EXTRACTION AND CHARACTERIZATION OF AVOCADO SEED (*PERSEA AMERICANA*) EXTRACT FOR BIOACTIVE PACKAGING FILM DEVELOPMENT

NUREDIN SEID ALI

A thesis submitted to the Department of Chemical Engineering

School of Mechanical, Chemical, and Material Engineering

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Chemical Engineering (Process Engineering)

Office of Graduate Studies

Adama Science and Technology University

February, 2023

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DECLARATION

I hereby declare that this Master Thesis entitled “Extraction and characterization of avocado seed (*Persea Americana mill*) extract for bioactive packaging film development” is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation

NUREDIN SEID ALI _____ _____

Name of the student Signature Date

RECOMMENDATION

I the advisor of this thesis, hereby certify that I have read the revised version of the thesis entitled "Extraction and characterization of avocado seed (Persea Americana mill.) extract for bioactive packaging film development" prepared under my guidance by NUREDIN SEID ALI submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Chemical Engineering (Process Engineering). Therefore, I recommend the submission of revised version of the thesis to the department following the applicable procedures.

_____ _____ _____

Major Advisor Signature Date

_____ _____ _____

Co-advisor Signature Date

APPROVAL

We, the advisors of the thesis entitled **"Extraction and characterization of avocado seed (*Persea Americana mill.*) Extract for bioactive packaging film development"** and developed by Nuredin Seid Ali, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

P. Selvakumar (PhD) _____ _____

Major Advisor Signature Date

Zelalem Tumsa(PhD) _____ _____

Co-advisor Signature Date

We, the undersigned, members of the Board of Examiners of the thesis by Nuredin Seid have read and evaluated the thesis entitled Extraction and characterization of avocado seed (Persea Americana mill.) extract for bioactive packaging film development and examined the candidate during the open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Chemical Engineering (Process Engineering).

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Chairperson Signature Date

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Internal Examiner Signature Date

Dr. Wondalem Misganaw _____ 03-04-2023

External Examiner Signature Date

Finally, approval and acceptance of the thesis is contingent upon submission of its final

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ACKNOWLEDGEMENT

All special thanks and praises to Almighty God (A.S.W). This paper is fully dedicated to the in loving memory of my mom. (Worknesh A.), who left us far too soon. I would like to convey my deepest gratitude to my principal advisor P. Selvakumar (PhD) and Co-advisor Zelalem Tumsa (PhD); for their guidance and help in all the time of research and writing of this thesis. Their communicative enthusiasm, motivation, and encouragements have been a great help. I express my great thanks to my family and my friends for their moral support and love during the time of inconvenience and my work.

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LISTS OF ABBREVIATIONS AND ACRONYMS

AAE	Ascorbic acid equivalent
ASE	Avocado Seed Extract
BH	Butylated Hydroxyl Anisol
BT	Butylated Hydroxyl Tolune
DPP	2, 2-diphenyl-1-picrylhydrazyl
DW	Dry weight
FCR	Folin-Ciocalteu Reagent
FTIR	Fourier Transform Infrared
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography- mass spectroscopy
HPLC	High Performance Liquid Chromatography
OH•	Hydroxyl radicals
RSA	Radical scavenging activity
Std. Dev.	Standard deviation
TPC	Total phenol content
CSA	Central Statistics Agency

ABSTRACT

A new generation of packaging films is now being developed specifically for the incorporation of antimicrobials, antioxidants, enzymes, and functional additives. These active films bioactivity as antioxidants or antimicrobials was the main focus of the research. On the other side, fruit and vegetable wastes have a wide range of biological activities related to their composition, including antioxidant, anticancer, antibacterial, anti-inflammatory, and anti-hypertensive properties. So, valorization of extract of avocado seed (ASE) into bioactive packaging film was the focus area of the study. Under this work the effects of extraction conditions on the Soxhlet extraction of polyphenolic compounds from avocado seed (Persea Americana mill.) and the development of bioactive packaging film from gelatin polymers and avocado seed extract (a bioactive component) were investigated. Dried avocado seed extract (ASE) was extracted using different conditions, including ethanol concentration (0%–100%), sample-to-solvent ratio (1:8.3–1: 16.6 g/ml), extraction temperature (60–90 °C), and time (2–8 h). The effects of the parameters on total phenolic content (TPC) and radical scavenging activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) were examined via a single-factor design experiment. The results showed all extraction conditions to have significant effects on the ASE yield of extract and antioxidant activities. The optimum extraction conditions of 100% ethanol, 1:12.5 g/mL solid-to-solvent, at 70 °C for 4 hours favored ASE polyphenolic potential and higher antioxidant activity. The maximum amounts for extract yield, total phenolic content, and radical scavenging activity were 14.30 ± 0.20 %, *64.61* ± 0.72 *mg GAE/g extract, and 71.65% respectively.* IC_{50} *value is also 1.97 mg/ml. In addition, the gelatin film-based bioactive film containing different concentration (0, 1, 3, 5, 7, and 10 wt. %) of ASE was successfully prepared by the solution casting technique. The incorporation of ASE into gelatin films increased the performance and radical scavenging activity from 13.49% to 60.29% at the highest percentage of ASE (10 wt. %). The water solubility of ASE incorporated gelatin-based film improved from 100% to 36.4%. Therefore, this study showed that gelatin film incorporated with avocado seed extract is a potential material for developing active packaging film, as it boasts superior antioxidant characteristics.*

Key words: Radical scavenging activity, DPPH, total phenolic content (TPC), Gallic acid equivalent, Avocado seed extract, crude extract.

CHAPTER ONE

1. INTRODUCTION

1.1 Background of the Study

Recently, fruit waste has become one of the main sources of municipal solid wastes, which have been a great environmental threat. One of the solutions to this problem is to use fruit wastes as a source of valuable compounds - the bioactive constituents, especially phenolic compounds, and use them in the food, pharmaceutical, as well as cosmetics industry. In general, these by-products would be discarded as waste, which would to some extent increase the burden on the environment and economic development (Mirabella, Castellani, & Sala, 2014). However, most by-products especially plant-derived food by-products (seeds and peels) contain abundant high added-value substances that deserve our attention. In recent years, numerous efforts have been put into the reutilization of these wastes as developing novel functional foods, supplements and active packaging (Comunian, Silva, & Souza, 2021; Grasso, 2020; Jiang, Zhang, Li, Shu, et al., 2021).

Natural bioactive compounds are classified as polyphenols, triterpenes and phytosterols, terpenoids carotenoids, alkaloids and others (Makkar et al.2021). They are also extracted from fungi, animals and bacteria (Camara et al.2020), as well as from agro-industrial residues such as avocado peel, avocado seed, mango seeds and grape peels (Shirahigue and Antonini 2020). They manifest antioxidant, ant-allergic, anti-inflammatory, antimicrobial and antimutagenic properties and are essential for the human body. Moreover, they are also vital in the pharmaceutical, food and chemical industries (Camara et al. 2020). Among the above-mentioned fruits and vegetables avocado seeds represent about 20% from its mass and are considered as waste at a volume more than one million tons per year. According to numerous studies (Arajo et al., 2018; Figueroa et al., 2018; Rosero et al., 2019), these extracts have a wide range of biological activities related to their composition, including antioxidant, anticancer, antibacterial, anti-inflammatory, and anti-hypertensive properties. According to CSA, 2019, with a yield of 4.2 tons/ha, 84,793.7 tons of avocados were produced in Ethiopia on an estimated 19,758.75 ha of land. Among this around 20 percent (16,958.74 tons) of this potential is classified as a seed component and is disposed of as waste to the environment without any benefits.

Diverse types of extraction strategies have been employed over the years, considering properties such as the bioactive compounds and solvents used, as well as the effect of temperature, time, yield and selectivity of extraction (Azmir et al.2013). Conventional methods of extraction include maceration and extraction. laboratory scale Soxhlet elevated temperature is used to recirculate solvent with in an apparatus and to aid the extraction of compounds from samples placed in the thimble (Muala et al. 2021, Raynie et al.2019)

Active packaging films with enhanced functional attributes can be created by incorporating other types of natural ingredients within them, such as antioxidants, antimicrobials, colorants, flavourants, or nutraceuticals (Umaraw et al. 2020). Well-known biopolymers that are frequently utilized as raw materials for biodegradable films include starch, proteins, gums, and derivatives of cellulose, as well as gums, lipids, and proteins. One of the main biopolymers used for the preparation of biodegradable films is gelatin, a high molecular weight polypeptide, which is naturally present in different animal sources, including pig, cattle, and fish skin (Pahoff et al., 2019). Extracts that rich in phenolic compounds obtained from pomegranate peel could be incorporated into edible packaging film, the preservation effects of which would be improved (Gull et al., 2021). Therefore, it's critical to create bioactive components from this waste material and incorporate them into a bioactive film in order to transform this waste into a useful product.

Gelatin is one of the most interesting biomaterials for the development of biopolymers, mainly due to its good film-forming properties and abundance in nature. However, its hydrophilic characteristics result in a rapid degradation process as the hydrogen bonds easily rupture in humid environments and temperatures above 30–35 °C, destroying the physical network of gelatin (Rosseto et al., 2021; Rigueto et al., 2021). Nevertheless, the addition of other compounds to the gelatin network makes it possible to obtain materials with improved characteristics that overcome the limitations of gelatin (Rigueto et al., 2021). The objective of this study was to investigate how different process variables affected the extraction of avocado seed extract in order to identify the ideal conditions for producing extracts with the highest levels of bioactive component (TPC) and antioxidant activity and to evaluate the antioxidant activity of films made from gelatin polymer and avocado seed extract.

1.2 Statement of the Problem

Oxidative reactions in foods represent a serious deteriorative process, leading to significant waste. Oxidative reactions promote discoloration and the development of rancidity and off flavors, negatively affecting the appearance, nutritional value, and quality of foodstuffs. To overcome these problems, the food industry often uses synthetic antioxidants, which have dubious health properties, to prevent these undesirable processes. So, new natural antioxidant compounds are necessary to control oxidative reactions in foods. Active packaging based on natural polymers such as proteins, lipids, and polysaccharides and their combinations has been pointed out as the most promising, eco-friendly solution. Using natural materials for the preparation of bioactive packaging films has many benefits, such as biocompatibility, thermostability, antimicrobial and antioxidant effects, etc. Packaging films incorporating different types of bioactive found in plants, such as fruit, leaves, seeds, stems, etc., have resulted in an improvement of the bioactive properties (antioxidant) of the biofilms. Bioactive components obtained from residues and byproducts of the food production chain are highly feasible since they are a cheap natural source of these compounds and have no economic value.

The gelatin polymer is a good film-forming material despite its low bioactivity and high solubility in water. So, to overcome those challenges, conducting research on non-edible parts of fruits with high polyphenolic compounds is an emerging trend to enhance the bioactive property of the gelatin packaging film and reduce the solubility of the film. The seed of the avocado is one of the underutilized, non-edible parts of the fruit, which are usually discarded as residue. The phenolic compounds present in the extracts of avocado seeds for the development of antioxidant packaging films could terminate the propagation reactions, induced by free radical intermediates, by reacting either directly with the free radicals or by preventing hydroperoxides from decomposing into free radicals. In this work, the bioactive components present in the crude extract of avocado seed will be analyzed. A gelatin polymer was utilized as a base matrix for the preparation of active packaging film incorporating the crude extract of avocado seed. In addition, its antioxidant activity was evaluated.

1.3 Research Questions

- What are the operating conditions that affect the amount of total phenolic content (TPC) and antioxidant activity of the extract?
- What is the composition of bioactive compounds available in the crude extracts of avocado seed?
- Do the bioactive compounds have an effect on the characteristics of bio active packaging film?

Avocado seed extract is selected as a bioactive material due to: The potential of high-level poly phenolic compounds responsible for antioxidant activity of the bioactive packaging film.

Hypothesis: The successful incorporation of avocado seed extract (Bioactive active component) with a gelatin polymer matrix and glycerol as a plasticizer may improve the antioxidant activity of the bioactive packaging film.

1.4 Objectives

1.4.1 General Objective

The main objective of this work is to extract and characterize crude extract from avocado seed (Persea Americana mill.) for developing the bioactive packaging film and evaluate its antioxidant activity.

1.4.2 Specific Objectives

The specific objectives of the study were to: -

- Extract and characterize avocado seed crude extract
- To investigate the effect of extraction conditions on the total phenolic content and evaluate the antioxidant activity of the crude extract of the avocado seed
- Develop the packaging film incorporating the crude extract of the avocado seed extract with the gelatin polymer
- Characterize the developed bioactive film in terms of antioxidant activity

1.5 Significance of the study

The most promising, environmentally acceptable alternative has been identified as active packaging based on natural polymers such proteins, lipids, and polysaccharides and their mixtures. The development of bioactive packaging films using natural materials has several advantages, including biocompatibility, thermostability, antibacterial and antioxidant properties, etc. The bioactive characteristics (antioxidant) of the biofilms have improved as a result of packaging films combining various forms of bioactive found in plants, such as fruit, leaves, seeds, stems, etc. It is extremely feasible to extract bioactive components from food production chain residues and byproducts because they are a cheap natural source of these substances and have little commercial value. Preparing a bioactive extract from avocado seeds and creating a bioactive packaging material using a gelatin polymer matrix and various proportions of avocado seed extract are important aspects of this study. The development of bioactive packaging using a gelatin polymer matrix and avocado seed extract increased the value of the raw material and enhanced the packaging material's antioxidant activity. The avocado seed's enhanced antioxidant capacity makes it a better alternative to synthetic packaging material for use in food packaging.

1.6 Scope of the Study

The following points are used to explain the scopes of this research. This research is mainly focus on the: -

- Extracting of bioactive compounds from Avocado seed biomass based on operating condition such as temperature, time, solvent and solid-to-solvent ratio
- Evaluating the antioxidant activity of the crude extract of the avocado seed by using total phenolic content (TPC) and DPPH (-2,2 Diphenyl-1-picryl hydroxyl radical) assay
- Developing the packaging film incorporating the crude extract of the avocado seed with the gelatin polymer by solution casting method
- Characterizing the developed bioactive film in terms of antioxidant activity

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Overview of Avocado Fruit (*Persea Americana* Mill.)

Avocado (Persea Americana Mill.) is a subtropical/tropical fruit native to Mexico and Central America, and widely produced and consumed worldwide. It belongs to the Lauraceae family and the genus Persea, of which there are more than 150 known species (Arajo, Rodriguez-Jasso, Ruiz, Pintado, & Aguilar, 2018). Avocados are mostly consumed fresh, but are also processed to extract oil and other products. However, several components of the fruit, including peel, seed, and defatted paste are commonly not used and are instead discarded, making them a source of environmental contamination. However, these components are rich in protein, fiber, and numerous bioactive compounds (Dalle Mulle Santos et al., 2016; Rodríguez Carpena, Morcuende & Estevez, 2011). An average avocado fruit weighs approximately 150 to 400 g, of which the pulp, seed, and peel weigh about 73 %, 16 %, and 11 %, respectively, although these proportions vary among cultivars. For example, the seed and peel of 'Hass' avocado represent about 15 % and 14 %, respectively, of a fruit's weight, while the seed and peel of 'Fuerte' avocado represent about 21 % and 8 %, respectively (Calderón-Oliver et al., 2016; Melgar et al., 2018; RodríguezCarpena, Morcuende, Andrade, Kylli, & Estévez, 2011).

Botanical Description

Scientific Name: Persea Americana Mill.

Family Name: Lauraceae

English Name: Avocado

Genus: Persea

Species: P. Americana

Part Used: Seed

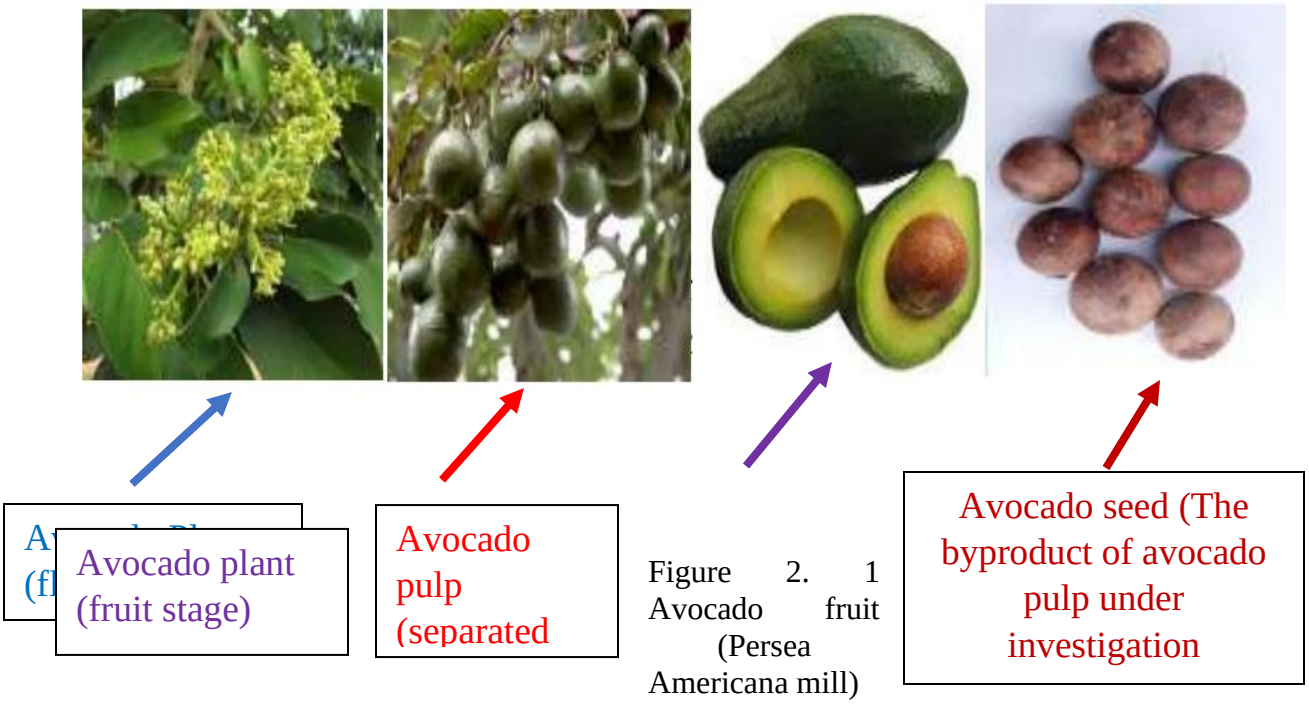


Figure 2. 1 Avocado fruit (Persea Americana mill)

2.1.1 Avocado Production Potential

The recent (FAOSTAT data of 2018) revealed that avocado is produced globally on 918, 531 ha and production of more than 6.4 million tons showing about the productivity of 7 tons per ha. About six million tons of avocados are produced annually around the world, with 62 % harvested from five main countries; Mexico (33 %), the Dominican Republic (10.5 %), Peru (7.8 %), Indonesia (5.7 %) and Colombia (5.1 %) (FAOSTAT - Food and Agriculture Organization of the United Nations, 2020). Avocado was first introduced to Ethiopia in 1938 by private orchardists in Hirna and Wondo-genet and production gradually spread into the countryside where the crop was adapted to different agro-ecologies (Edossa, 1997; Woyessa and Berhanu, 2010; and Zekarias, 2010). Avocados are second in total volume of production, next to banana, in Ethiopia (Joosten, 2007). In Ethiopia, currently, 84,793.7 tons of avocado was produced on an estimated area of 19,758.75 ha of land with a productivity of 4.2 tons/ha. Horticultural fruits generally have about a 2% proportion of the total crops. And Oromia region shares 34% area coverage and production contributing 34.6% in Ethiopia (CSA, 2019)

2.1.2 Physico-Chemical Characteristics of Avocado Fruit

An average avocado fruit weighs approximately 150 to 400 g, of which the pulp, seed, and peel weigh about 73 %, 16 %, and 11 %, respectively, although these proportions vary among cultivars. For example, the seed and peel of 'Hass' avocado represent about 15 % and 14 %, respectively, of a fruit's weight, while the seed and peel of 'Fuerte' avocado represent about 21 % and 8 %, respectively (Calderón-Oliver et al., 2016; Melgar et al., 2018; RodríguezCarpena, Morcuende, Andrade, Kylli, & Estévez, 2011). This is equivalent to at least 1.6 million tons of annually discarded avocado seeds and peels globally, in addition to other related industrial waste.

As for the physical characteristics to identify the different varieties of the avocado fruit, each is popular for its shape, color, skin, and size (Salunkhe and Kadam, 1995).

