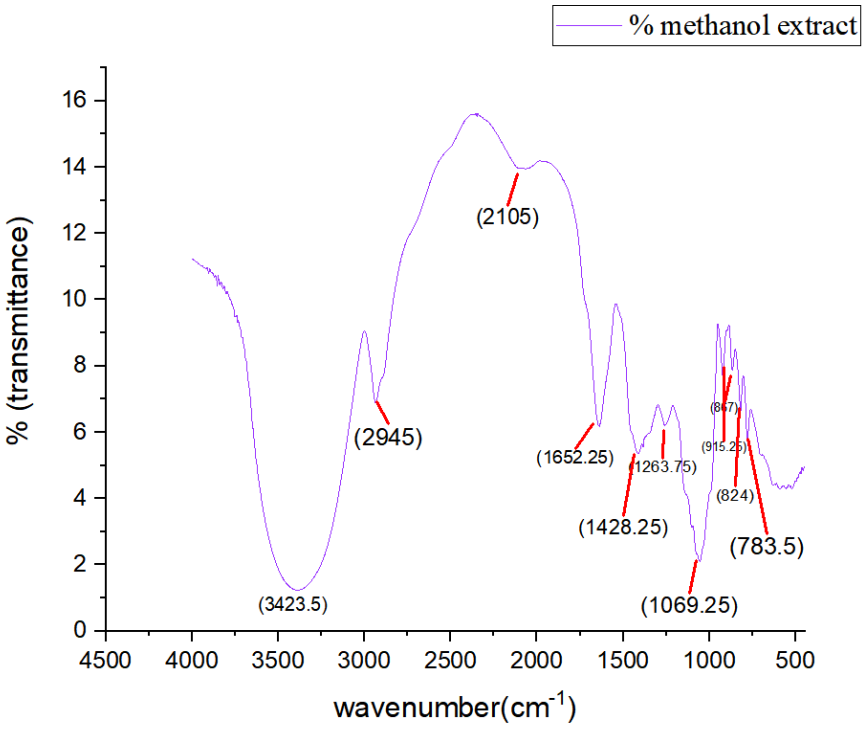


Figure 4.15 FTIR Spectrum of BP methanol extract

4.7 preservation effect of the banana peel extract

The extract preservative effect was tested on soybean oil. The potential preservative effect was evaluated based on the chemical analysis of rancidity parameters. This rancidity parameter is Peroxide Value (PV) which indicates the deterioration of the model foods, edible oil. Accelerated oxidation tests or Schaal Oven tests were conducted. Normally Schaal oven test is used for determination of oxidation of oils. The study was carried out for 8 days at a daily interval of 2 consecutive days. Every 2days the rancidity parameters, PV of the soybean oils were measured.

4.7.1 Peroxide value (PV)

The degree of primary oxidation of soybean oil was determined by measuring peroxide value (PV) in the presence and absence of antioxidants at 60°C for 8 days. As presented in table 4.18, the PV of all the treated samples increased with time. PV is a measurement of the concentration of hydro peroxides and peroxides that are created in the initial stages of lipid oxidation, represents primary reaction products. It is determined based by their ability to liberate iodine from potassium iodide (Ling *et al.*, 2015).

Table 4.18 Effect of BHT and banana peel extracts on peroxide value (meq/kg) of soybean oil

Time (days)	Control	0.05% BHT	0.1%acetone BP extract	0.2%acetone BP extract	0.1%methanol BP extract	0.2%methanol BP extract
0	3.86	3.86	3.86	3.86	3.86	3.86
2	14.91	9.02	12.30	11.13	11.70	10.24
4	28.96	16.14	21.78	18.7	19.3	18.26
6	39.13	20.10	35.61	33.07	34.03	32.2
8	54.73	27.07	49.57	47.78	48.34	46.98

The effect of the addition of banana peel extract on soybean oil's peroxide value is shown in Table 4.18. Compared with the control, the added antioxidant reduced the peroxide value of the oil. Initially, the peroxide value the oil was 3.86mEq/kg of soybean oil. From the results obtained, the peroxide value increased with storage time. For soybean oil, without the addition of BP antioxidant, the peroxide value significantly increased from 3.86 to 54.73 within 8 day of storage time. While oil samples treated with 0.1% and 0.2% of extracts have PV ranges from 3.86-49.57, and the positive control BHT range from 3.86-

27.07 within 8 days in both extracts. The PV of the extracts is relatively less than the negative control but BHT most effective compare to the extracts at storage time. Each sample with and without antioxidant has taken different induction time to reach at the targeted peroxide value of 20mEq /kg soybean oil. Induction time required for reaching the targeted peroxide value of control was 2 days after that it passes the targeted value, for BP extract treated with 0.1% acetone extract pass the targeted peroxide value after 2 days, for methanol extract it was 4 days. The BP extract treated with 0.2% both extracts pass the targeted peroxide value after 4 days and for BHT it was 4 days similar to the BP extract treated with 0.2% but the PV of BHT is much less. This show that oxidative stability of oils decreased with storage conditions.

In this test, the oxidation of oils is accelerated by keeping the oil at 60°C. By increasing the temperature of storage, the oxidation rate is increased (accelerated). One day of storage at this condition is equal to one month of storage on ambient temperature (Terrence al., 2018). Therefore, in the current study can conclude that 0.2% BP extract treatment has longer shelf life than the control sample in 2 months. So BP extract can be effective used as a preservative.

CHAPTER FIVE

CONCULSION AND RECOMMANDTION

5.1 Conclusion

In both organic solvents extraction conditions (solvent ratio, temperature and time) increased from lower to higher level. The extraction yield was increasing, as extraction conditions increased from lower to high level. The extraction parameter of solvent ratio, extraction time and extraction temperature had significant effect on the extract yield of banana peel extracts. It was confirmed that the factors solvent ratio, extraction temperature and time, investigated in this study do affect the yield of extract from banana peel individually. In addition, extraction yield was influenced by interactive extraction conditions; increasing the engagement of extraction conditions from low to high levels improved extraction yield.

Extraction yield was positively affected by linear effects for both solvents (acetone and methanol) BP extracts. Extraction yield was negatively affected by all quadratic (pure and interaction quadratic) effects for acetone and methanol BP extracts. It is known that significant model terms on extract yield are the individual variables and their interaction effects. The maximum yield obtained after conducting the experiment at selected optimum condition was 24.63 at 81.92v/v, 55.69 ºC and 3.79 hrs. for acetone and 32.46 at 88.56 v/v, 53.35 ºC and 2.62 h for methanol extract with a high value of combined desirability.

Banana peel extract using methanol as a solvent has more phenolic compounds (phenol and flavonoid contents) compared to banana peel acetone extract. In addition, the methanol extract had higher DPPH radical scavenging activity than banana peel acetone extract.

A peroxide value of control treatments (without extract) has increased for oil during the storage. Both treatments of 0.1 and 0.2% banana peel extracts significantly retard the values of these oxidative quality parameters of oil. The positive control (BHT) most effective compare to the BP extracts at storage time. The addition of 0.1% and 0.2% banana peel extract has the beneficial effect of regulating the oil's oxidative stability.

Finally, it can be concluded that banana peel contains significant amount of phenolic compounds (phenol and flavonoid contents) also its shows higher antioxidant activity that was measured by ability to reduce free radical scavenging method. So that antioxidant extracted from banana peel can be a strong source of natural antioxidants may substitute synthetic antioxidants plus its preservative effect on soybean oil by preliminary test by

measuring peroxide value was effective.so can be used to prolong the storability of food items.

5.2 Recommendation

The following study is recommended based on these research findings.