Table 2. 1 presents the Physical characteristics of the four selected varieties

Varieties	**Shape**	**Average Wt.(g)**	**Skin**	**Seed Size(cm)**
Ettinger	Pear, longer and broad neck	150-300	Smooth, medium glossy and green	12-15
Fuerte	Pear	250-400	Green and slightly pebbled	10-12
Hass	Oval	120-200	Dark green bumpy	6.5-9
Reed	Round	300-400	Medium to thick and easy peeling	10-11

Source: (Salunkhe and Kadam, 1995).

2.2 Characteristics of Avocado (*Persea americana* Mill.) Seed

The avocado industrial processing unit generates large amounts of peels and seeds (Segovia F J et.al 2018). The seeds are underutilized as a non-edible part of the fruit and discarded as wastes

(Adaramola B et.al 2016). Conducting research on non-edible parts of fruits is an emerging trend, which may prove to be very profitable in the near future. Mostly, because it involves an important reduction in the production of wastes and the fact that the non-edible parts of many fruits like avocado have high levels of valuable bioactive, particularly natural antioxidants (Oluwaniyi O, Jimenez P et.al 2017,2020).

2.2.1 Phyto-chemical composition of Avocado Seed

Avocado by-products are rich sources of carbohydrates (seed 42-81 %, peel 43-81 %), lipids (seed 3-15 %; peel 2-9 %), proteins (seed 0.14-9 %; peel 0.17-8 %), fibers (seed 2-4.2; peel 1.3-55 %), minerals (seed 1.3-4.3 %; peel 1.5-6.0 %), and various other bioactive compounds (Araújo et al., 2018). For example, hydroxycinnamic acids, hydroxybenzoic acids, organic acids, phenolic-alcohol derivatives, flavonoids, proanthocyanins, terpenoids, alkaloids, saponins, acetogenins, phytosterols and other polar and non-polar compounds have been identified.

2.3 Bioactive compounds in avocado seed

Bioactive compounds are chemicals separated from plants. The components of avocado seed are protein, sugar, starch, fat, and water. The bioactive compounds found in avocado are phenolics, flavonoids, carotenoids, vitamin C, and vitamin E (Vinha A F et.al 2013). Table 1 presents the composition of phytochemicals in the avocado seed. Phytochemicals present in avocado seed include flavonoids, tannins, saponins, phenolics, antioxidant capacity, oxalates, phytates, and alkaloids (Bahru T B 2019).

Table 2. 2 Compositions of phytochemicals in avocado seed (Oluwaniyi O, 2017, Bahru T B 2019)

No.	Phytochemicals	Content (mg/100g)
1	Flavonoids	20.33 $\pm$ 0.01
2	Tannins	0.76 $\pm$ 017
3	Saponins	0.52 $\pm$ 0.42

4	Oxalates	4.4 ± 0.3
5	Phytates	0.44 ± 0.01
6	Alkaloids	5.4 ± 0.00

Table 2 lists the polyphenols in the avocado seed. The other polyphenol compounds found in avocado seeds are 3-O-caffeoylquinic acid, 3-O-p-coumaroylquinic acid, procyanidin trimer A(I), procyanidin trimer A(II), catechin, or epicatechin gallate (Dabas D et.al 2013). Other research stated that avocado seeds have tannins, saponins, and flavonoids, which are parts of polyphenols (Henry L N et.al 2015)

Table 2. 3 The identified polyphenols in avocado seed (Henry L N et.al 2015, Jimenez P.et al,2020)

No.	**Phenolic Compound**	**Content (mg/100g)**
1	Catechin	24.3-2,000
2	Caffeic acid	13.7-22.5
3	Chlorogenic acid	0.0516-1,953
4	(Epi)catechin	1,106-2,906
5	Ferulic acid	0.09-1.2
6	Kaempferol	10.74
7	Kaempferide	10.74

8	Procyanidins	152-5,560
9	Rutin	0.22
10	Trans-5-O-caffeoyl-D-quinic acid	163-574
11	Vanillic acid	286

2.3.1 Bioactive Compounds Present in different types of plant origin

In recent years, bioactive compounds have attracted the attention of scientists, researchers and the world's population, as their consumption has been associated with beneficial effects on physical and mental health because they provide crucial biological effects in the prevention and treatment against a wide range of diseases. Some bioactive compounds have antimicrobial (Zahan et al.2020, Jafarzadeh et al. 2020), antioxidant (Taghavi Kevij et al.2020), anticancer, anti-inflammatory and/or anti-neurodegenerative activities (Sakthivel et al.2019)

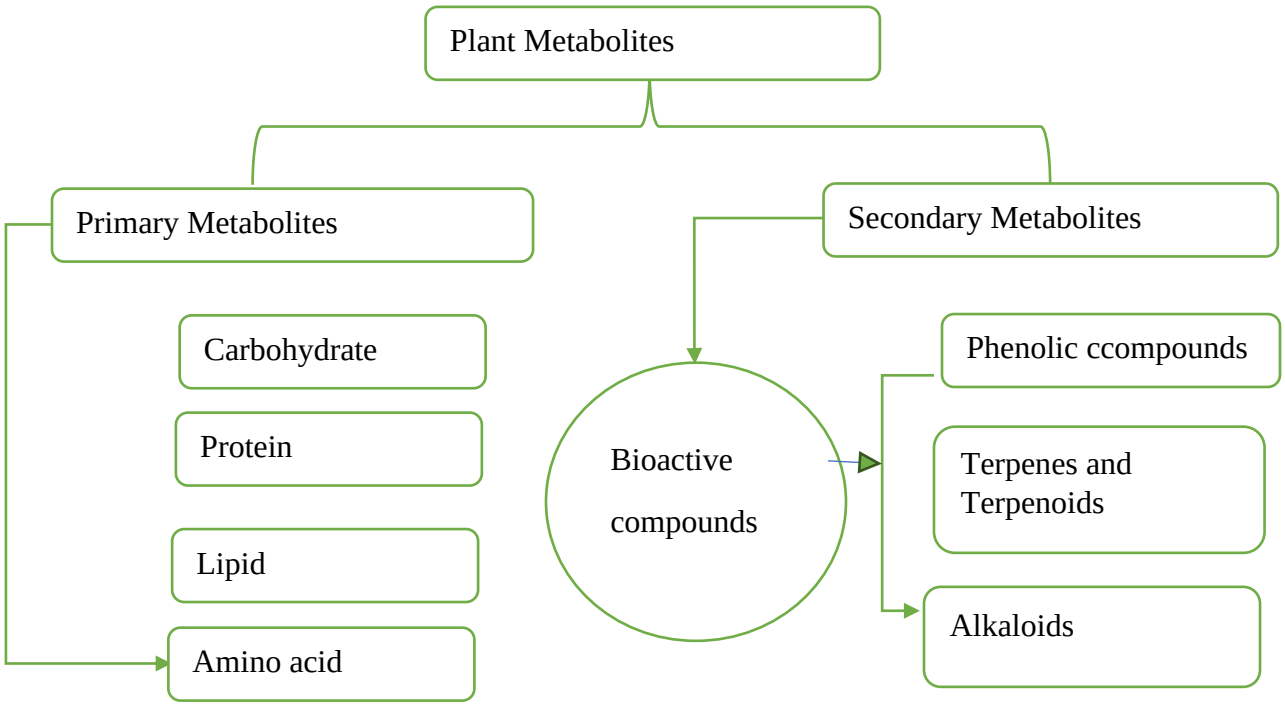


Figure 2. 2 Classifications of plant metabolites

Plant-derived bioactive compounds are being considered interesting ingredients for the production of biodegradable and bioactive films due to their natural origin and functionality (Jafarzadeh et al. 2020). Studies have shown that the incorporation of plant extracts and fruit pulps as sources of bioactive compounds or isolated bioactive compounds into film-forming solutions cause antioxidant and antimicrobial effects on the resulting films, extending their application in bioactive and biodegradable films or packaging (Chen et al. 2019, Genskowsky. et al.2015).

2.3.2 Bioactive Compounds from Fruit/Vegetable Processing Wastes and By-Products

From the available research data, it is evident that much of the wastes and by-products of fruit industry arises after pressing the juice or after producing value-added products. Non-edible parts of fruits such as peels or skin portion and twigs often contain higher amounts of bioactive compounds when compared to the edible parts. For example, peels of apple, grapes, citrus fruits

and seeds of jackfruit, avocado and mango, are reported to have more than 15% higher content of polyphenolic compounds than pulp (Soong et al.2004).

Besides, fruits after the production of beverages (in the food industry) generates huge volume of wastes, which are in the form of pomace (a mixture of pulp, skin, seeds, and stem). Owing to high perishability of the pomace, severe technical and environmental problems are incurred (Majerska et al.2019).

Table 2. 4 Bioactive compounds from popular fruits processing wastes and by-products

Fruit	**Waste Type**	**Bioactive compound**		**Main compounds**	**Reference**
		Class	**Concentration mg/100g**		
Apple	Pomace	Phenolic acid	523-1542	Chlorogenic acid Caffeic acid Ferulic acid p-coumaric acid Sinapic acid p-coumaroyl- quinic acid	Barreira et al.2019
		Flavonoids	2153-3734	Isorhamnetin Kaempferol Quercetin Rhamnetin glycoconju- gates Procyanidin B2 (−)-Epicatechin	
		Anthocyanins	50-150	Cyanidin-3-O- galactoside	
		Flavones	1659 (Lemon) 55 (Orange)	Apigenin- glucoside Diosmetin- glucoside	

		Flavanones	10646 (Lemon) 22298(Orange)	Eriocitrin Hesperidin Narirutin	
	Seed	Limonoids	375 (Lemon) 114 (Orange)	Limonin Nomilin Obacunone Ichangin	
		Phenolic acids	N. A	Gallic acid and its derivatives	
		Flavonoids	7200–13000	Quercetin	
Mango	Seed Kernel			Isoquercetin	
				Fisetin	
		Catechins	N. A	Epicatechin	
				Epigallocatechin	
				Epicatechin gallate	
		Phenolic acids	99.5	Ferulic acid p-Coumaric acid Caffeic acid Sinapic acids	
		Flavonols	1019.6	Rutin, Quercetin Kaempferol Myricitin Laricitrin	
Banana	Peel	Catechins	N. A	Catechin Epicatechin Gallocatechin	Vu et al.2018
		Catecholamines	4720	Dopamine, L- dopa	

Elderberry	Branche waste	Phenolic acids	45,600	Chlorogenic acid	Silva et al.2017
		Flavonols	468,200	Quercetin and its glycoconjugates	
		Anthocyanins	2530	Cyanidin and its glycoconjugates	

2.4 Extraction methods of different bioactive compounds

As phenolic compounds are found in all plants, almost all research related to the extraction of phenolic compounds focuses on bioprospecting for new plant varieties to serve as sources of these compounds. Phenolic compounds could be valuable components of products that decrease the risk of cardiovascular and neurological diseases as well as cancer. Meanwhile, the antioxidant power of phenolic compounds suggests they could be used as natural additives to functional foods. For example, phenolic compounds from olive mill wastewater were able to prolong the shelf life of bakery products due to its antimicrobial properties (Charis M. et al. 2018). For instance, phenolic compounds are widely used as natural antioxidants and antimicrobial agents (Tanase et al. 2019). In the same way, (Basanta et al. 2018) developed a colored film containing phenolics compounds extracted from cherry that constituted a food preserving antioxidant barrier.

2.4.2 Conventional extraction methods

Phenolic compounds have been extracted for decades using conventional extraction methods such as Soxhlet, maceration, infusion, and digestion (Wong-Paz et al. 2017). Soxhlet extraction and maceration are the most common techniques. For example, the extraction of phenolic compounds using maceration and Soxhlet from grape skin (Caldas et al. 2018), Vernonia cinerea leaves (Alara et al. 2018a, b, c) and feijoa peel (Henrique et al. 2019), produces a total phenolic content (TPC) of between 48.6 and 71 mg of gallic acid equivalent (GAE) per gram.

2.4.2.1 Soxhlet Extraction

Soxhlet extraction and maceration generally use high solvent/feed (S/F) ratios (above 20) and long extraction times for exhaustive extraction of all compounds from a matrix. Thus, these methods are commonly used as models to compare the efficiencies of alternative methods. Extraction times of up to 360 and 720 min have been used for extraction of phenolic compounds using Soxhlet extraction and maceration, respectively.

In Soxhlet extraction, a dry sample is placed in a thimble. The thimble and the solvent are then placed in a distillation flask, which is heated to evaporate the solvent. The condensate and reflux return to the thimble-holder until they reach an overflow level and can be aspirated by a siphon. The compounds are extracted to the bulk liquid. As the flask is continuously heated, the solvent is continually refluxed and the compounds remain in the flask. The reflux process is repeated several times until extraction is complete (Azmir et al. 2013).

One of major advantages of Soxhlet extraction is that, unlike maceration, the matrix can be in contact with fresh solvent repeatedly and, as the sample is packed in a thimble, extract filtration is not necessary. However, as the extraction of phenolic compounds is performed using longer extraction times and higher temperatures, the bioactivity of the extracts is diminished due to the degradation of the compounds.

2.4.2.2 Maceration

In maceration, the raw material is extracted in a specific solvent for a period of time. Maceration can be performed with or without agitation. Contrary to Soxhlet extraction, maceration is performed using lower temperatures. For example, the extraction of phenolic compounds from oil mixtures by maceration is performed under optimized conditions at room temperature (Ji et al. 2018). Thus, the main advantages of maceration include that the process is performed using low temperatures and as the equipment required is not complicated, the process is inexpensive. However, maceration has lower yields and it uses longer extraction times.

2.4.3 Alternative Extraction Method

Unlike conventional methods, alternative methods can produce extracts rich in phenolic compounds using moderate temperatures with shorter extraction times and solvents generally recognized as safe. Choice of solvent, temperature, and extraction time can exhibit similar behavior in all extraction processes.

The solubility of phenolic compounds is higher in polar solvents such as water and ethanol or their mixtures, the diffusion of compounds and mass transfer rates are enhanced by increased temperature, and longer extraction times allow for a more intimate and effective contact between solvent and matrix

2.4.3.1 Microwave-assisted extraction

MAE is an efficient technique due to its ability to heat a matrix internally and externally without a thermal gradient (Calle and Costas-Rodriguez 2017). Molecules with a permanent dipole moment such as phenolic compounds and ionic solutions strongly absorb microwave energy However, there is a limit above which microwave power can cause a decrease in extraction yield. This behavior was observed by (Alara et al. 2017) in the extraction of phenolic compounds from Vernonia amygdalina leaves. Generally, overexposure to microwave radiation leads to overheating and the degradation of phenolic compounds, reducing bioactivity and decreasing extraction yields

2.4.3.2 Ultrasonic-assisted extraction

The raw material is placed in an extraction vessel, then the solvent at the desired temperature is added and the ultrasonic process begins. After extraction time, the extract is recovered after filtration. In UAE of phenolic compounds from defatted oat bran (Chen et al. 2018), TPC increased when the temperature was increased from 20 to 70 C and TPC obtained at 70 0C was almost two times higher than that obtained at 20 0C after 5 min of UAE.

2.4.3.3 Supercritical fluid extraction

SFE uses fluids above critical pressures and temperatures. Supercritical extraction is then performed and the CO2 as a co-solvent and extract mixture is recovered through decreasing

pressure and temperature (Hatami et al. 2018). After the extraction, if a co-solvent was used, it can be evaporated in a rotary evaporator. Ethanol is the most recommended co-solvent because it increases the solubility of the compounds. For example, ethanol has been employed to increase the extraction yield of phenolic compounds from H. sabdariff dried calyces (Pimentel-Moral et al. 2019) and feijoa peel (Henrique et al. 2019) In general, lower temperatures and lower ultrasound power or amplitude results in lower phenolic extraction yields. When the value of these process parameters is raised, the phenolic extraction yield increases up to a certain value, after which the phenolic compounds are degraded and extraction yields falls. Therefore, although the phenolic content depends on the raw material source, extraction efficiency is related to the optimization of the process parameters.

comparative study between UAE and maceration in the extraction of phenolic compounds from baobab seeds. The results indicated that UAE resulted in a significant higher TPC (418.01 mg GA/100 g db.) compared with maceration (357.34 mg GA/100 g db.). Maceration had a lower TPC than UAE, and it involved an extraction time of 24 h whereas UAE required only 20 min, which represents an extraction time 72 times longer. Despite these disadvantages, conventional methods remain in use because the extraction units are widely available and are less expensive than those for MAE, PLE, SFE and UAE alternatives.

2.5 Extraction Parameters for bioactive compounds

The aim of this study is to extract of antioxidant compounds from avocado seed extract and to evaluate its ability to limit food oxidation as a potential alternative to commercial antioxidants. The extraction conditions are extraction temperature, type of solvent, extraction time and solid to solvent ratio studied to optimize the yields of total phenolics and antioxidant activity and the main phenolic compounds identified.

2.5.1 Extraction temperature

The temperature, during extraction, affects the compound stability due to chemical and enzymatic degradation, losses by volatilization or thermal decomposition. Researchers investigated the effects of extraction temperatures (25 – 80 ºC) on total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (AA) and yield of extract. The used of 40 ºC to 80 ºC of

extraction temperature showed a gradual reduction in TPC, thus decreased in AA for DPPH (Moure, 2000).

2.5.2 Type of Extraction Solvent

Solvent extraction is more frequently used for isolation of antioxidants and both extraction yield and anti- oxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potential of compounds with different polarity. Polar solvents are among the most employed solvents for removing polyphenols from water. Ethanol and water are the most widely employed solvents for hygienic and abundance reasons, respectively. Selective extraction of more a polar compound was reported to enhance the antioxidant activity of lentil husk extracts (Moure, 2000).

Table 2. 5 Effect of different solvents on yield of extract and TPC of orange peel extract

	Yield of extract (%)	TPC (mg GAE/g de)	DPPH scavenging activity (%)
Methanol	28.32	165.38	73.42
Ethanol	27.92	169.56	78.14
Acetone	18.21	145.79	68.38
Hexane	8.27	63.20	58.78

Source**: (Ibrahim, 2012).**

2.5.3 Extraction Time

Extraction time is one of the factors that affect the extraction of antioxidant from different plant materials and the extraction yield is dependent on the extraction time. This could have been due to the longer amount of time the solute and solvent were in contact with each other. Longer contact time favored the system to have more mass transfer. However, excessive extraction time would be unnecessary as the solvent and sample would be in final equilibrium after certain duration. This is

based on Fick's second law of diffusion by then; the rate of extraction of compounds would decelerate (Teixeira et al., 2014).

2.5.4 Solid to solvent ratio

Generally, different solid to solvent ratio significantly affect extract yield (Sheng et al., 2011). If ratio of ethanol to seed is too small, the active ingredient in the seed cannot be completely extracted up. When ratio of ethanol to seed powder is increased, the extraction rate of active ingredient also increases. Therefore, suitable ratio of ethanol to avocado seed powder should be selected for extraction of active ingredient.

2.6 Lipid Oxidation in food

Lipids are the primary components of many foods, and their presence is often needed for the development of flavor, texture and color attributes. However, lipids are highly unstable and are readily attacked by oxygen, leading to a chain of chemical reactions that generate undesirable flavor and odor compounds. These oxidative reactions can be accelerated by metals (e.g., iron, copper), light, temperature, enzymes and bacteria. Foods containing high levels of unsaturated fats such as meat and meat products, dairy, fish and oils are especially susceptible to oxidative reactions as oxygen is able to attack those double bonds, leading to the formation of free-radicals and other oxidation products. The food industry suffers significant losses as a result of decreased product shelf-life caused by "warmed-over flavor" (WOF) development, rancidity and diminished nutritional quality, all of which stem from lipid oxidation (Ballard, 2008).

2.6.1 Oxidation Mechanisms of Fats and Oil

Different chemical mechanisms are responsible for the oxidation of fats and oils during processing, storage, and cooking. Two types of oxygen, atmospheric triplet oxygen and singlet oxygen can react with fats and oils. Triplet oxygen, having a radical character, reacts with radicals and causes autoxidation. The non-radical electrophilic singlet oxygen does not require radicals to react with; it directly reacts with the double bonds of unsaturated fats and oils with high electron densities, which is called type II photosensitized oxidation (Choe and Min, 2009). In this case the lipid radical reacts with triplet oxygen very quickly at normal oxygen pressure and forms lipid peroxyl

radical. The lipid peroxyl radical abstracts hydrogen from other lipid molecules to form lipid hydro-peroxide and another lipid radical.

2.6.2 Formation of free radicals

Normally, bonds would not split in a way that leaves a molecule with odd, unpaired electrons. However, when weak bond split, free radicals are formed. Free-radicals are very unstable and react quickly with other compound trying to gain stability. When the attacked molecule loses its electron, it becomes a free-radical itself, these formations of free-radicals continue on and on and finally result in the disruption of the substance especially in fatty foods. Of particular interest is the free radical damage in the body system, fatty foods and other substance like polymer and antioxidants mechanism of action in inhibiting these damages (Hamid et al., 2010).

2.6.3 Antioxidants Activities

Antioxidants are substances capable of slowing or preventing the oxidation of other molecules and may protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols. Some of antioxidants include beta-carotene, lycopene, vitamins C, E, A, and other substances (Sies, 1997).

2.6.4 Types of Antioxidants

2.6.4.1 Natural Antioxidant

Natural antioxidants are compounds from plant or animal sources that retard oxidative rancidity of oils, fats, and fat-soluble components, thus protecting them while delaying the development of unpleasant flavors and odors resulting from oxidation. Natural antioxidants are primarily phenolic compounds that may occur in all parts of a plant. They are multifunctional and can act as free radical terminators, metal chelators, and singlet oxygen quenchers. Another widespread antioxidant in nature is ascorbic acid, but today its extraction from natural sources is not significant

as all ascorbic acid used for food is chemically synthesized. To be considered as an antioxidant for practical use, a compound extracted from a natural source must meet several criteria, including (a) absence of any toxic or physiological effect, (b) no impartation of any strong odor, flavor, or color to the product, and (c) considerable antioxidant activity at small concentrations in the product. It has been reported that plant polysaccharides or their derivatives have strong antioxidant activities and can be explored as novel potential antioxidants (Jiang et al., 2005).

2.6.4.2 Synthetic Antioxidants

Synthetic antioxidants have been widely used to extend the shelf-life of various food materials. The current preference for synthetic antioxidants can be attributed to their proven effectiveness in a variety of food systems and their relative low cost when compared to natural antioxidants. The most commonly used synthetic antioxidants in the food industry include BHT, BHA and TBHQ. Although synthetic antioxidants are extremely effective at slowing oxidation, there have been recent consumer concerns over potential adverse health effects associated with these compounds. Many processors wish to avoid adding synthetic antioxidants to foods to eliminate these health concerns and to be able to state on the label that the product is “all natural” (Ballard, 2008).

2.6.5 Source of Natural Antioxidants

In recent years, much research has been focused on identifying sources of natural antioxidants that can be used to replace their synthetic counterparts. Strong natural antioxidant compounds were found in fruits, vegetables, trees and leaves. The most common plant sources are cereals, citrus fruits, cocoa and coffee beans, herbs and spices, oilseeds, olives, onion and garlic, tea, as well as some miscellaneous products.

2.6.6 The chemistry of antioxidants

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

Chain reactions of free radicals

- Initiation stage
  1. $RH \rightarrow R^{\cdot} + H^{\cdot}$
  2. $R^{\cdot} \rightarrow R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$
  3. $2ROOH \rightarrow ROO^{\cdot}\ RO^{\cdot} + H_2O$
- Propagation stage
  1. $R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$
  2. $ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$
  3. $RO^{\cdot} + RH \rightarrow ROH + R^{\cdot}$
- Termination stage
  1. $R^{\cdot} + R^{\cdot} \rightarrow R - R$
  2. $R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$
  3. $ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + O_2$
  4. Antioxidant + $O_2 \rightarrow$ oxidized antioxidant ((Hamid et al., 2010). The antioxidants added to it, will neutralize the free radicals by donating one of their own electrons ending the reactions.

2.6.7 Mechanisms of Antioxidants in the Oxidation of Food products

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

2.6.7.1 Free radical scavenging

Antioxidants scavenge free radicals of foods by donating hydrogen to them, and they produce relatively stable antioxidant radicals with low standard reduction potential, less than 500 mV (Choe and Min, 2005). The effectiveness of antioxidants to scavenge free radicals of foods depends on the bond dissociation energy between oxygen and phenolic hydrogen, pH related to the acid dissociation constant, and reduction potential and delocalization of the antioxidant radicals. The bond dissociation energy for O–H of the phenolic antioxidants also predicts the stabilization of antioxidant radicals. The lower the bond dissociation energy for the O–H group of the antioxidants,

the more stable the antioxidant radical. The O–H bond strength of phenolic antioxidants is affected by substitution of hydrogen in a benzene ring.

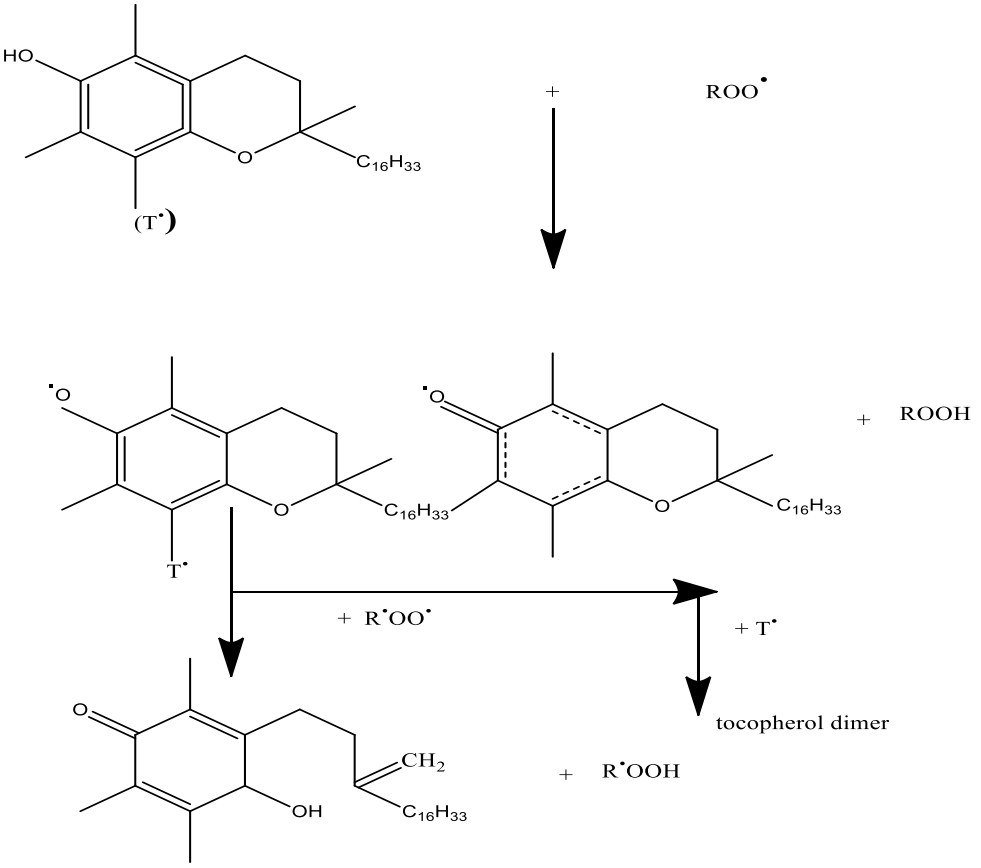


Figure 2. 3 Reaction of α-tocopherol with lipid peroxyl radical

2.6.7.2 Metal chelating

Metals reduce the activation energy of the oxidation, especially in the initiation step, to accelerate oil oxidation. Metals catalyze food radical formation by abstracting hydrogen. Crude oil contains transition metals such as iron or copper, often existing in chelated form rather than in a free form. Oil refining decreases metal contents. Most chelators are water-soluble, but citric acid can be dissolved in oils with some limitation to chelate metals in the oil system (Choe and Min, 2009).

2.6.7.3 Singlet oxygen quenching

Tocopherols, carotenoids, curcumin, phenolics, urate, and ascorbate can quench singlet oxygen. Singlet oxygen quenching includes both physical and chemical quenching. Physical quenching leads to deactivation of singlet oxygen to the ground state triplet oxygen by energy transfer or charge transfer. There is neither oxygen consumption nor product formation. Singlet oxygen quenching by energy transfer occurs when the energy level of a quencher (Q) is very near or below that of singlet oxygen

Figure 2. 4 Formations of ascorbic acid hydro peroxides by singlet oxygen

Chemical quenching of singlet oxygen is a reaction involving the oxidation of a quencher rather than a quenching, thus producing breakdown or oxidation products of a quencher. β-Carotene, tocopherols, ascorbic acid, amino acids (such as histidine, tryptophan, cysteine, and methionine), peptides, and phenolic are oxidized by singlet oxygen, and they are all chemical quenchers of singlet oxygen

2.6.8 Structure of antioxidants

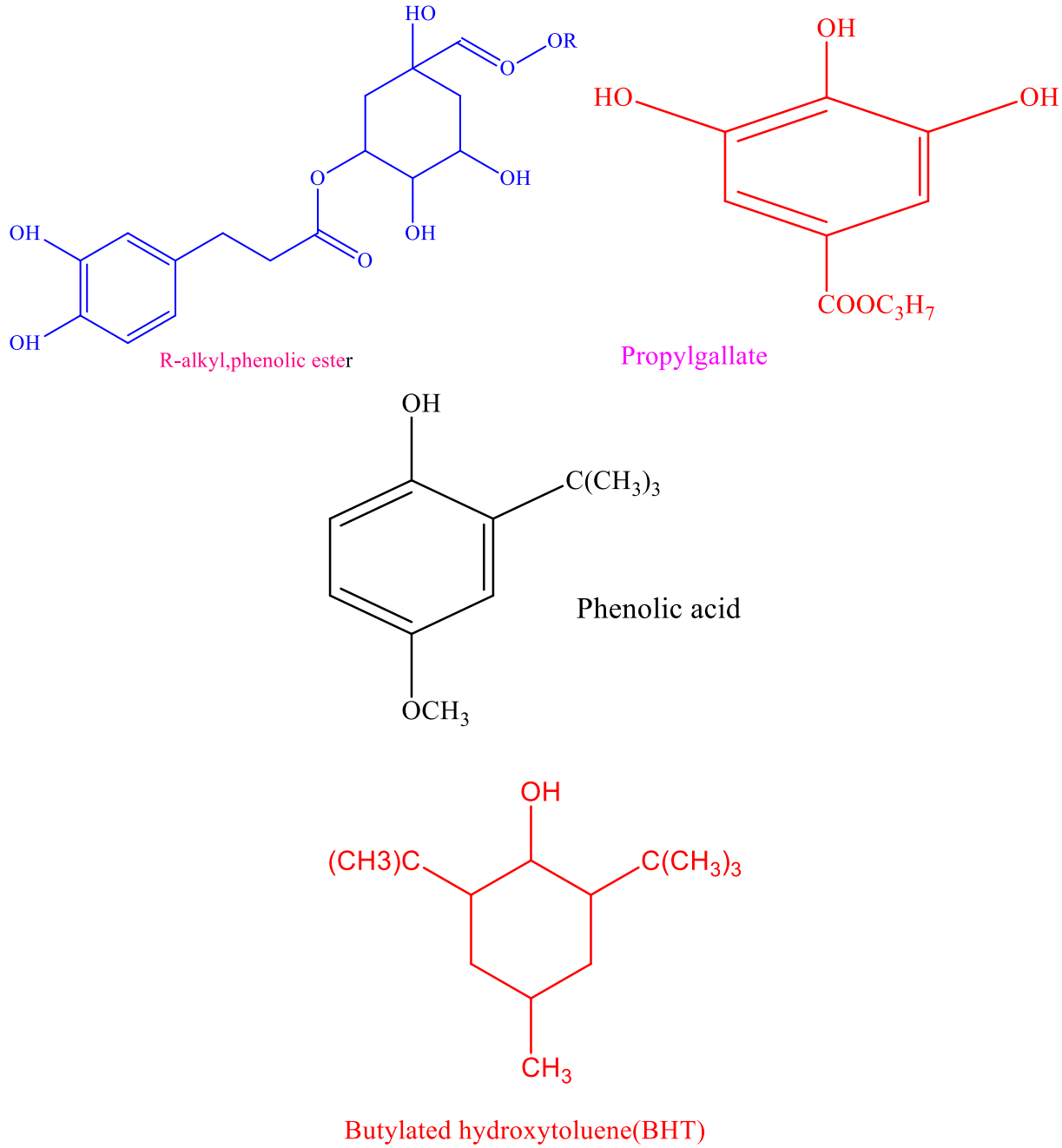


Figure 2. 5 Different types of antioxidants and their structure

2.7 Bioactive film ingredient and film forming materials

2.7.1 Bioactive film ingredients

A variety of natural bioactive ingredients can be added to edible films to improve their functional properties. In particular, there has been a focus on ingredients that can protect foods from degradation due to their antioxidant and antimicrobial potentials.

2.7.1.1 Polyphenols

Studies have shown that these polyphenols have good antioxidant, antimicrobial, anti-browning, and protein cross-linking properties, which may be useful for edible film applications. For instance, polyphenols have been shown to effectively delay lipid oxidation in foods (Maqsood, Benjakul, and Shahidi 2013), which has been attributed to various effects, including their scavenging of free radicals, quenching of active oxygen, inhibition of oxidases, and binding of transition metals.

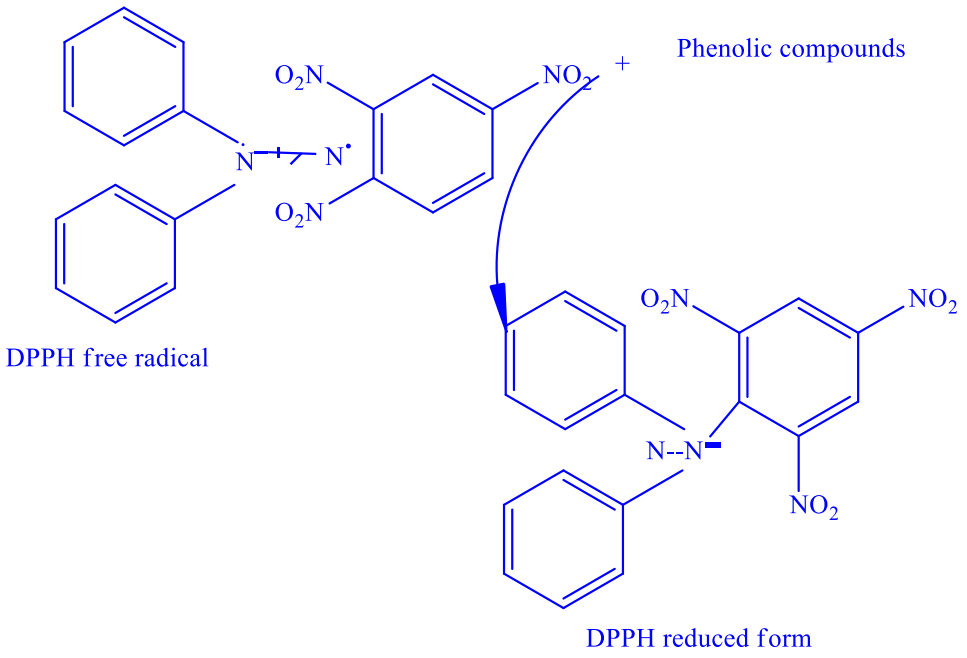


Figure 2. 6 Reaction of α-tocopherol with lipid peroxyl radical.

2.7.1.2 Essential oils

In recent years, essential oils (EO) have been widely utilized as active ingredients in edible films and coatings. Essential oils extracted from plants and spices have good antibacterial and antioxidant properties, and may improve the physicochemical properties and functional performance of edible films, which makes them good candidates for many applications.). However, the application of essential oils in food preservation is often limited because of their strong aroma, since the direct addition of essential oils to food may adversely affect sensory perception (Saricaoglu and Turhan, 2019)

2.7.1.3 Other active ingredients

Other kinds of active ingredients can also be incorporated into edible films to enhance their functional properties, including natural pigments, flavors, polypeptides, vitamins, and nutraceuticals. For instance, natural pigments, such as anthocyanin's, carotenoids and curcumin, can be used to create active films with desirable appearances (Stoll et al. 2016；Assis et al. 2018；Jung et al. 2020).

2.7.2 Film-forming materials for bioactive packaging films

2.7.2.1 Polysaccharides

Many food-grade polysaccharides are also good film-forming materials. Polysaccharides contain a large number of hydroxyl groups and other polar groups, which means that hydrogen bonding often plays an important role in film formation and final film properties (Gomez and Roman 2018). Some of the most commonly used film-forming polysaccharides are chitosan, alginate, carrageenan, cellulose, and starch (Nesic et al. 2019).

2.7.2.2 Lipids

Edible lipid sources include triglycerides, fatty acids, waxes and resins, which may be used in bulk or emulsified forms. Due to the strong hydrophobicity, lipids have good moisture resistance, which is important to prevent food spoilage. Nevertheless, lipid-based films are often relatively fragile,

have poor mechanical properties, and have weak adhesion to foods that have hydrophilic surfaces (Falguera et al. 2011)

2.7.2.3 Composite materials

The shortcomings of edible films made from a single film-forming material can be overcome by using combinations of different film-forming materials, such as proteins, polysaccharides, and lipids. For example, the addition of lipid droplets to protein- or polysaccharide-based films can be used to create films with good mechanical strength and low moisture permeability (Dhall 2013).

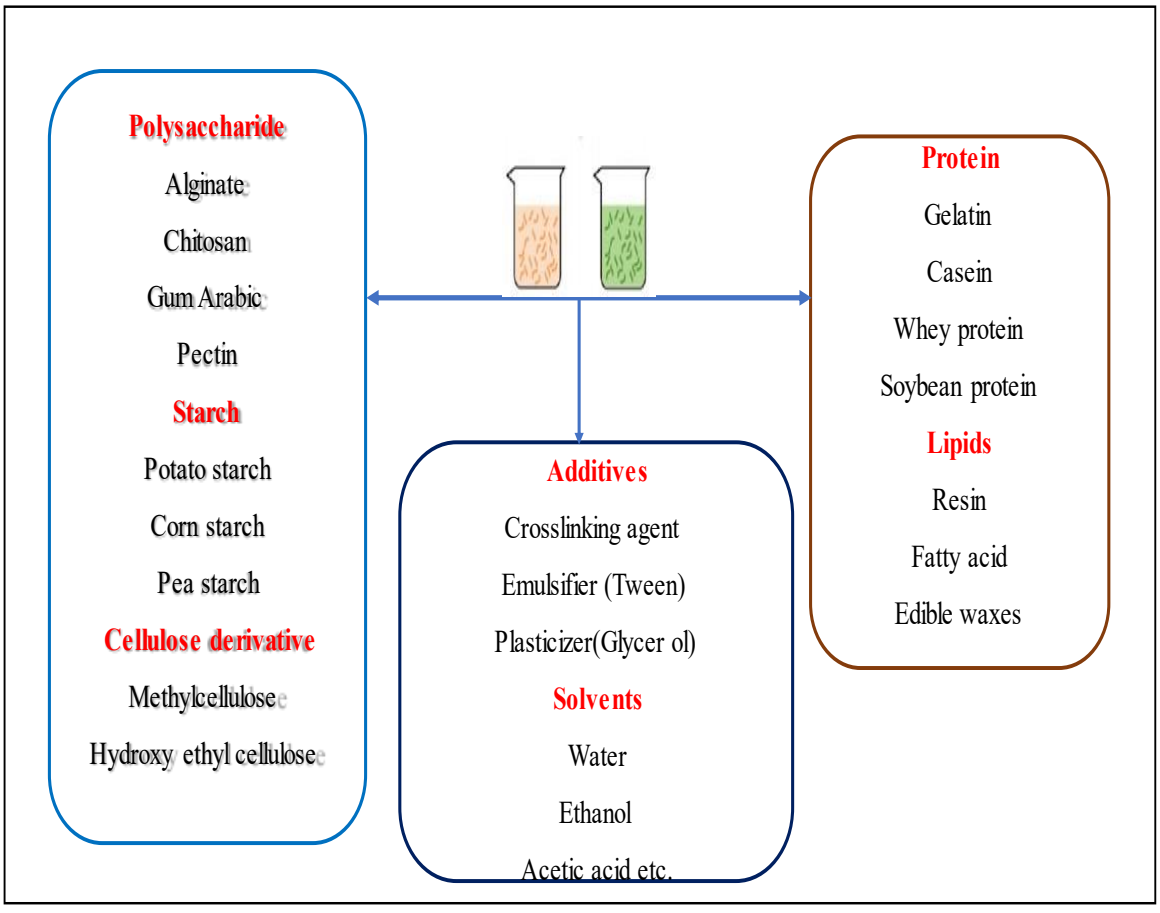


Figure 2. 7 Polymer materials for developing bioactive films

2.7.3 Preparation methods of packaging film containing active ingredients

Both wet and dry processes are commonly used in the preparation of edible films. The wet method involves dispersion or solubilization of the polymers in a solvent medium prior to film formation, whereas the dry method involves hot press molding or melt extrusion processes of powdered polymers (Ciannamea, Stefani, and Ruseckaite 2016).