- In this research, the variety of banana used is Musa Cavendish; further analysis should be done on the other varieties, also the Antimicrobial potential of the banana peels extract should be studied.
- Since the phenolic compounds in banana peel vary, different extraction conditions are needed for complete extraction of phenolic compounds from banana peel.
- further studies should be conducted on the banana pulp too, to evaluate the presence of other bioactive compounds, including the evaluation of their antioxidant activity of the phenolic compounds.
- In future study, the Antioxidant activity of banana peel extract could also be determined using other antioxidant activity evaluation methods like ferric reducing ability of plasma (FRAP) assay and Rancimat methods.
- The preservative capacity of banana peel extracts can also be tested on various food products like butter, cheese, salad, and meat for further work.

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APPENDIXES

Appendix A: Raw data for characterization of antioxidants from banana peel by UV Spectrophotometer

A. For Total Phenolic Content

Table A1 Gallic acid standard solution preparation and corresponding absorbance

Concentration (mg/ml)	Gallic acid(µl)	Methanol (µl)	FC (ml)	Na_2CO_3 (ml)	Distil water (ml)	Absorbance	Absorbance Mean ± SD
0	0	1000	2	2	10	0.0143	
1.0	20	980	2	2	10	0.0781	
1.0	20	980	2	2	10	0.0824	0.0790±0.002
1.0	20	980	2	2	10	0.0768	
2.0	40	960	2	2	10	0.1736	
2.0	40	960	2	2	10	0.1690	0.1675±0.0053
2.0	40	960	2	2	10	0.1599	
4.0	80	920	2	2	10	0.2975	
4.0	80	920	2	2	10	0.3153	0.2992±0.0104
4.0	80	920	2	2	10	0.2848	
6.0	120	880	2	2	10	0.4613	
6.0	120	880	2	2	10	0.4325	0.4558±0.0160
6.0	120	880	2	2	10	0.4736	
8.0	160	840	2	2	10	0.6217	
8.0	160	840	2	2	10	0.5523	0.6025±0.0347
8.0	160	840	2	2	10	0.6335	
10.0	200	800	2	2	10	0.7438	
10.0	200	800	2	2	10	0.7296	0.7358±0.0142
10.0	200	800	2	2	10	0.7340	
12.0	240	760	2	2	10	0.9402	
12.0	240	760	2	2	10	0.8839	0.9176±0.01
12.0	240	760	2	2	10	0.9287	

Table A2 Sample solution preparation and corresponding absorbance for BP acetone extract

Concentration (mg/ml)	Gallic acid(µl)	Methanol (µl)	FC (ml)	Na_2CO_3 (ml)	Distil water (ml)	Absorbane	Absorbance Mean ± SD
0	0	1000	2	2	10	0.0143	
1.0	20	980	2	2	10	0.1769	
1.0	20	980	2	2	10	0.1942	0.187±0.0101
1.0	20	980	2	2	10	0.1899	
2.0	40	960	2	2	10	0.0447	
2.0	40	960	2	2	10	0.0524	0.0493±0.0015
2.0	40	960	2	2	10	0.0508	
4.0	80	920	2	2	10	0.1161	
4.0	80	920	2	2	10	0.1347	0.119±0.0104
4.0	80	920	2	2	10	0.1062	
6.0	120	880	2	2	10	0.1627	
6.0	120	880	2	2	10	0.1530	0.1586±0.0205
6.0	120	880	2	2	10	0.1601	
8.0	160	840	2	2	10	0.1953	
8.0	160	840	2	2	10	0.1865	0.1947±0.0158
8.0	160	840	2	2	10	0.2023	
10.0	200	800	2	2	10	0.2129	
10.0	200	800	2	2	10	0.2218	0.2160±0.0443
10.0	200	800	2	2	10	0.2133	
12.0	240	760	2	2	10	0.2941	
12.0	240	760	2	2	10	0.3125	0.2975±0.0184
12.0	240	760	2	2	10	0.2859	