2.7.3.1 Wet process

The wet process, also known as solution casting, involves dissolving or dispersing the polymers and other ingredients in a suitable solution, casting them on a surface, and then drying them. Because of its simplicity, edible films prepared by the wet process are widely used under laboratory conditions. Although the wet process is simple to perform, it is not easy to carry out continuously on a large scale. At the same time, the water content of the film-forming solution is high, the drying time is long, and the energy consumption is high (Chevalier, Chaabani, et al. 2018), which increases production costs. The three main steps involved in the wet process are described in more detail here

- **Solubilization:** The film-forming materials, plasticizers, active compounds, and any other additives are weighed into a suitable solvent (such as water, alcohol, or an organic solvent) at a certain temperature and stirred until fully dissolved or dispersed. Any undissolved gasses in the resulting film-forming solution are then removed by sonication, centrifugation, and/or vacuum treatment to prevent bubbles forming in the final film.
- **Casting**: A fixed quantity of film-forming solution is then poured onto a planar level surface and allowed to spread evenly, so that a uniform film with a well-defined thickness will be produced.
- **Drying:** Finally, the film is formed and shaped, which may be achieved by drying, cooling, and rolling processes. In laboratory studies, simple air drying is often used

2.7.3.2 Dry process

Dry processing is based on the thermoplastic characteristics of biopolymers. Dry processing is often used in the preparation of thermoplastic packaging films such as starch and protein, for

example, corn starch (Li et al. 2011), wheat bran gluten protein (Zubeldıa, Ansorena, and Marcovich 2015), and soy protein (Nilsuwan et al. 2019).

- **Mixing and hydration**: The film-forming materials, plasticizers, cross-linking agents, and active compounds are mixed with water at a certain temperature, which causes them to become hydrated and swell. The wet components are then defoam
- **Extrusion:** The wet and dry components are added to the input screw of an extruder, which is set to operate at a certain speed ratio. The screw mixes and heats the various components under high pressure, and then forces them through a die to form a film. After extrusion the prepared film can be dried and cooled to obtain the finished edible film.

2.8 Properties of packaging film with active ingredients

Some bioactive ingredients can improve the structural properties of edible films due to their covalent and non-covalent interactions with the reactive groups on the biopolymer chains, thereby affecting the structural, optical, mechanical, and barrier properties of packaging films. Therefore, this section focuses on the effects of bioactive ingredients on the optical, mechanical, barrier, antioxidant, and antibacterial properties of edible films.

2.8.1 Bioactive Properties of packaging film with active ingredients

2.8.1.1 Antimicrobial properties

Adding active ingredients to edible films can significantly improve their antimicrobial properties. For example, when studying the antibacterial effect of eugenol in chitosan/acorn starch (CS/AS) films against E. coli and S. aureus, it was found that the control group (no eugenol) showed no significant effect on the two tested bacteria, while the test group (with eugenol) showed good inhibition in a dose dependent manner. The inhibitory zones of E. coli and S. aureus were 67.5 ± 1.0 and 73.1 ± 1.3 mm^2 for the test group with 3% eugenol (Zheng et al. 2019).

2.8.1.2 Antioxidant properties

Adding antioxidants to active packaging film films can improve their anti-corrosion properties, inhibit browning reactions, and reduce the oxidation of nutrients (Bonilla, Talon, et al. 2013).

There are many similarities between the study of the anti- oxidation and antimicrobial of active components in edible films. In particular, the antioxidant properties of a film depend on active ingredient type, film-forming material type, encapsulation, and food type. Many natural food ingredients exhibit good antioxidant properties, including polyphenols, essential oils, tocopherols, carotenoids, and plant extracts. Films incorporated with mango, acerola (Malpighia glabra L. seriguela, Spendias purpurea et al. 2020) pulps caused an antioxidant effect on dendê oil during 40 days of storage and can be applied to control product oxidation.

Table 2. 6 Bioactive compounds from plant-derived incorporated into biodegradable films and their respective properties in active packaging

Source Material	**Bioactive Compounds**	**Film-Forming Material**	**Effect on Food Packaging**	**Reference**
Turmeric rhizome	Curcumin	Whey protein isolate	Antioxidant properties	Taghavi Kevij et al.2020
Extract of germinated fenugreek seeds	Phenolic compounds	Semi-refined κ-carrageenan	Antioxidant and antimicrobial	Farhan et al.2020
Green coffee oil	Fatty acids and phenolic compounds	Carboxymethyl cellulose	Antioxidant capacity	Lombo Vidal et al.2020
Blackberry pulp	Anthocyanins (phenolic compound)	Arrowroot starch	Antioxidant capacity	Nogueira et al.2019
Coconut water concentrate	Phenolic compounds	Coconut protein	Antioxidant capacity	Rodsamran et al. 2018

Spirulina extract	Phenolic compounds	Crab chitosan	Antioxidant activity	Balti et al. 2017

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Raw Materials and Reagents

This chapter aims to introduce the necessary materials, sample preparation methods and appropriate characterization techniques that had to be followed for various experiments in this research work. For convenience, the required materials, experimental protocols and characterization techniques were categorized into two sections. The first section describes the parameter optimization for the extraction of crude extract from avocado seed (*Persea americana Mill.*) and characterization of the crude extract and the second section describes the synthesis and characterization of bioactive film developed from avocado seed extract and gelatin matrix. Finally, the effect of incorporating of ASE on antioxidant property, water solubility capacity and FTIR analysis of the developed gelatin based bioactive film

Materials and Reagents: were Avocado seed kernel, Gelatin powder polymer, Glycerol, distilled water, Ethanol with MS grade, DPPH (-2,2 Diphenyl -1-picryl hydroxy radical), sodium carbonate, Methanol, Gallic acid are the different chemicals and materials that was used for extraction, characterization and film making. All reagents and solvents were analytical grade. The instrument

used for analysis were Soxhlet extractor, vacuum pump, chiller, water bath, centrifuge, condenser, oven, flask, beaker, balance, dissector, test tubes, UV- Visible spectrophotometer, sieve, miller.

Table *3.1* Lists of major chemicals and reagents used in this study with their purpose

Chemical name with specification	State	Purpose
Gelatin powder (ex. bovine type B)	Solid	Used as a film matrix for the development of bioactive film
Glycerol ($C_3H_5(OH)_3$, 99.8% purity)	Viscous liquid	Used as plasticizer in the production of bioactive film
Ethanol (CH_3CH_2OH, 96 %)	Liquid	Used as a solvent for the extraction of avocado seed extract from avocado seed ((Persea americana Mill.)
DPPH (-2,2 Diphenyl -1-picryl hydroxy radical), CAS No. 1898-66-4	Liquid	Used as a standard for the determination of antioxidant activity of the crude extract and the bioactive film
Distilled water	Liquid	For solution preparation, to wash instruments and for mixing all the ingredients during bioplastic film preparation
Gallic acid, (CAS 149-91-7)	Liquid	Used as a standard for the determination of total phenolic content of crude extract.

Sodium Carbonate (≥99.5 %, Ph. Eu)	Aqueous	Used to constant the intermediate ring compound which sensitive to light wave that uses to determine the amount of total phenolic content
Methanol (CH3OH), (ASTM A 99.85%)	Liquid	Used as a solution formation for the determination total phenolic content

3.2 Experimental frame work the thesis

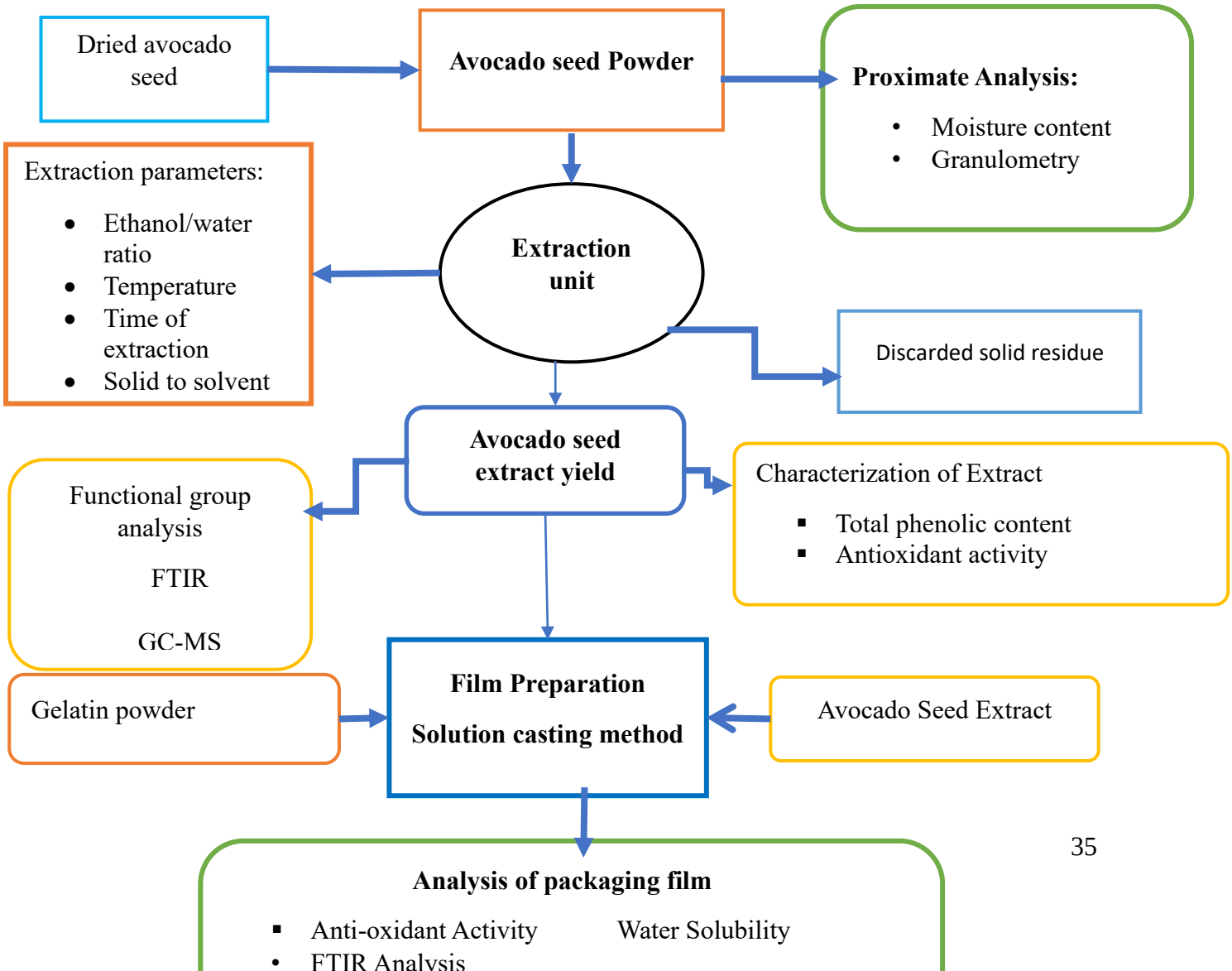


Figure 3. 1 The overview of experimental frame work of thesis

3.3 Experimental Methods

3.3.1 Preparation of Avocado seed powder for extraction

Avocado fruit (*Persia Americana Mill*.) was stored at room temperature until it reached full maturity. The avocado seeds were manually separated and cleaned under a continuous flow of tap water. The seeds were washed to remove pulp residues and cut into small pieces with a kitchen knife. After that, a portion of the sample was placed on a tray and dried in an oven. Oven drying conditions were 55 ^{0}C for 24 Hr. with forced air. The final point of the drying was assessed by sampling and evaluating the moisture content by weighing differences until it reached less than 5 percent (w/w) (in triplicate). Finally, the dried material was stored in dark packaged polyethylene at -10 ^{0}C for the next day's grinding. To prevent thermal stress of the material, a coffee grinder was used to reduce the size of particles at intervals of twenty seconds. The entire procedure was carried out while insulating the material from light. The flour was kept in a glass bottle that was completely closed and kept at room temperature, away from light and air.

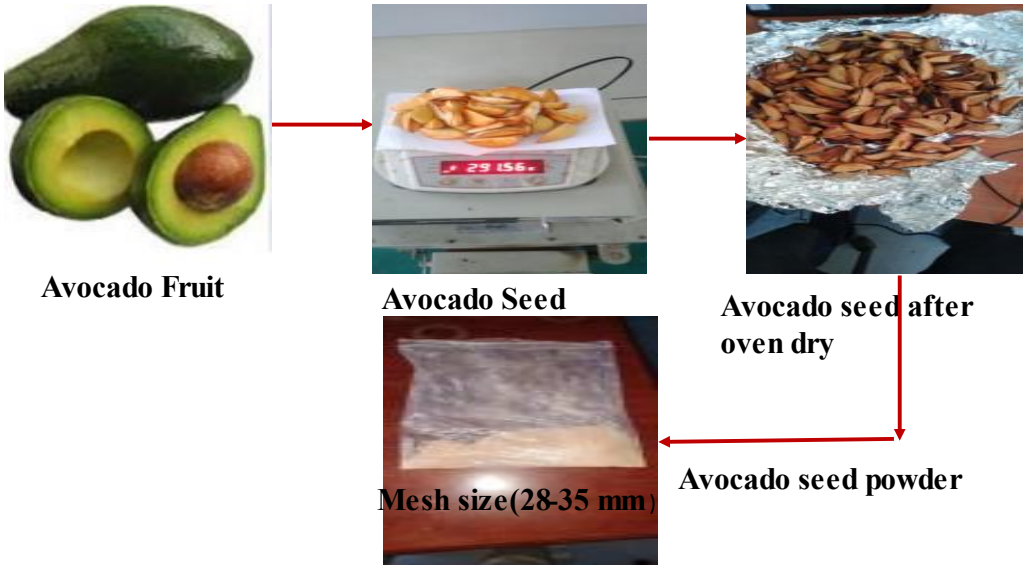


Figure 3. 2 Preparation of avocado seed powder for extraction

3.3.1.1 Granulometry

The seeds were milled in a coffee miller before being sorted by particle size using a Tyler series sieve system. A vertical-vibratory shaker was utilized to improve particle size classification using a set of sieves that included 9, 12, 24, 28, and 35 meshes. Once considerable quantities of seeds were produced after milling, the seeds kept between and below 35-mesh (mesh 28 = 0.60 mm and mesh 35 = 0.42 mm) sieves were selected for extraction. The milled samples were sealed in polyethylene vacuum bags and kept refrigerated at 4 °C until further testing was required.

3.3.1.2 Moisture Content

A weighed sample of avocado seed was placed in a ventilated oven where a temperature of 105oC was maintained in accordance with Association of Official Analytical Chemists (AOAC, 2002). Dried sample from the oven was withdrawn and weighed every 2 hours until readings confirmed no significant loss of mass. The moisture content (in percent) was calculated according to equation

$$\% \text{ Moisture } = \left[\left(\frac{\text{Mi } - \text{Mod}}{\text{Mi}} \times 100\right)\right] \ldots\ldots\ldots\ldots\ldots\ldots \text{Eq (3.1)}$$

Where M_i = Mass of sample before drying

M_{od}= Mass of sample after drying

3.4 Extraction of crude extract from avocado seed

The extraction was performed with a Soxhlet apparatus. The Soxhlet extraction was comprised of a condenser and an extractor, and followed a methodology similar to that used in earlier investigations (S. Suwal and A. Marciniak, et al., 2019) with some modifications. About 20 g of dry avocado seed was inserted inside the extractor (capacity of 250 ml) within a handmade filter paper (6.5 x 4.5 cm) bag to avoid clogging the siphon hole. Soxhlet extraction was performed on seeds of both sizes (28-35 mesh and below 35 mesh). The extractor was then filled with a pure solvent for about 250 mL and poured into a 1000 ml three-neck flask, which is convenient for adjustment of temperature for the heater by using thermometer

To prevent light from accessing the flask and causing deterioration of the bioactive components, the flask was covered with aluminum foil. The device was then connected to the Soxhlet apparatus and heated above its boiling point to achieve a consistent condensation rate. Solvents, EtOH (99.8% purity, Neon, Brazil) and distilled water were employed in these experiments. The studies were carried out with the solvent being refluxed in the Soxhlet apparatus for 6 hours, as confirmed by a subsequent kinetic investigation that involved removing the flasks from the device after 2, 4, 6, and 8 hours of extractions. The Petri dish containing the extract was stored in an oven at 55 ^{O}C for around 18 hours to guarantee complete elimination of leftover solvent. The sample was weighed to estimate the extraction yield after drying and cooling to room temperature, then transferred to a 2 ml vial and stored in the refrigerator (-10 ^{O}C) for chemical analysis.

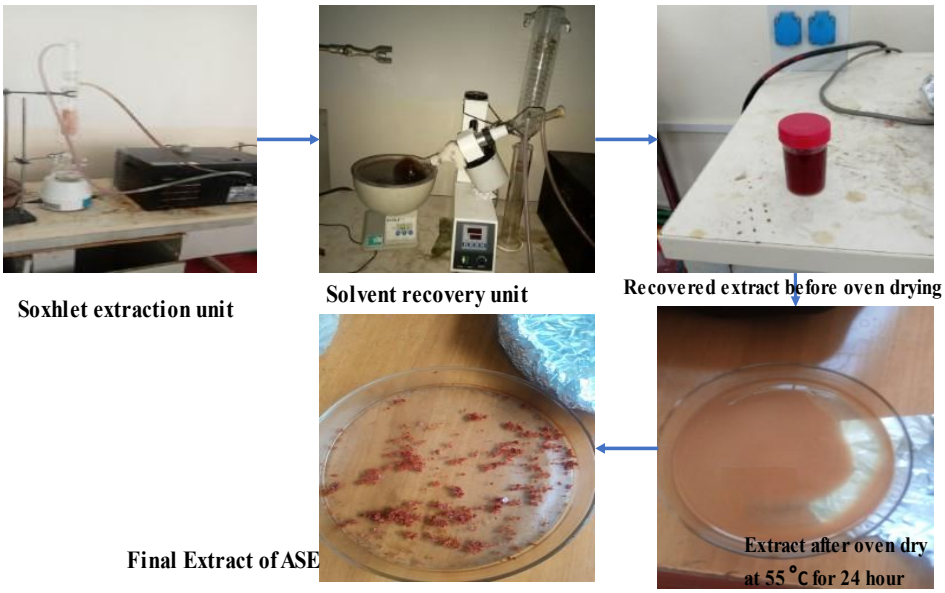


Figure 3. 3 Extraction of cruse extract from avocado seed

3.4.1 The effect of process parameters extraction of avocado seed

The most important factors affecting the extract yield and phenolic content of extraction of the seed are: extraction temperature, ratio of solvent, solid to solvent ratio, and extraction time. For this thesis work, the effect of factors which are temperature, solid to solvent ratio, solvent ratio and extraction time were investigated to optimize the extraction operating conditions for achieving maximum extract yield. The seed was extracted using the method described by Awolu et al. (2013) with minor modifications. In a Soxhlet apparatus, 20 g of avocado seed powder was weighed with 250 ml of solvent and defatted. Different solvents and solvent ratios were used in the extraction (100 % ethanol, 50 % aqueous ethanol, and 100 % water). Excess solvent was removed from the extraction in a rotary evaporator and then oven dried at 60 0_C for 18hours to remove the remaining moisture in the extract. Before further analysis, the extracts were stored in an airtight bottle in a laboratory fridge. The selection of parameters and their ranges was from the preliminary analysis and previous works which had a direct effect on the yield of extract and total phenolic content. The priority for studying the parameter effects was designed by a four-step experimental procedure for determining the parameters for obtaining maximum extracts, which will simplify the extraction operation and save time and expensive reagents. The parameters were the extraction solvent (ethanol/water ratio) at concentration levels of, 0:100, 50:50, 75:25 and100:0 v/v, extraction

temperature (60, 70, 80 and 90°C), extraction time (2 hr, 4 hr, 6 hr, and 8 hr.), and solid to solvent ratio (1:16.6g/ml(15 g to 250 ml EtOH) , 1:12.5g/ml(20g to 250ml EtOH), 1:10g/ml(25g to 250 ml EtOH) and 1:8.3g/ml(30g to 250 ml EtOH).

Table 3.*2* Ranges of parameters for extraction of avocado seed extract

No.	Extraction parameter	Range	Yield (%)	TPC(mgGAE/g)
1	Solvent ratio	0-100 % (EtOH: H2O)		
2	Temperature	60 – 90 (°C)		
3	Solid -to solvent ratio	1.83 – 1:16.6 (mg/ml)		
4	Time of extraction	2 - 8 (Hour)		

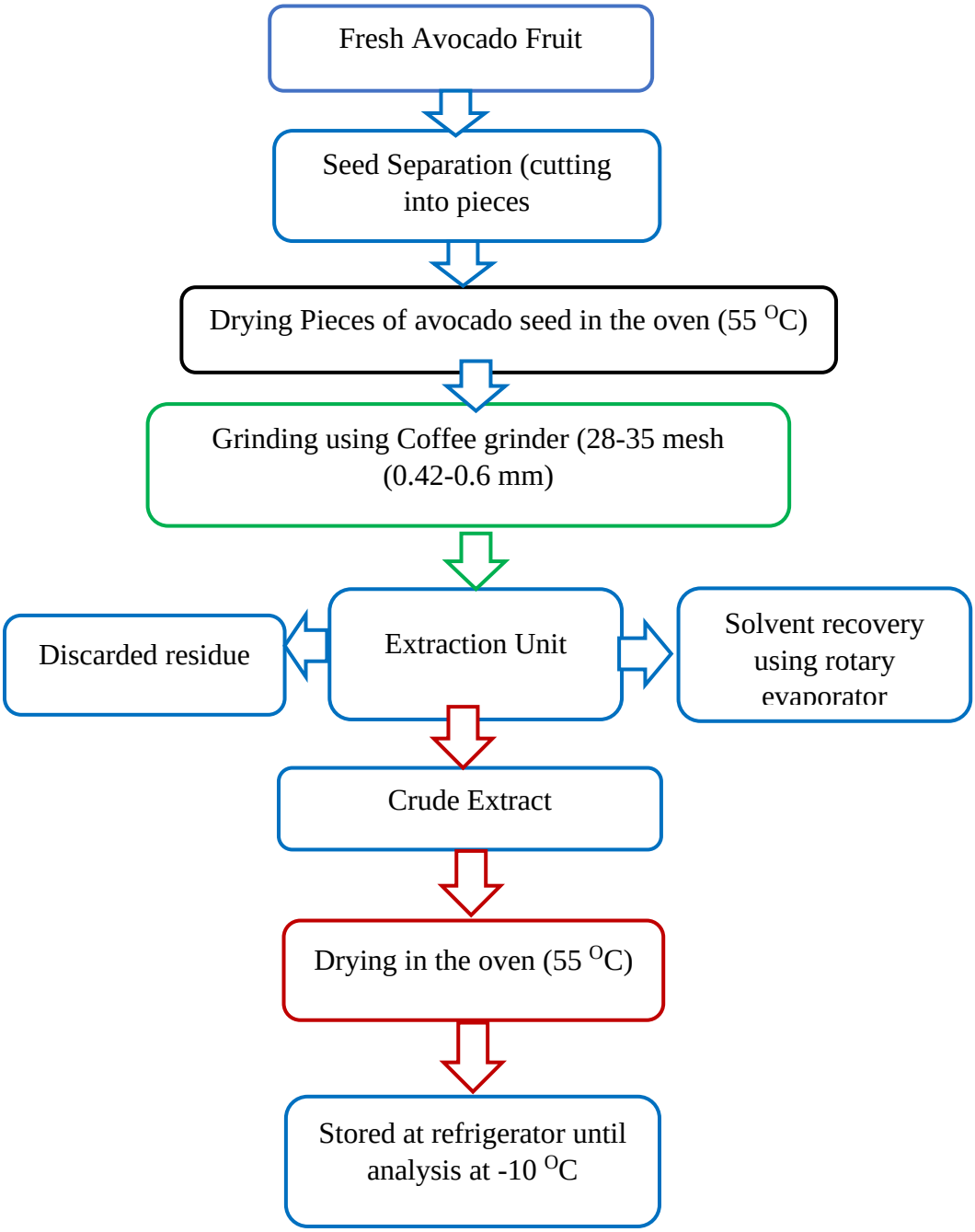


Figure 3. 4 Procedure of avocado seed extract from avocado seed powder

3.4.2 Determination of Extract Yield

The extraction yield is defined as the quantity of extract recovered in mass compared to the initial amount of whole seed powder and is a measure of the solvent's efficacy in extracting certain components from the original material. It was calculated for each approach evaluated and expressed as a percentage (%). The extraction yield was calculated by weighing the dry extract obtained after filtration.

$$\text{Extraction Yield (\%)} = \frac{\text{Dried wt. of extract}}{\text{Total Dried wt. seed powder}} * 100 \ldots\ldots \text{Eq (3.2)}$$

3.4.3 Determination of Total Phenolic Content

The total phenol content was measured using the modified Singh-Folin-Ciocalteu reagent technique (Singh, 2014). 1 ml of Gallic acid or extract solution and 1 ml of Folin-Ciocalteus reagent were mixed and incubated at room temperature for 3 minutes, resulting in the formation of a blue color complex and an increase in extract absorbance. The sodium carbonate solution was then added in 1 mL increments. The solution was adjusted to 10 mL with distil water or (7 mL with distil water), mixed thoroughly, and incubated at room temperature for 60 minutes before being measured at 760 nm with a Visible Spectrophotometer (UV-Vis)

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

$$\text{TPC} = \frac{C*V}{W} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Eq (3.3)}$$

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

V = Volume of stock solution of extract (ml)

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

TPC = Total phenolic content (mg GAE/g dry seed)

If dilution of the solution was used for correct concentration to obtain the required absorbance, Dilution factor was considered

$$T = \frac{C*V*DF}{W} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Eq\ (3.4)$$

Where DF= Dilution Factor

3.4.3.1 Preparation of standard solution

Gallic acid was used as a positive control or standard for determination of total phenolic content of the extract. A stock solution (50 mg/ml) of Gallic acid was prepared by weighing 2.5g of Gallic acid and dissolving in 5ml methanol and diluted to 50 ml Volumetric Measuring flask using distil water. Aliquots of 10, 20, 40, 60, 80, 100 µL of standard Gallic acid were withdrawn from the stock solution and mixed with 990, 980, 960, 940, 920, 900 µL of methanol solvent to get Gallic acid concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg/ml respectively in separate test tubes. Then 1 ml of Folin-Ciocalteu reagent was added, mixed well and incubated for 3 min. After 3 min, 1ml of saturated Na_2CO_3 solution was added and adjusted the solution to 10 ml with distil water (added 7ml distil water), mixed well and incubated in the dark for 90 min at room temperature. After 90 min incubation, the absorbance was measured against the blank (the same mixture without the Gallic acid) at 760nm using a UV-Visible Spectrophotometer. The experiment was carried out in triplicate.

3.4.3.2 Preparation of sample solution

Sample stock solution of extract (50 mg/ml) was prepared by dissolving 1.0 g of extract in to 20 ml of ethanol from stock solution 10, 20, 40, 60, 80, 100 and 120 µL of sample solution were withdrawn and mixed with 990, 980, 960, 940, 920, 900 µL of methanol to get sample concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg/ml respectively in separate test tubes. Then 1 ml of Folin-Ciocalteu reagent was added, mixed well and incubated for 3 min. After 3 min, 1ml of saturated Na_2CO_3 solution was added and adjusted the solution to 10 ml with distil water (added 7ml distil water), mixed well and incubated in the dark for 60 min at room temperature. After 60 min incubation, the absorbance was measured against the blank (the same mixture without the extract) at 760nm using a UV-Visible Spectrophotometer. The experiment was carried out in triplicate. The total phenol content was calculated and expressed as milligrams of Gallic acid

equivalents (mg of GAE/g dry extract) using the Gallic acid calibration curve and the curve used to determine the corresponding Gallic acid concentration of the samples.

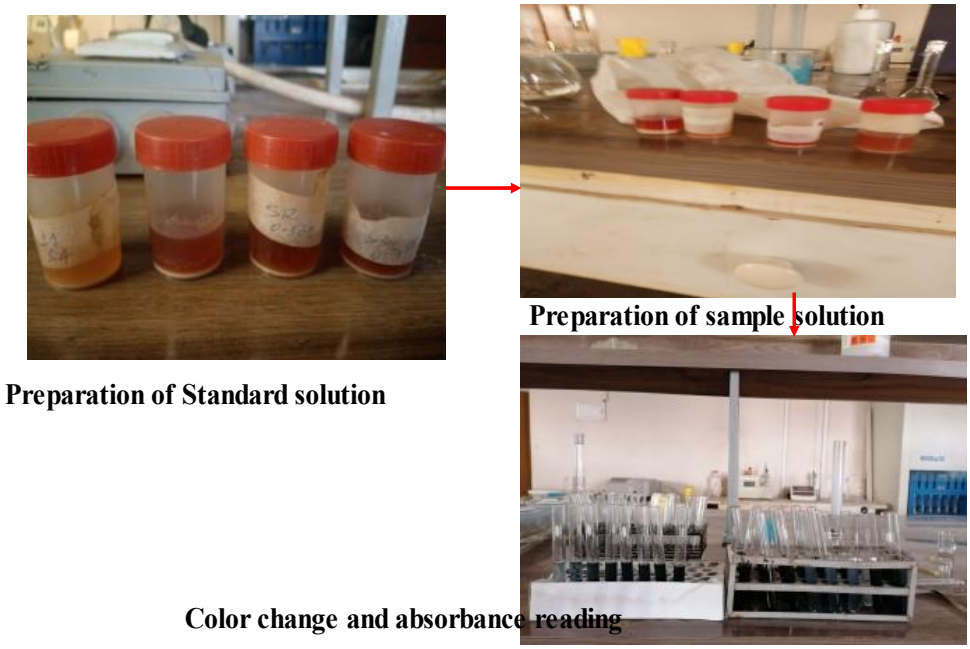


Figure 3. 5 Preparation of standard solution and sample solution

3.4.4 Measurement of absorbance for Gallic acid standard solution and sample solution

The Folin-Ciocalteu reagent was used in a colorimetric test to measure phenolic chemicals. Phenolic compounds and the Folin-Ciocalteu reagent react to produce a blue color complex that absorbs light and enables the measurement of phenolic compounds using a UV spectrophotometer. The concentration of phenolic chemicals determines the maximal chromophore absorption in the process, which forms a blue chromophore made up of a phosphotungstic-phosphmolbdenum complex. The faster the absorbance increased, the higher the antioxidant activity of the extract. In an alkaline solution, the reagent quickly breaks down, enabling the use of excess reagent to achieve a full reaction. This excess can cause precipitates and severe turbidity, which limit spectrophotometer analysis. The addition of Na_2CO_3 salt neutralizes and prevents this problem.

Table *3. 3* Gallic acid standard solution preparation and corresponding absorbance

Gallic acid (µl)	Methanol (µl)	Concentration (mg/ml)	FC (ml)	Distilled Water(ml)	Absorbance
0	1000	0	1	7	0.001
10	990	1	1	7	0.2773 ± 0.13
20	980	2	1	7	0.4718 ± 0.34
40	960	3	1	7	0.7566 ± 0.21
60	940	4	1	7	0.9642 ± 0.019
80	920	5	1	7	1.2272 ± 0.213
100	900	6	1	7	1.4201 ± 0.014

Table *3. 4* Concentrations of Gallic acid standard solution and their corresponding absorbance.

No.	Concentration (mg/ml)	Absorbance (λmax =760 nm)
1	0	0.001
2	1	0.2773 ± 0.13
3	2	0.4718 ± 0.34

4	3	0.7566 ± 0.21
5	4	0.9642 ± 0.019
6	5	1.2272 ± 0.213
7	6	1.4201 ± 0.014

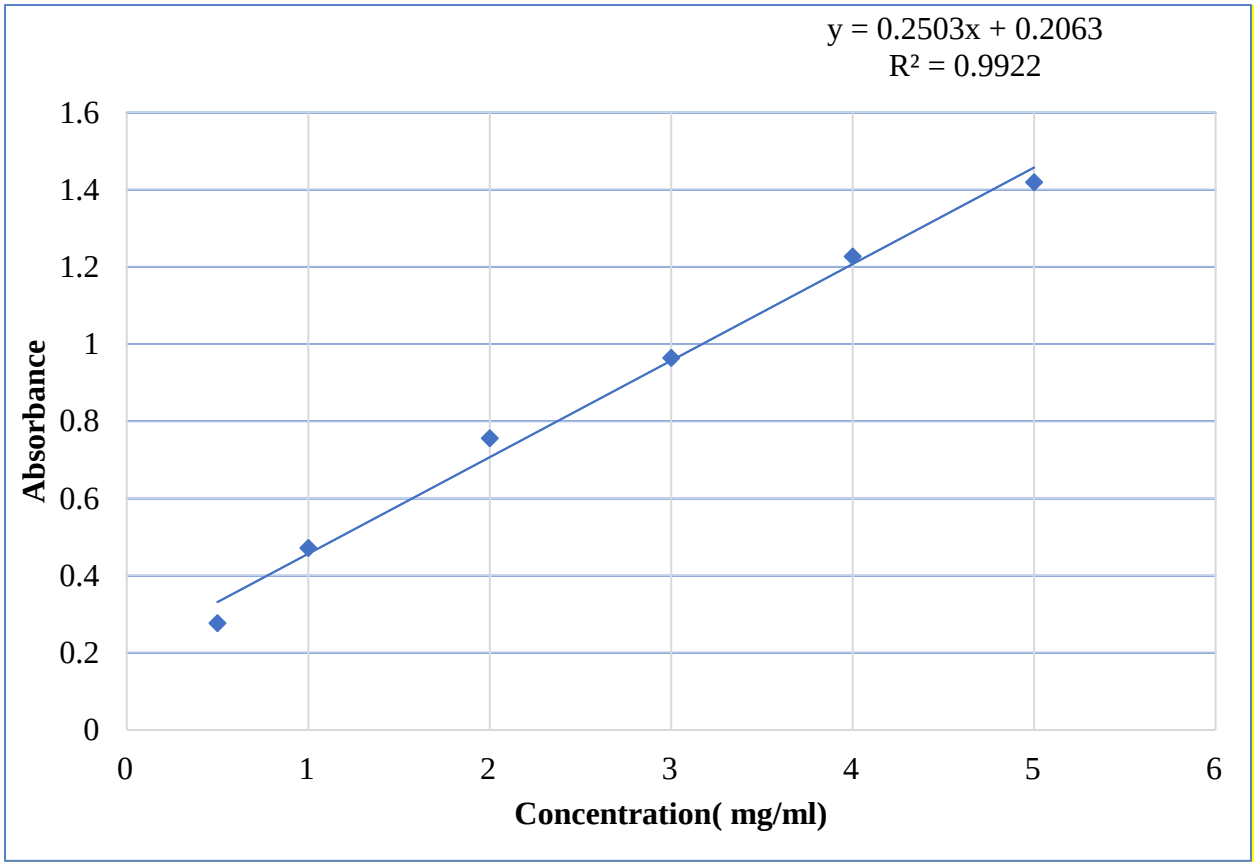


Figure *3.6* The Gallic acid equivalent concentration graph

3.4.5 Determination of the antioxidant property of avocado seed extract

Radical Scavenging Activity was determined according a modified version of the DPPH method by Singh (2014). Stock solution of the extract was prepared. 0.004% DPPH solution was prepared

by dissolving 0.01 g DPPH dissolve in 250 ml methanol in volumetric flask. Then 1 mL of extract or standard ascorbic acid from stock solution was added into 4 mL of DPPH solution. The mixture was shaken vigorously and was left to stand in the dark for 30 minutes. The absorbance of the resulting solution was measured using a UV-Visible Spectrophotometer at 517 nm by monitoring the decrease in absorbance. Ascorbic acid was used as standard. The scavenging activity of the extract was calculated using the formula:

$$\text{HHP radical scavenging activity}(\%) = \frac{\text{AC} - \text{AS}}{\text{AC}} * 100 \ldots\ldots \text{Eq (3.5)}$$

Where: AS=Absorbances solution with extract

AC= Absorbance of DPPH without extract

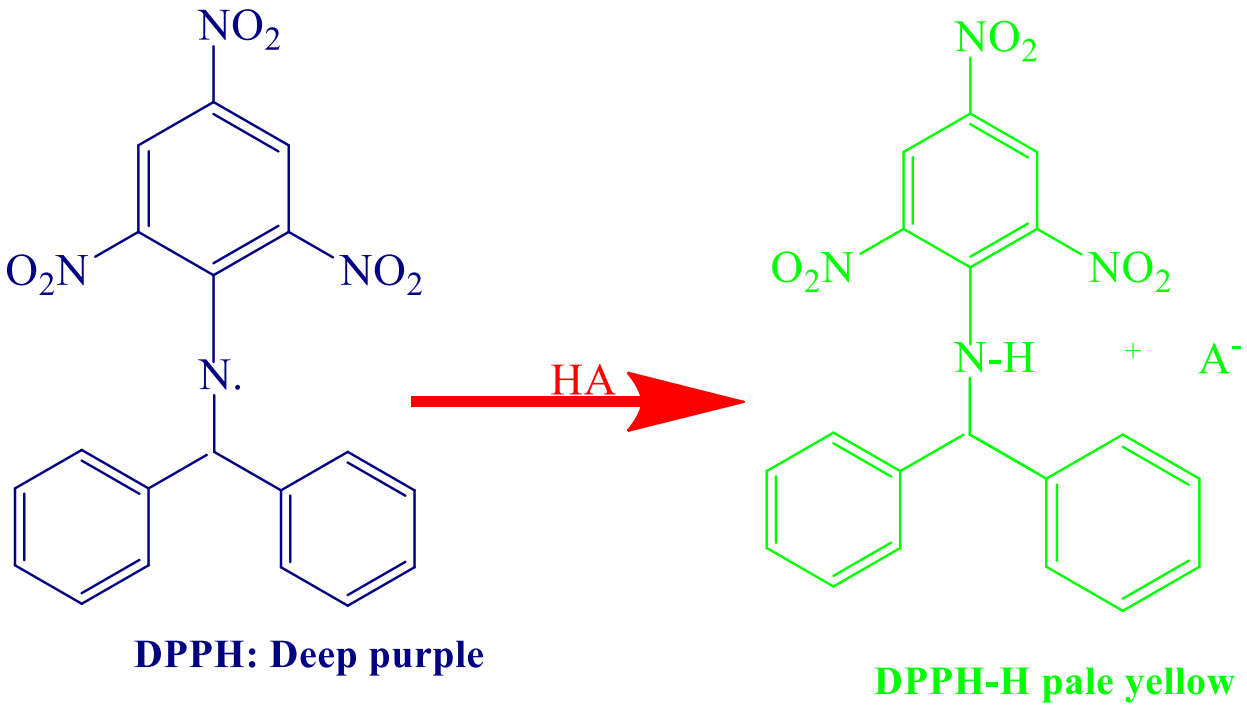


Figure 3. *7* Mechanism of DPPH (2, 2-diphenyl-1-pichrylhaydrazyl)

3.4.6 Measurement of absorbance for standard ascorbic acid solution.

Ascorbic acid was used as a positive control or standard for determination of radical scavenging activity. A stock solution (50mg/ml) of ascorbic acid was prepared by dissolving 2.5 g of ascorbic

acid in to 50 ml of methanol. Aliquots were withdrawn from the stock solution to get ascorbic acid concentrations 0.0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml by mixing 0, 20, 40, 60, 80, 100 and 120µL of standard ascorbic acid with 1000, 980, 960, 940, 920, 900 and 880µL of methanol solvent respectively in different 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 minutes 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. The experiment was carried out in duplicate

3.4.7 Measurement of absorbance for sample solution

Sample stock solution of extract (50 mg/ml) was prepared by dissolving 1 g of extract in to 20 ml of ethanol. From the stock solution, aliquots were withdrawn to get different concentrations. 0.0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml of sample solution were prepared by mixing 0, 20, 40, 60, 80, 100 and 120 µL of aliquots from the stock solution with 1000, 980, 960, 940, 920, 900 and 880µL 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. The experiment was carried out in triplicate

3.5 Characterization of avocado (*Persia Americana mil.*) crude extract

3.5.1 Functional group determination

The absorbed radiation is converted into rotational and/or vibrational energy by the sample molecules. The resulting signal at the detector presents as a spectrum, typically from 4000 cm^{-1} to 400cm^{-1}, representing a molecular fingerprint of the sample. Fourier Transport Infrard Spectroscopy (FTIR) transmission spectra was carried out through a spectrometer coupled to hyperion microscope. The obtained IR spectra were scanned from wave number ranging from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} (Alara et al. 2018). The spectra obtained for the extracts

were interpreted with a chart for characteristics infrared absorption frequencies of functional groups.

3.5.2 Compositional Analysis

Bioactive compound from crude extract was determined using Gas chromatography mass spectroscopy (GC-MS). Mass spectra of the column eluate was recorded in Dynamic MRM mode. The instrument was operated with a capillary voltage of 3500 V and a nozzle voltage of 1000 V. Nitrogen was used as the nebulizer gas at 60 psi, a carrier gas of 8 L/min at 350 °C and a sheath gas of 10 L/min at 400 °C. GC-MS were acquired in negative ionization mode to obtain the m/z of the fragment ion.