Table A3 Sample solution preparation and corresponding absorbance for BP methanol extract

Concentration (mg/ml)	Gallic acid(µl)	Methanol (µl)	FC (ml)	Na_2CO_3 (ml)	Distil water (ml)	Absorbane	Absorbance Mean ± SD
0	0	1000	2	2	10	0.0143	
1.0	20	980	2	2	10	0.0253	

1.0	20	980	2	2	10	0.0182	0.0215±0.019
1.0	20	980	2	2	10	0.0210	
2.0	40	960	2	2	10	0.0435	
2.0	40	960	2	2	10	0.0529	0.0452±0.0137
2.0	40	960	2	2	10	0.0392	
4.0	80	920	2	2	10	0.0912	
4.0	80	920	2	2	10	0.10	0.0989±0.0077
4.0	80	920	2	2	10	0.1055	
6.0	120	880	2	2	10	0.1446	
6.0	120	880	2	2	10	0.1391	0.1426±0.0028
6.0	120	880	2	2	10	0.1441	
8.0	160	840	2	2	10	0.1992	
8.0	160	840	2	2	10	0.1928	0.1938±0.0044
8.0	160	840	2	2	10	0.1894	
10.0	200	800	2	2	10	0.2093	
10.0	200	800	2	2	10	0.2140	0.2224±0.0215
10.0	200	800	2	2	10	0.2439	
12.0	240	760	2	2	10	0.2834	
12.0	240	760	2	2	10	0.3125	0.2975±0.024
12.0	240	760	2	2	10	0.2966	

B. For Antioxidant activity determination

Table A4 Ascorbic acid standard solution preparation and the corresponding absorbance

Concentration (mg/ml)	Ascorbic acid(µl)	Methanol (µl)	DPPH (ml)	Absorbance	Absorbance Mean ± SD
0	0	1000	4	0.5211	
1.0	20	980	4	0.4107	0.4206±0.012
1.0	20	980	4	0.4326	
1.0	20	980	4	0.4107	
2.0	40	960	4	0.3580	0.3193±0.0387
2.0	40	960	4	0.2988	
2.0	40	960	4	0.3011	

4.0	80	920	4	0.1319	0.120±0.0119
4.0	80	920	4	0.1020	
4.0	80	920	4	0.1261	
6.0	120	880	4	0.0403	
6.0	120	880	4	0.0381	0.0395±0.0014
6.0	120	880	4	0.0401	
8.0	160	840	4	0.0195	
8.0	160	840	4	0.0172	0.0185±0.003
8.0	160	840	4	0.0188	
10.0	200	800	4	0.0167	
10.0	200	800	4	0.0204	0.0189±0.0015
10.0	200	800	4	0.0196	
12.0	240	760	4	0.0190	
12.0	240	760	4	0.0289	0.0194±0.0095
12.0	240	760	4	0.0103	

Table A5 Preparation of DPPH absorbance for BP acetone extract

Concentration (mg/ml)	Standard (μL)	Methanol (μL)	DPPH (ml)	Absorbance	Absorbance Mean ± SD
0	0	1000	4	0.5211	
1.0	20	980	4	0.5193	
1.0	20	980	4	0.4726	0.4837±0.0356
1.0	20	980	4	0.4592	
2.0	40	960	4	0.4322	
2.0	40	960	4	0.4153	0.4098±0.0279
2.0	40	960	4	0.3819	
4.0	80	920	4	0.3284	
4.0	80	920	4	0.2792	0.2916±0.0368
4.0	80	920	4	0.2672	
6.0	120	880	4	0.2486	
6.0	120	880	4	0.1994	0.2046±0.0386
6.0	120	880	4	0.1660	