3.6 Bioactive film development and its characterization

3.6.1 Preparation of gelatin based bioactive film

The active films can be made from natural biopolymers, such as proteins, lipids and polysaccharides or the combination of these materials (Tharanathan, 2003). Among proteins, gelatin has been impressively used for the development of active packaging due to its relative abundance and good film forming ability (Arfat et al., 2014). Gelatin film was prepared using the casting technique as described by Suderman et al. (2016) with some modifications. (4 % w/v) of gelatin powder was mixed with different amounts of avocado seed extract (0, 1,3,5,7 and 10 w/w %) in 100 mL of distilled water under mechanical stirring until it was completely dissolved. The film-forming solution was then added with 30 % glycerol as a plasticizer. All mixtures were stirred at 45° C for 30 mins in order to obtain a homogeneous gelatin film solution. Thereafter, the film-forming solutions were put into a Petri dish and dried at 35°C in an oven for 36 days. Dried films were then removed from the Petri dish and conditioned in desiccator prior to characterization.

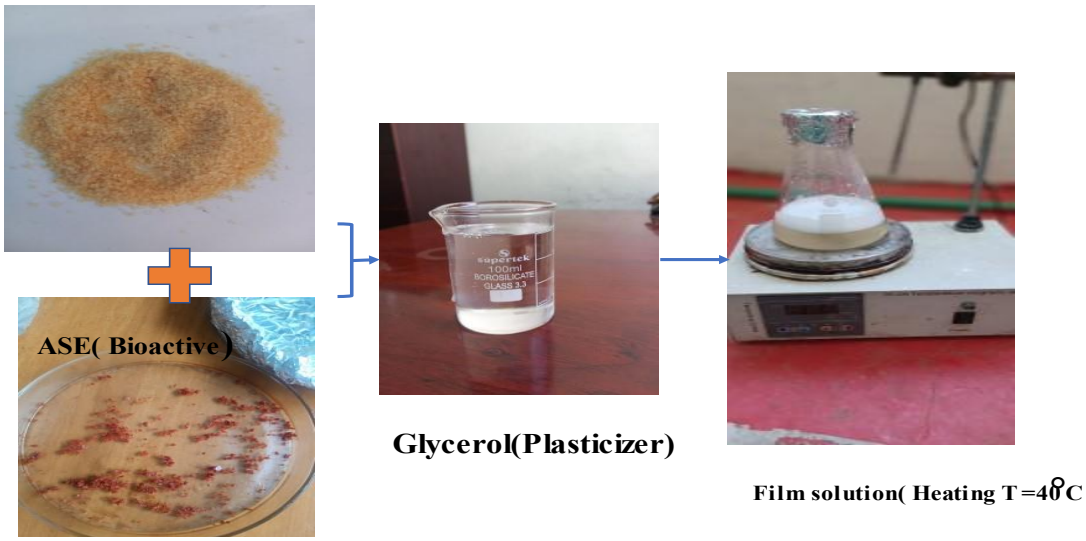


Figure 3. *8* Preparation of gelatin-based film incorporating with ASE

3.6.2 Characterization of gelatin-based film incorporated with avocado seed extract (ASE)

3.6.2.1 Determination of Fourier transform infrared spectroscopy (FTIR)

FTIR spectrum was used to measure the functional group that arose from the blending of film materials. Infrared spectra were recorded using a Thermo Nicolet 380 Spectrometer (Fisher Scientific Inc, USA) with a deuterated triglycerine sulphate (DTGS) detector as described by Suderman et al. (2016). The sample holder comprised a multi-ounce horizontal attenuated total reflectance (HATR) plate of zinc selenite (ZnSe) crystal. The plate was cleansed with acetone and a background spectrum (without sample) was collected. Film samples were then affixed to the plate and spectra recorded. Resolutions varied between 4000 to 400cm^{-1} over 32 scans. Each analysis was repeated three times.

3.6.2.1 Antioxidant Activity of film incorporated with avocado seed extract (ASE)

The antioxidant activities of the films incorporated with C. asiatica were measured by the method stated by (Li et al. 2014). Approximately 25 mg of each film composite was dissolved in 5 ml of distilled water by continuous stirring. Then 0.1 mL of film extract solutions were added to 4 mL of the DPPH solution (0.1 mM methanol solution) followed by 60 minutes incubation in the dark

area at ambient temperature. The absorbance was measured at 517 nm against pure methanol. The percentage of DPPH radical-scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity}(\%) = \frac{\text{Ab C} - \text{Ab s}}{\text{Ab C}} * 100 \ldots\ldots\ldots \text{Eq (3.6)}$$

Where Ab c is the absorbance of DPPH before adding the antioxidant agent (ASE)

Abs is the absorbance of DPPH after adding antioxidant (ASE)

Absorbance at 517 nm.

3.6.2.2 Water solubility (WS)

Water solubility of the films was carried out according to (Nouraddini et al. 2018). The weights of the 2 × 2 cm sections of the film samples were measured and then stirred in 30 mL of distilled water. The samples were weighed after drying at 105 ◦C for 24 h and removing the excess water. The following equation was used:

$$\text{WS}(\%) = \frac{\text{Wf} - \text{Wi}}{\text{Wi}} * \ 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Eq} \ \ (3.7)$$

Where WS is water solubility of film

Wi and Wf is the initial and final mass (g) of film sample respectively

CHAPTER FOUR

4. RESULT AND DISCUSSION

4.1 Characterization of avocado seed for extraction

4.1.1 Moisture Content Determination

Moisture content on a dry basis (only determined on seeds) is most commonly used for describing moisture changes during drying. After the flesh part was removed and 300 g of fresh avocado seed were dried by oven dryer 55 ^{0}C and by taking three samples in each 2-hr. difference in the last drying time as shown in the table below, and the average moisture content was calculated as Mean $\pm$ SD. According to the findings, the liquid moisture content weighed 48.7 % (w/w).

Table 4.1The moisture content of avocado seed powder

No.	Time (Hr.)	Wt.(g) of sample	Moisture content (%)
1	Initial wt.	300	
2	Last 2 hours	155.8 ± 0.03	48.0
3	Last 4 hours	154.9 ± 0.02	48.3
4	Last hours	154.2 ± 0.04	48.6
5	Last 8 hours	153.7 ± 0.06	48.7
6	Last 10 hours	153.7 ± 0.08	48.7

The result obtained 48.7± 0.08 and was higher than the moisture content obtained with avocado seed by Páramos et al. (2020), which was 46 ± 1 wt%.

4.2 Effect of Extraction Process Variables on Yield of extract and Total phenolic content (TPC)

Extraction is the first step in studying plants with bioactive properties because it is necessary to separate and characterize the desired active compounds from plant materials (S. Sasidharan1 et al. 2011). The solid-liquid extraction is greatly influenced by a variety of variables, including solvent composition, extraction time, temperature, and solid-to-liquid ratio. Because of the large difference in polarity between phenolic compounds, alternative polarity solvents were required (water, acetone, methanol, ethanol, or their mixtures with water). Due to the different polarity of the bioactive ingredients and the suitability of this solvent system for human consumption, ethanol and water/ethanol mixtures were perhaps the best solvent systems for the extraction of phenolic compounds (M. Dent et al. 2013). An experimental approach is developed, which allows the optimal process parameters to be found by a reduced number of experiments.

4.2.1 The effect of solvent ratio on yield of extract and total phenolic content (TPC)

The effect of ethanol concentration on extraction yield grew with increasing concentration for a wide range. This operation was performed by keeping the temperature, time, and solid-to-solvent ratio constant. Due to an increase in the hydrolysis rate of ethanol soluble components, the extraction of phytochemical compounds from avocado seeds increased dramatically as the concentration of ethanol increased from low to high. In addition, as the concentration of ethanol further increased, concentration of OH- present in the solvent was increasing and led to the degradation of phytochemical compounds. Selection of appropriate concentration of a solvent, which dissolves maximum amount of antioxidant compounds (phenolic compounds). Usually, solvents as methanol, ethanol, acetone, ethyl acetate, etc., at different volume fractions in water have commonly been used for extraction of polyphenols from different plants (O. A. Folasade et al.2016). In this research water, ethanol, and aqueous solutions of ethanol (0%, 50%, 75% and 100%) were used for the extraction of avocado seed polyphenols.

Table 4. *2* The effect of solvent ratio on the yield of extract and total phenolic content (at constant temperature (*80* ^{O}C), time of extraction (6 Hr.) and solid to solvent ratio (1:12.5)

Run	Solvent Ratio (EtOH: H_2O (%)	Mass of extract	Gallic acid equivalent concentration (mg/ml)	Absorbance (λmax =760 nm) Mean ± S. D	Df.	Extract of yield(wt.%)	TPC (mg GAE/g
1	0:100	2.12 ± 0.21	2.74 ± 0.24	0.854 ± 0.16		10.6 ± 0.43	54.8 ± 0.21
2	50:50	2.25 ±0.32	2.98±0.46	0.95±0.27	-	11.27±0.22	59.58±0.32
3	75 :25	2.64±0.23	3.112 ± 0.25	0.985 ± 0.41		13.20 ± 0.31	62.24 ± 0.32
4	100:0	2.81 ± 0.23	3.22 ± 0.14	1.013± 0.33	-	14.05 ± 0.35	64.45 ± 0.43

It can be seen that as the ethanol ratio rises and the water ratio falls, the yield rises as well. This study revealed that reducing the water content of ethanol boosted the extract yield and TPC. it is evident that an increase in the volume fraction of water does not have positive influence on the extraction efficiency of phenolic compound. However, overall extract yield decreased as water content increased Figure 4.1 showed that the yield of extract increased from the less ethanolic extract (0/100 %) which was 10.6 ± 0.43 % to higher ethanolic extract (100% EtOH) which was 14.05 ± 0.35 %. So, the maximum extract yield was 14.05 ± 0.35 %. % at 100 % ethanolic extract. The reason for this is that when the ethanol ratio rises, the solubility of the extracted antioxidants also rises, resulting in a faster extraction rate. Other authors reported similar results, such as Yilmaz and Toledo (2006), who found that lowering the amount of water in the combination from 30% to 10% enhanced the yield of ethanol extracts from grape seed powder. In a separate investigation, Cacace and Mazza (2003) discovered that the extraction of anthocyanins from black currants using aqueous ethanol increased with ethanol concentration, peaking at around 90%.

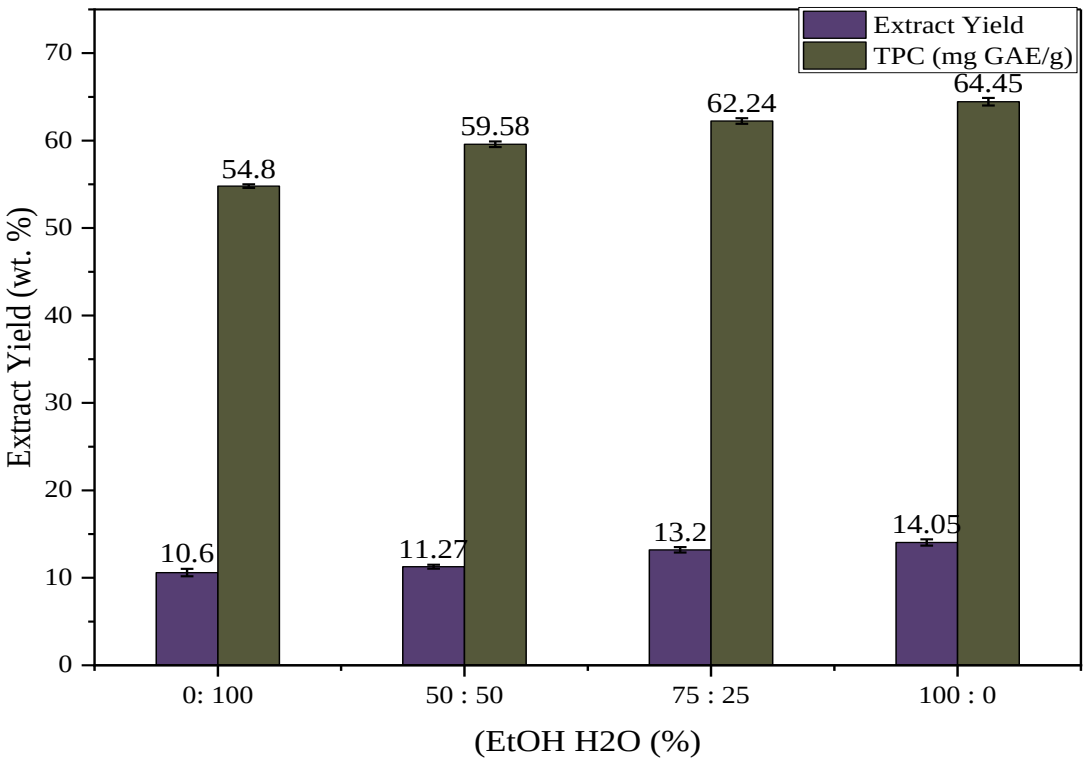


Figure 4. *1* The effect of solvent ratio on yield of extract and total phenolic content (TPC) of avocado seed extract constant temperature (80 ^{O}C), extraction time (6 hour) and solid to solvent ratio (1:12.5)

Figure 4.1 showed that all solvents were capable of extracting phenolics but ethanol (100%) was a more effective solvent than ethanol (50%), ethanol (75%) and (water 100%) for extracting avocado seed extract samples. Ethanol (100%) solvent gave the highest TPC which was (64.45 ± 0.43 (mg GAE/ g dw) followed by ethanol (75%) (62.24 ± 0.32 mg GAE/ g dw) and ethanol (50%) was (59.58 ± 0.35 mg GAE/ g DW). (Y.Y. Soong et al. 2004), studied the capacity of total phenolic content and antioxidant capacity of edible parts and seed kernels of different fruits. In this study the amount of total phenolic content (TPC)only by aqueous ethanolic extract in mango kernel seed was 117 ±13.5, tamarined seeds 94 ±4.9 and avocado seed kernels (88.2 ± 2.2).

It can be seen from the result with rising of the ethanol ratio and decreasing water ratio yield was generally increased. The reason was that as the ethanol ratio increased would increase the solubility of the extracted antioxidants, giving a higher rate of extraction. J.G. Rodríguez-Carpena et al. (2011), studied the effects of verities and types of solvent (dilution of ethyl acetate, acetone and

methanol) on the total phenolic content and antioxidant activities of avocado peels and seed kernels. The result obtained for peel of 'Hass' variety was ranged from 32.93 ±9.25 to 89.97 ±31.03 and for 'fruete' variety the result was ranged from 40.54 ± 10.08 to 172.18 ± 14.46 (mg GAE/g). The result obtained for avocado seed for Hass avocado was ranged from 35.11 ± 9.98 to 60.82 ±8.63 while for 'frute' avocado it was ranged from 20.29 ± 7.15 to 69.12 ±16.9 (mg GAE /g). Based on these experimental results, it was believed that highly active phenolic compounds presented in avocado seed were moderately polar. According to the principle of "like dissolve like", solvents would only extract those compounds which have similar polarity with the solvents (Spigno and De Faveri, 2007; Zhang and Wang, 2005).

4.2.2 The effect of temperature on yield of extract and TPC

Temperature has a great influence on the extraction yield of the active component. But higher extraction temperature could produce degradation of bioactive constituents. To overcome such problems the choice of extraction temperature plays a significant role depending on the final application of the product. The extraction yield of antioxidant extracts and TPC from avocado seeds is influenced by a number of parameters. In this research Extraction temperature (60 ^{O}C, 70 ^{O}C, 80 ^{O}C and 90 ^{O}C) were used for the extraction of phenolic compound of avocado seed (Persea americana mill.) extract. In order to achieve complete extraction, long extraction time (6 hour), extraction solvent (Ethanol) and medium solid to solvent ratio (1:12.5) were used.

Table 4. *3* The effect of temperature on the yield of extract and TPC of avocado seed extract (at constant time (6 Hr.), solvent ratio (100% ethanol) and solid to solvent ratio (1:12.5 mg/ml)

Run	**Temperature (^{O}C)**	**Mass of extract(g)**	**Gallic acid equivalent concentration (mg/ml)**	**Absorbance ((λmax =760 nm) Mean± S. D**	**Df**	**Extract of yield (wt. %)**	**TPC (mg GAE/g)**
1	60	2.19 ± 1.12	1.289 ± 0.32	0.529 ± 0.43	2	10.95 ± 0.26	51.57 ± 0.32

2	70	2.72 ± 0.56	3.218 ± 0.21	1.012 ± 0.29	_	13.6 ± 0.21	64.36 ± 0.26
3	80	2.86 ± 0.35	1.425 ± 0.101	0.563 ± 0.38	2	14.3 ± 0.20	57.00 ± 0.43
4	90	2.82 ± 0.45	1.385 ± 0.153	0.553 ± 0.1	2	14.15 ± 0.12	55.40 ± 0.24

Table 4.3 showed the effect of temperature on the yield of avocado seed extract and TPC. During extraction all the process parameters are kept constant. It was observed that the yield slightly decreased when temperature increased from 80 to 90 which was 14.3 % and 14.15 % respectively and increases when increased from 60 to 80 which was 10.95 % and 13.6 % respectively. The reason is that as the extraction temperature increased, the rate of solubility of phytochemical compounds was increasing and gave high rate of extraction. According to (Wells 2003), increasing the temperature improves the extraction efficiency because it makes the cell permeable, increases the solubility and diffusion coefficients of the compounds to be extracted, and lowers the viscosity of the solvent, allowing it to pass through the solid substrate mass more easily. This result was in agreement with Cruz et al. (2004), who found that increasing the temperature improves solvent extraction of antioxidant compounds, enhancing both diffusion coefficients and solubility. The results obtained in this study were better than the extraction yield of avocado seed using Soxhlet Extraction with EtOH at 80 ^{0}C. (10.3 % Wt.) Patricia Renata et al. (2019). From the present investigation, maximum yield was found to be 14.3 % at a temperature of 80 ^{O}C. extraction time and solvent ratio.

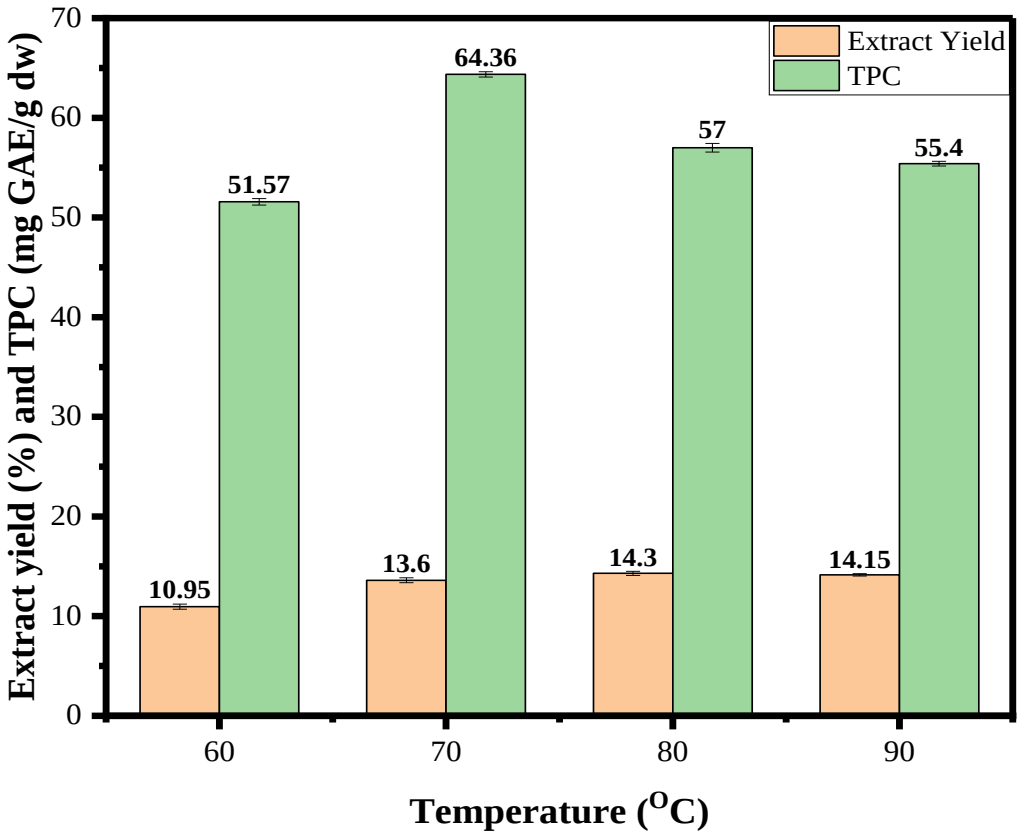


Figure 4. 1 The effect of temperature on the yield of extract and total phenolic content (TPC) at constant time (6 Hour), solvent ratio (100% EtOH) and solid to solvent ratio (1:12.5)

It is well known that a higher temperature would likely improve the solid matter's solubility. The objective of this study was to determine the ideal temperature that would produce a higher TPC value and how the temperature would affect the phenolic compounds in the seed extract. Fig. 4.2 represent the effects of temperature on extraction yield and total phenolic content (TPC) and the extraction yield was found to be the highest under a maximum temperature of 80 °C which was 14.3 % and the minimum extraction yield was recorded at a minimum temperature of 60 °C which was 10.95%. However, higher temperature decreased the total phenolic content because the high temperature causes the oxidation and degradation of the desired compounds (Silva EM.et al.2007). The highest total phenolic content was recorded at a temperature of 70 °C which was 64.36 ± 0.26 (mg GAE/g de) and the minimum TPC recorded was at a temperature of 60 °C which was 51.57 ± 0.32 mg GAE/g dw). It is observed that after 70 ^{O}C increasing extraction temperature does not

have appositive impact on the production of TPC. The reduction of TPC after 70 ^{O}C was the indication of phenolic compound degradation with increasing temperature. The result obtained at 70 ^{O}C had a significant increase in the amount of TPC extracted, which was 64.36 mg GAE per gram of dry extract, compared to the extraction temperature at 60 ^{O}C, which was 51.57 mg GAE per gram of dry extract or a 24.8% increase. Therefore, moderate extraction temperatures of 70 °C were chosen as the temperature for obtaining highest TPC from avocado seed extract under investigation.

4.2.3 The effect of solid to solvent ratio on the yield of extract and TPC

The volume of extracting solvent is another important factor, a large volume of solvent requires more energy and time to condense extraction solution in the purification process. Plant matrix needs to be totally immersed in the solvent for higher recovery during the extraction process. In general, higher volume of solvent will increase the extraction yields in conventional extraction methods (Mojzer et al., 2016).

Table 4. *4* The effect of solid to solvent ratio on the yield of extract and TPC at constant temperature (70 ^{O}C), time of extraction(6Hr.) and solvent ratio (100% EtOH)

Run	Solid to solvent ratio	Mass of extract(g)	Gallic acid equivalent concentration (mg/ml)	Absorbane (λmax=760 nm) Mean± S. D	Extract of yield(wt.%)	TPC mg GAE/g
1	1:10	2.52 ± 0.04	3.09 ± 0.13	0.98 ± 0.48	10.63 ± 0.13	61.8 ± 0.26
2	1:16.6	2.72 ± 0.02	2.97 ± 0.08	0.951 ± 0.62	13.6 ± 0.16	59.41 ± 0.44

3	1:8.3	2.490 ± 0.12	3.03 ± 0.17	0.967 ± 0.35	9.96 ± 0.11	60.78 ± 0.63
4	1:12.5	2.1 ± 0.14	3.23 ± 0.25	1.015± 0.28	14.0 ± 0.1 2	64.61 ± 0.72

The highest extraction yield obtained was at a solid to solvent ratio of 1:12.5(20g for 250 ml solvent) which was (14.0%) and the minimum yield obtained was at solid to solvent ratio 1:8.3(30 g to 250 ml solvent) which was (9.96%). On a similar matter the highest TPC also recorded at a solid to solvent ratio of 1:12.5 which was (64.61 ± 0.72mg GAE/g. dw) This can be noted in these results, extraction yield increased as solvent to sample ratio increased (the higher solid to solvent ratio produced the highest yield of extract), which was about 14.0 ± 0.12 %. The most effective ratio was 1:12.5 and 1:16.6 which produced 14.0 ± 0.12 % and 13.6± 0.16% of avocado seed extract respectively, But the amount of TPC was higher at a solid to solvent ratio of 1:12.5 and 1:10 which was 64.61 ± 0.72mg GAE/g. dw and 61.8 ± 0.26 mg GAE/g dw. The increase of extraction yields with the increase of solvent to solid ratio is consistent with mass transfer principles. Smaller volumes of solvent can lead to incomplete target extraction while larger volumes can make the extraction procedure becomes complex and wasteful (Touati and Meniai, 2011)

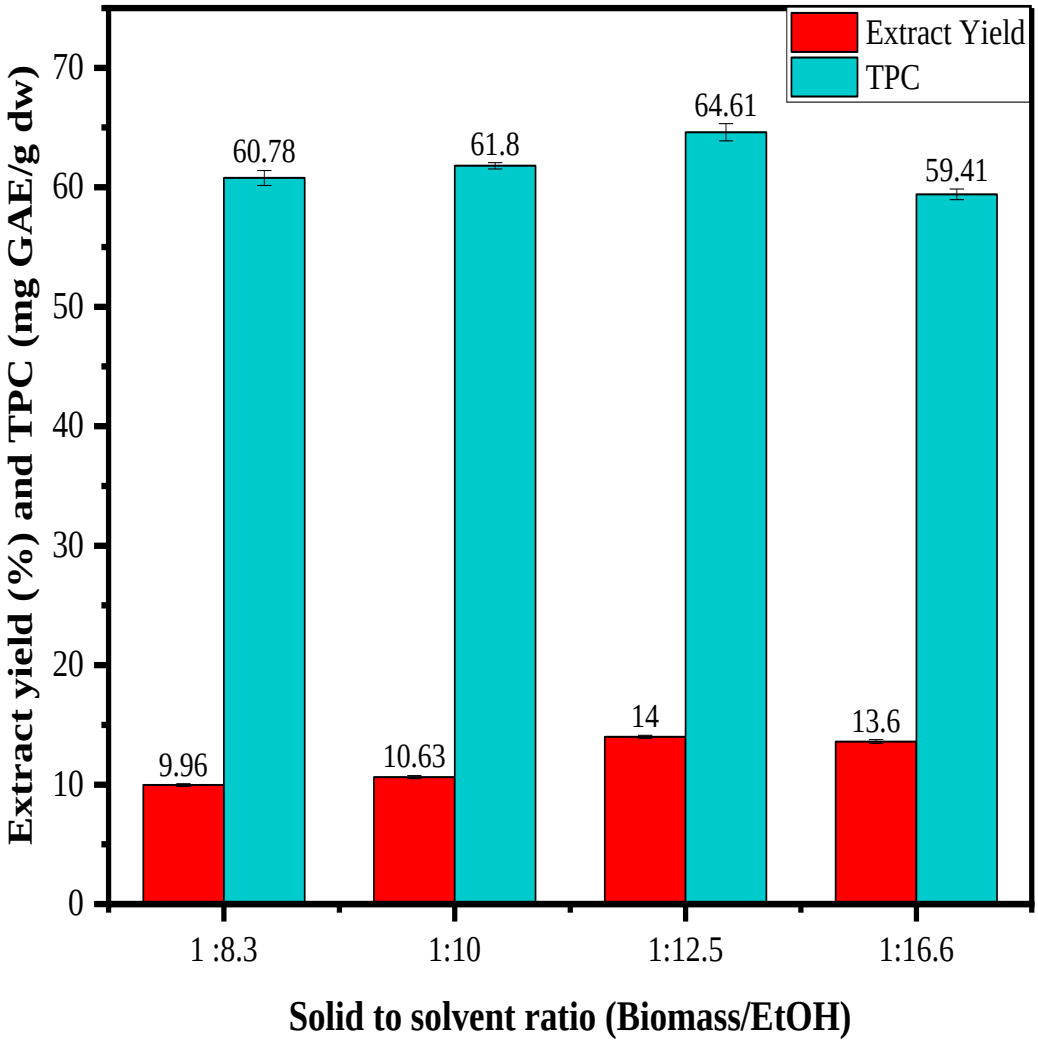


Figure 4. *3* The effects of solid to solvent ratio on the total phenolic content constant temperature (70 ^{O}C) extraction time (6 hour) and solvent ratio (100 % EtOH)

The aim was to minimize the amount of solvent and determine the appropriate proportion of biomass to solvent ratio, at which maximum amount of target components is derived. It can be clearly seen that the recoveries of extracts and phenolic compounds tend to increase as the feed/solvent increases from 1:8.3 g/mL through 1:12.5 g/ ml. Beyond 1:12.5 g/mL of feed/solvent, the yields were declined. The maximum recovery yields of extracts and TPC were obtained at 1:12.5 g/ ml and further increase reduced the yields. The solid-to-solvent ratio between 10:1 (ml/g)

and 20:1 (ml/g) had been reported to give optimal yields (Vegga masginani et al., 2013). Therefore, the feed-to-solvent ratio of 1:12.5 g/ml was considered as optimal for the other parameters (temperature, time and solvent ratio).

4.2.4 The effect of extraction time on the yield of extract and TPC

Extraction time is the time required to extract the active ingredient present in the avocado seed. Extraction time plays significant role on the yield. In a general way, extraction rate will be increased along with extraction time increase. Excessive extraction time, on the other hand, would be unnecessary because the solvent and sample would be in final equilibrium after a specific period of time. This was based on Fick's second law of diffusion; the rate of compound extraction would slow down through time (Teixeira et al., 2014).

Table 4. *5* The effect of extraction time on the yield of extract and total phenolic content (TPC) of extract at constant temperature(70^{O}C), solvent ratio (100 %EtOH) and solid to solvent ratio (1:12.5)

Run	Time (Hr.)	Mass of extract(g)	Gallic acid equivalent concentration /9g/ml)	Absorbance (λmax=765 nm) Mean± S. D	Yield of extract (wt.%)	TPC (mg GAE/g
1	2	2.226 ± 0.21	2.911± 0.34	0.86 ±0.36	12.85 ± 0.12	58.2 ± 0.33
2	4	2.815 ± 0.26	3.202 ± 0.23	1.018 ± 0.14	14.10 ± 0.45	64.04 ± 0.34
3	6	2.81 ± 0.11	3.201 ± 0.41	1.005 ± 0.41	14.05 ± 0.33	64.02 ± 0.56

4	8	2.79 ± 0.12	3.198± 0.21	0.89 ± 0.24	14.01 ± 0.11	63.98 ± 0.51

Time is one of the most important factors that alter the recovery of phenolic compounds from plant matrix. This is due to the fact that over-exposure of plant sample to localize heating tends to degrade phenolic compounds (Mojzer et al., 2016). Thus, it is essential to determine the appropriate extraction time for higher recovery. As shown in the Figure 4.4 The yield was increased when extraction time increase from 2 to 4 hour and the yield was nearly similar increased from 4 to 8 hour. Hence, a prolonged exposure of the sample in the solvent, allowed sufficient time for the desired compounds to migrate into the solvent. The maximum yield was found when the extraction process was going to 4 hours and it was 14.10 %.

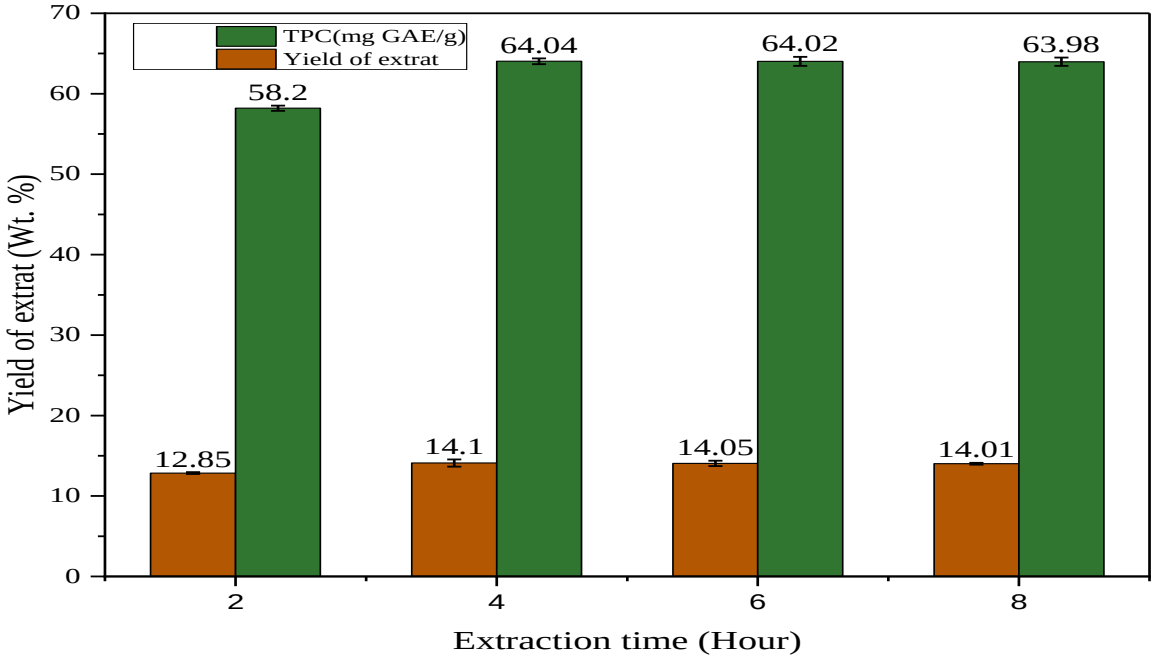


Figure 4. *4* The effect of extraction time on yield of extract and total phenolic content (TPC) at constant temperature (70 ^{O}C), solvent ratio (100 % EtOH) and solid to solvent ratio (1:12.5)

Figure 4.4 showed the TPC extracted from avocado seed extract samples using various range of extraction time. As observed above, the highest value of TPC for extraction time in extraction of phenolic compound was at 4 hours accompanied followed by a nearly similar TPC up to 8-hour extraction time. The total phenolic content (TPC) of avocado seed extract at 4 hours and 2 hours

was 64.04 ± 0.34 and 58.2 ± 0.33 mg GAE/ g respectively. As seen in Figure 4.4 the lowest total phenolic content (TPC) reading was at 2 hours extraction time 58.2 ± 0.33 mg GAE/ g and the maximum total phenolic content (TPC) was 64.04 ± 0.34 at extraction temperature of 4 hours. Tremocoldi MA et al. (2018), also studied the total phenolic content and antioxidant activity of different variety of avocado peels and seed kernels. The result obtained was ranged from (63.5 ±7.2 to 120.3 ± 7.8 mg GAE/g) for seeds and for peels it was ranged from (57.3 ± 2.7 to 59.2 ± 6.9 mg GAE/g).

The range of time was determined based on the practical and economical aspects. It was probably because longer time will increase cost. However, even at longer time, there was not much difference in extraction of phenolic compounds when compared to shorter time. Time does have a significant effect on extraction of phenolic compound as shown in Figure 4.4. Excessive extraction time is not useful to extract more phenolic antioxidants (Silva et al. 2007). Taking into account of these facts 4 Hours extraction time was taken as a time for extraction of higher phenolic compounds from avocado seed extract.

4.2.5 Maximum extraction conditions

Better extraction condition for the simultaneous maximum extraction yields, TPC and DPPH radical scavenging activity were determined. From analysis, the extraction conditions using an extraction temperature of 70 °C, an extraction time of 4 hour, a solvent ratio (Ethanol: water) of 100:0 v/v% and solid to solvent ratio of 1:12.5 could produce the maximum extraction yields, TPC and DPPH radical scavenging activity of 14.30 ± 0.20 %, 64.61 ± 0.72 mg GAE/g extract 71.65 %, respectively. These data are close to those found in the previous study mentioned above and it showed that the quantifications of TPC can vary according to the different protocols of extraction and species analyzed even though they belong to the same genus. In a study by Wang et al. (2010), who assessed antioxidant capacity and phenolic compounds of eight varieties of avocado, the Hass variety presented phenolic compound levels of 12.6, 4.9, and 51.5 mg GAE/g of dry sample for peel, pulp, and seed, respectively. These results were lower than those of present study for the peel and higher for the pulp and seed. This difference is probably due to the different solvents and conditions used in the extraction. According to Soares et al. (1998), a number of factors are important during the extraction process such as the solvent penetration in the cells, dissolution of

extractable substances and solution diffusion outside the plant cell, temperature, pH, the nature of the solvent, extraction time, and the presence of pro-oxidant agents such as metals or light. Daiuto et al. 2014 obtained 63.5 and 57.3 mg GAE/g dry extract for the peel and seed extracts, respectively, of Hass variety avocados extracted using ethanol: water (80:20 v/v). Thus, the ideal method is to choose the most appropriate protocol according to the purpose of active principles application, generating savings and efficiency in the processes. (Embuscado 2015) also showed that the use of different extraction solvents might lead to different TPC responses, which can also vary according to plant genotype, geographic cultivation location, type of crop, and climate

4.3 Anti-oxidant activity of avocado seed extract

The radical scavenging of extracts was tested using a methanolic solution of the "stable" free radical DPPH at room temperature and produce a violet solution in alcohol. It is reduced in the presence of antioxidant molecule. The antiradical activities or free radical-scavenging capacities of essential oil and various extracts from fruits were determined by comparing with the activities of known antioxidants, such as trolox and ascorbic acid by 2,2 -diphenyl-1-picrylhydrazyl (DPPH) radical assay. The reduction ability of DPPH radicals' formation was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The evaluation of the antioxidant activity of extract was determined based on the scavenging activity against the free radical DPPH through IC_{50} parameter. Under this work the investigation of antioxidant activity of the extract was based on the optimal values taken from the total phenolic content (TPC) optimization of different extraction parameters of the extract. The lower IC_{50} indicates a greater ability of neutralize the free radicals.

A freshly prepared DPPH solution exhibited as a deep purple color complex with absorption medium. Thus, antioxidants quenched DPPH free radicals (i.e., by providing hydrogen atom or by electron donation, conceiving via free radical attack on the DPPH molecule) & converted them to a color less/bleached product resulting in a decrease in absorbance. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract. Antioxidant activities of Avocado seed powder were expressed as percentage of DPPH radical inhibition and IC_{50} values (μg/mL). IC_{50} values of the samples were calculated from the concentration Vs percent inhibition curve

Table 4.*6* Absorbance value and % of radical scavenging activity for standard ascorbic acid

Ascorbic acid Concentration (mg/ml)	**Absorbance (λmax=517nm)**	**RSA (%)**	**IC_{50} (mg/ml)**
Control (0)	1.03 ± 0.001	0	__
1	0.509 ± 0.043	50.24	
2	0.463 ± 0.058	55.5	
3	0.366 ± 0.039	64.4	0.9079
4	0.353 ± 0.067	65.72	
5	0.329± 0.019	68.6	
6	0.203 ± 0.032	80.2	

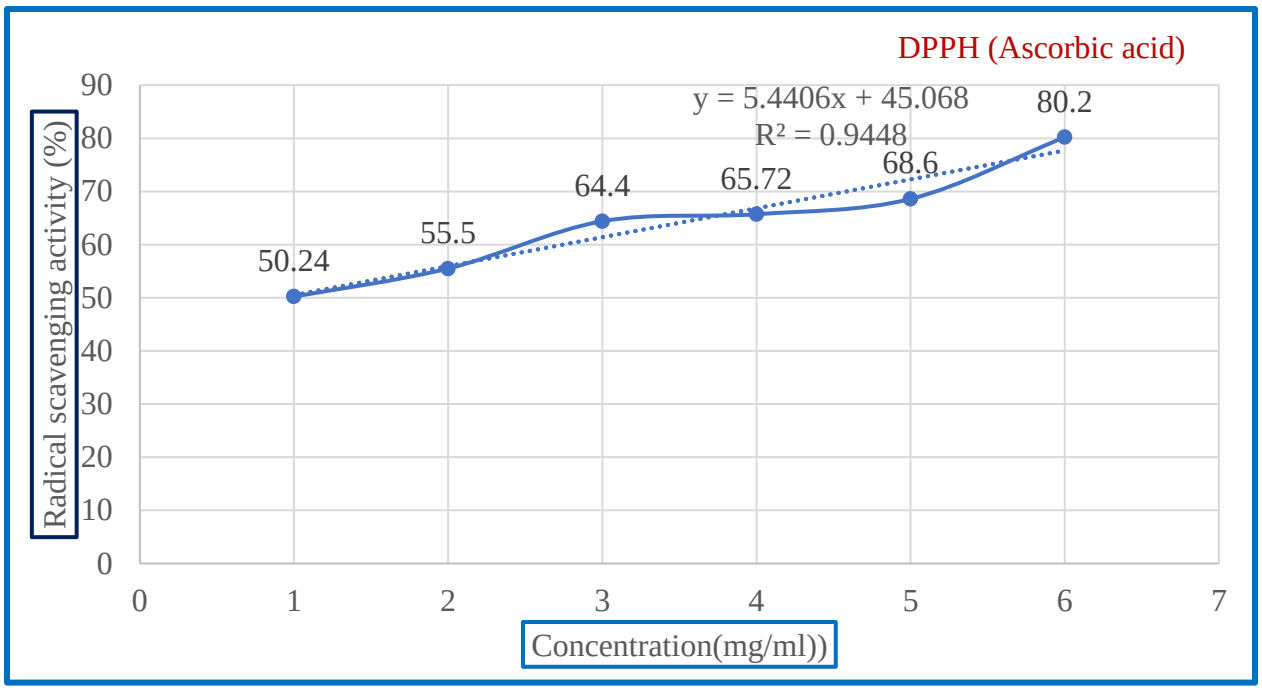


Figure 4. *5* Absorbance Values and % Inhibition of standard ascorbic acid

IC50 value gives the0 concentration required for 50% inhibition [86] was evaluated using the following equation given below.