8.0	160	840	4	0.1213	
8.0	160	840	4	0.1038	0.1085±0.0128
8.0	160	840	4	0.1004	
10.0	200	800	4	0.1325	
10.0	200	800	4	0.1194	0.1098±0.0323
10.0	200	800	4	0.0775	
12.0	240	760	4	0.1249	
12.0	240	760	4	0.1552	0.1352±0.02
12.0	240	760	4	0.1255	

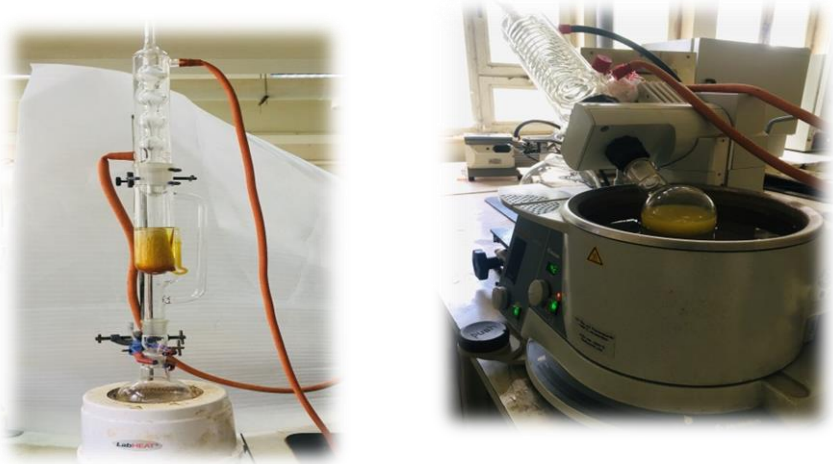
Table A6 Preparation of DPPH absorbance for BP methanol extract

Concentration (mg/ml)	Sample (µl)	Methanol (µl)	DPPH (ml)	Absorbance	Absorbance Mean ± SD
0	0	1000	4	0.5211	
1.0	20	980	4	0.5245	
1.0	20	980	4	0.5125	0.5111±0.0148
1.0	20	980	4	0.4963	
2.0	40	960	4	0.4586	
2.0	40	960	4	0.3857	0.4021±0.0565
2.0	40	960	4	0.3620	
4.0	80	920	4	0.2056	
4.0	80	920	4	0.1632	0.1852±0.022
4.0	80	920	4	0.1868	
6.0	120	880	4	0.0687	
6.0	120	880	4	0.0546	0.0568±0.0097
6.0	120	880	4	0.0471	
8.0	160	840	4	0.0548	
8.0	160	840	4	0.0496	0.0531±0.0035
8.0	160	840	4	0.0549	
10.0	200	800	4	0.0493	
10.0	200	800	4	0.0536	0.0536±0.0043
10.0	200	800	4	0.0579	

12.0	240	760	4	0.0610	
12.0	240	760	4	0.0589	0.0606±0.0017
12.0	240	760	4	0.0619	

Appendix B: Some important photo during experimental session

Figure B1 Preparation of banana peel powder



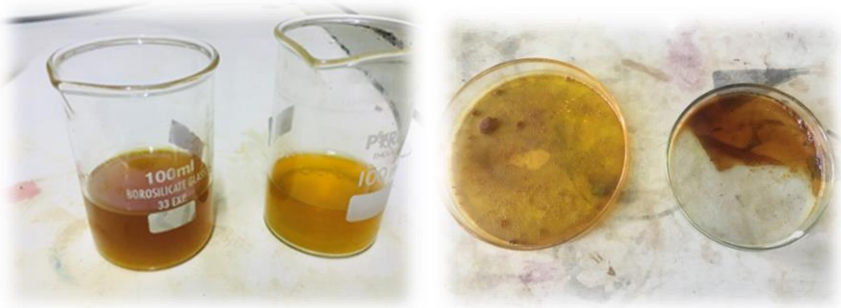


Figure B2 Extracted sample Preparation of banana peel

Figure B3 Testing antioxidant potential of BP extracts Extract