$$IC\ 50\% = 50 - y\frac{intercept}{slope(m)} \ldots\ldots\ldots\ldots\ldots\ldots Eq$$

IC 50% - The concentration at 50% radical scavenging activity

Y-intercept - y value from the linear equation

m – slope from the equation

Table 4. 7 Absorbance value and percent of radical scavenging activity (RSA%) for avocado seed extract (Persea americana mill)

Sample Concentration (mg/ml)	**Absorbance (λmax=517nm)**	**radical scavenging activity (%)**	**IC_{50} (mg/ml)**

0	1.03	0	
1	0.560 ± 0.057	45.8	
2	0.526 ±0.029	50.8	
3	0.476 ± 0.01	53.78	1.97
4	0.408 ± 0.04	60.38	
5	0.401 ± 0.12	61.02	
6	0.292 ± 0.25	71.65	

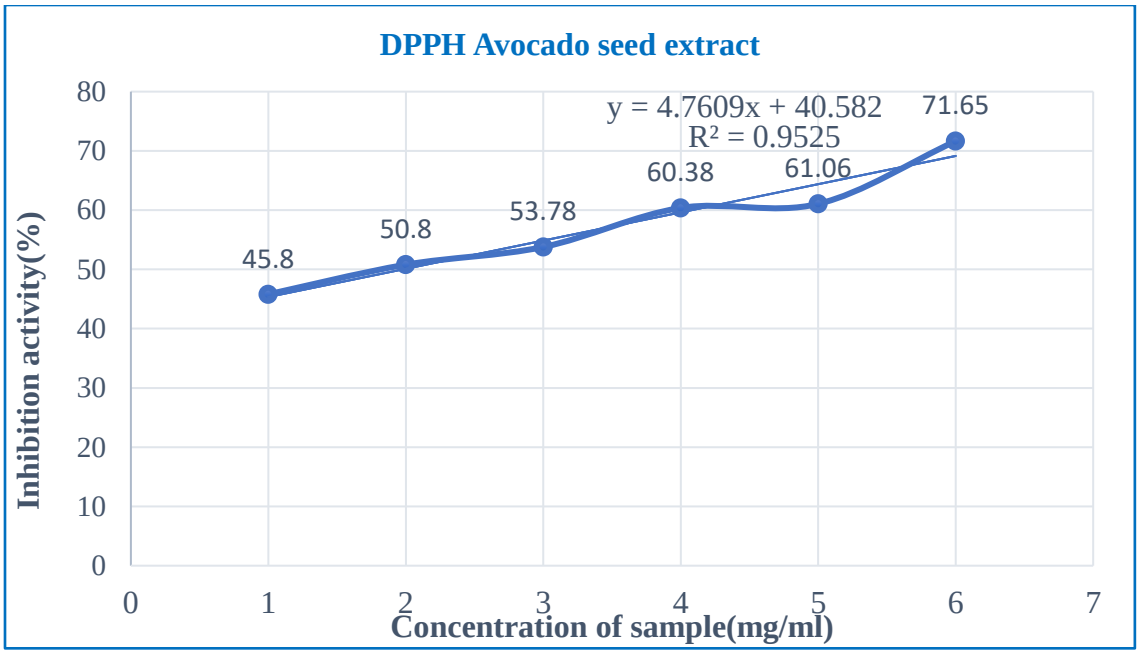


Figure 4. *6* Absorbance value and percent of radical scavenging activity (RSA%) for avocado seed extract.

The radical scavenging activity percentages of extracts (persea americana avocado seed) were presented in the table 4.7 and also illustrated in the figure 4.6. The concentrations for both ascorbic acid and avocado seed extracts were ranged in the same interval and their percentage of free radical scavenging activity was compared each other. In this study, different concentration was prepared and ranged from (1.0 to 6.0 mg/ml) for its respective antioxidant activity or free radical scavenging activity was ranged from 50.24 to 80.2 % for standard ascorbic acid (AA) and from 45.8 to 71.65% for avocado seed extract (ASE). At concentration (1.0mg/ml), the lowest capacity of free radical scavenging activity for standard ascorbic acid and avocado seed extract was 50.24 % and 45.58 % respectively was achieved. On the other extreme at the concentration of 6.0 mg/ml, the highest capacity of free radical scavenging activity for standard ascorbic acid (AA) and avocado seed extract was 80.2 % and 71.65 % respectively. The percentage scavenging activities of the of extracts was compared to the percentage of radical scavenging activity of the positive control or standard ascorbic acid, to investigate that the extracts had the capacity to remove free radicals or prevent the formation of radicals.

The other parameter used to compare potent of antioxidant activities of extract with standard ascorbic acid was IC_{50}, the concentration of samples (ascorbic acid or avocado seed extract) in

which the samples had the capacity to remove 50% of free radicals. IC_{50} was calculated using linear regression. As illustrated in the fig 4.6, ascorbic acid had the capacity to scavenge 50% of free radicals at the concentration of (0.9079 mg/ml), similarly extract had the capacities of removing 50% free radicals at the concentration of (1.97 mg/ml). Low IC_{50} indicates more potent in antioxidant activity. According to (S. Phongpaichit et.al,2007), extracts which possess IC50 values >2500- 3000 µg/mLis considered to exhibit intermediate antioxidant activity. Meanwhile, extracts with IC50 value <2500 µg/mL is considered to possess strong antioxidant activity. Therefore, the result obtained in this work was categorized under strong antioxidant activity. The present study was compared with the past investigation in terms of IC_{50}. According to the investigation of (Park et al.,2014), 50% antioxidant activity was achieved at the concentration (IC_{50}) of 1.1379 mg/ml for variety of Auranthium orange peel extract. The result obtained in this study was in better agreement with (Cid-Pérez Teresa Soledad et.al 2021), which was 61.05 % with ethanolic extract of avocado seed extract and 56.35 % of acetone extract of avocado seed extract. This may be the investigation for antioxidant activity under this work was taken from the maximum values of total phenolic content (TPC) among the different extraction parameters of temperature, time, types of solvent and solid to solvent ratio.

4.4 Characterization of Avocado seed extract

4.4.1 Fourier Transform Infrared (FTIR) Analysis of Avocado seed extract

Fourier transform infrared (FTIR) is one of the widely used characterizations for the identification and elucidation of functional groups in the plant extracts. This characterization was used to identify functional groups in the extract from Avocado (Persea americana) seed extract at the maximum conditions of Soxhlet extraction based on the peak value in the region of infrared radiation.

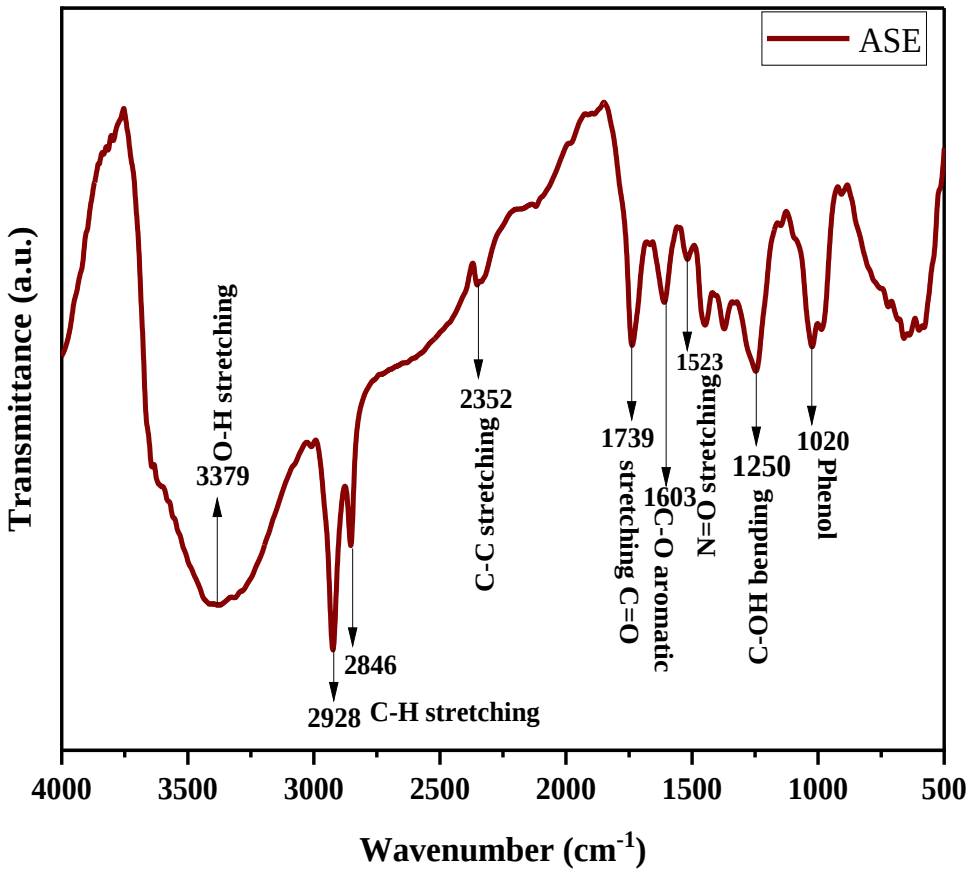


Figure 4. *7* FTIR analysis of avocado seed (Persea Americana mill.) extract

The FTIR spectra of the avocado seed extract (Fig 4.7) revealed the characteristic bands located around 3379 cm^{-1} with a broad peak was the –OH stretching group which presence shows high concentrations of amides amines, phenol and alcohols, 2928 cm^{-1} with sharp peak and 2846 cm^{-} shows C-H stretching which is the presence of alkanes and carboxylic acid overlapped. 1739 cm^{-} with sharp peak corresponds to C=O asymmetric stretching Esters, 1250 cm^{-1} with sharp peak corresponds to C-OH bending, 1603 with sharp peak correspond to C-O aromatic and 1020 corresponds to phenols (N. K. R. Bogireddy et al. 2018). Thus, the observed characteristic fingerprints in the extract from avocado seed extract using Soxhlet extraction method reflected the presence functional characteristics associated with phenolic compounds. The corresponding bands have been attributed to the presence of phytochemicals in the AS extract.

Table 4. *8* Functional groups and their corresponding peak intensities of avocado seed extract

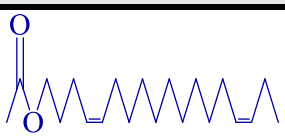
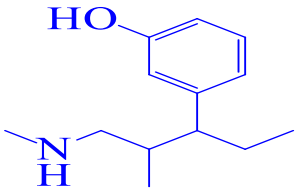
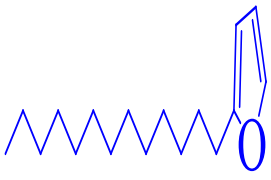
No.	Peaks (Cm^{-1})	Stretching frequency (Cm^{-1})	Bond	Name of compound
1	3374	3200-3600	O-H	Alcohols, Phenols
2	2928	2700-3300	C-H	Alkanes and carboxylic acid overlapped
3	2352		C-C	
4	1739	1630-1820	C=O	Esters, saturated, aliphatic, aldehydes
5	1603		C-O	Aromatic
6	1523		N =O	stretching amide
7	1250	1000-1250	C-O	Stretching Alcohols
8	1020	1000-1250	C-O	Phenols

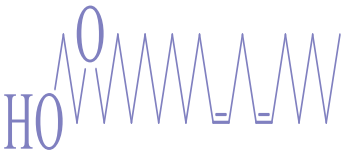
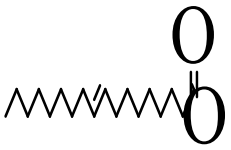
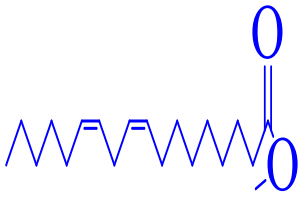
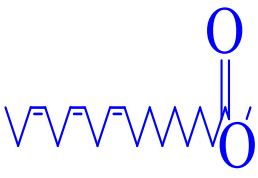
4.4.2 Gas Chromatography- mass spectroscopy (GC-MS) analysis of avocado seed extract

The results pertaining to GC-MS analysis of the ethanolic extract of avocado seed (Persea Americana mill) extract led to the identification of a number of compounds. These compounds

were identified through mass spectrometry attached with GC. The various components present in the extract were detected by the GC-MS are shown in the table below

Table 4. *9* Bioactive compounds (Persea present in avocado seed extract Americana mill).

Compound	**RT**	**Molecular formula**	**Peak area (%)**	**Structure of compounds**
Z,Z-4,15-Octadecadien-1-ol acetate **MW: 308.5**	10.2757	$C_{20}H_{36}O_2$	11.181	
N-Desmethyltapentadol **MW:207.31**	10.7211	$C_{13}H_{21}NO$	1.3443	
n-Hexadecanoic acid, methyl ester **MW:270.45**	11.4106	$C_{17}H_{34}O_2$	22.9349	
2-Tridecylfuran	11.9191	$C_{17}H_{30}O$	2.987	

MW:250.41				
Ethanol, 2-(9,12-octadecadienyloxy)-, (Z, Z)- **MW:310.51**	13.151	$C_{20}H_{38}O_2$	0.9844	
9-Octadecenoic acid, methyl ester, (E)- **MW:296.48**	13.2751	$C_{19}H_{36}O_2$	7.976	
9,12-Octadecadienoic acid (Z, Z)-, methyl ester **MW:294.47**	13.3407	$C_{19}H_{34}O_2$	44.9062	 ,
9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)- **MW:292.45**	13.5373	$C_{19}H_{32}O_2$	3.7453	

2H-Pyran, 2-(7-heptadecynyloxy) tetrahydro- **MW:336.55**		14.0204	$C_{22}H_{40}O_2$	3.941	

MW: Molecular weight, RT: Retention time

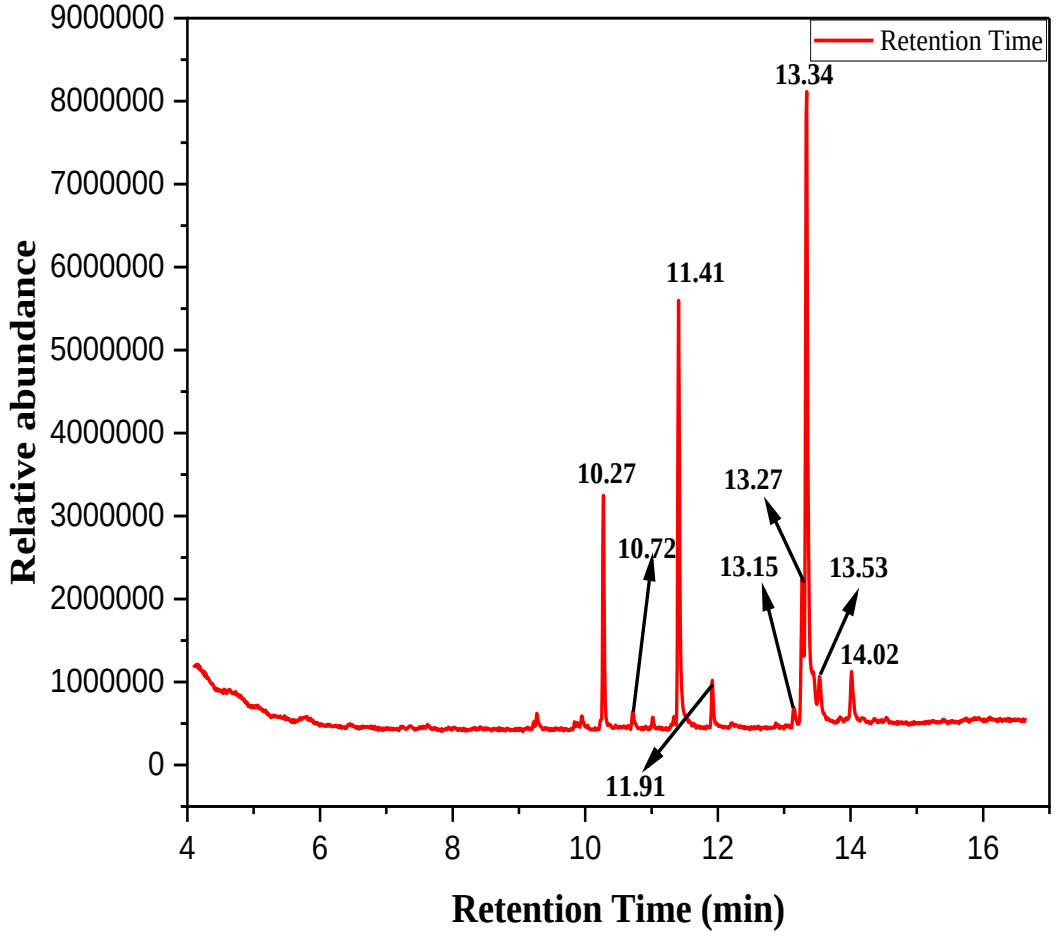


Figure 4. 8 GC-MS chromatograms of ethanolic extract of avocado seed (Persea Americana mill) extract

A total of 9 compounds were identified from the GC-MS analysis of ethanol extracts of avocado seed extract exhibiting various phytochemical activities. The chromatogram was presented in Fig. 4.8, while the chemical constituents with their retention time (RT), molecular formula, molecular weight (MW), and concentration or peak area in (%) in the were presented in table 4.9. The following bioactive compounds were present in the GC-MS analysis carried on ethanol extracts of avocado seed extract. The existence of these phytochemicals in ethanolic extracts of avocado seed (Persea americana mill extract) possibly indicates its numerous biological properties such as anti-inflammatory, anti-microbial and anti-oxidative properties, among others. Among the identified bioactive components 9 ,12-Octadecadienoic acid Z, Z methyl easter has highest percent peak area was (44.99%). This compound has antioxidant, anti-inflammatory, cancer preventive, hepatoprotective, and hypocholesterolemic properties (Starlin T et al.2019). The second most abundance among the compounds was n-hexadecanoic acid, methyl easter which was (22.93%) has antioxidant, 5- alpha-reductase inhibitor, anti-fibrinolytic, hemolytic, antimicrobial activity, hypocholesterolemic nematicide, pesticide, antiandrogenic flavor, and hemolytic properties (Starlin T et al.2019). 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- has antioxidant and antibacterial properties(Gopalakrishnan K et al. 2014).

4.5 Antioxidant activity of Gelatin film incorporated with avocado seed extract

At present, it is generally considered that an important method to determine the antioxidant activity is the scavenging ability of DPPH free radicals. Table 4.10 showed the antioxidant activity of gelatin films with different concentrations of avocado seed (*Persea americana mill*.) extract. The films incorporated with ASE showed higher antioxidant activities than the control film.

Table 4. *10* The effect of different concentration of ASE on the antioxidant activity of the gelatin-based packaging film

Film formulation (wt./wt.% of ASE)	**Absorbance (λmax=517nm)**	**Radical scavenging Activity (%)**

Control	1.03	0
0	0.891 ± 0.42	13.49 ± 0.59
1	0.682 ± 1.32	33.78 ± 1.24
3	0.643 ± 0.1.43	37.57 ± 0.86
5	0.536 ± 1.54	47.96 ± 1.35
7	0.514 ± 1.23	50.09 ± 0.65
10	0.429 ± 1.72	60.29 ± 0.51

Table 4.10 showed the radical scavenging activity in DPPH on gelatin films incorporated with avocado seed (*Persea americana mill*) extract. Control gelatin films showed some scavenging activity on DPPH. This may due to the scavenging activity that occurs between DPPH and gelatin molecules. This is because the radical scavenging mechanism of gelatin occurred when the free radical reacts with the residual free amino (NH_2) groups to form stable macromolecule radicals, and the NH_2 groups can form ammonium groups by absorbing a hydrogen ion from the solution

(Siripatrawan and Harte, 2010). In films containing avocado seed (*Persea americana mill*) extract, the antioxidant activity increased as the amount of avocado seed extract increased from 0.1 g (1%) to 0. 4 g (10%). From the results obtained, gelatin film with 0.4 g avocado seed extract showed the highest scavenging activity against DPPH radicals. So, the higher antioxidant activities in gelatin film with avocado seed extract were due to high amounts of phenolic compound contained in the extract

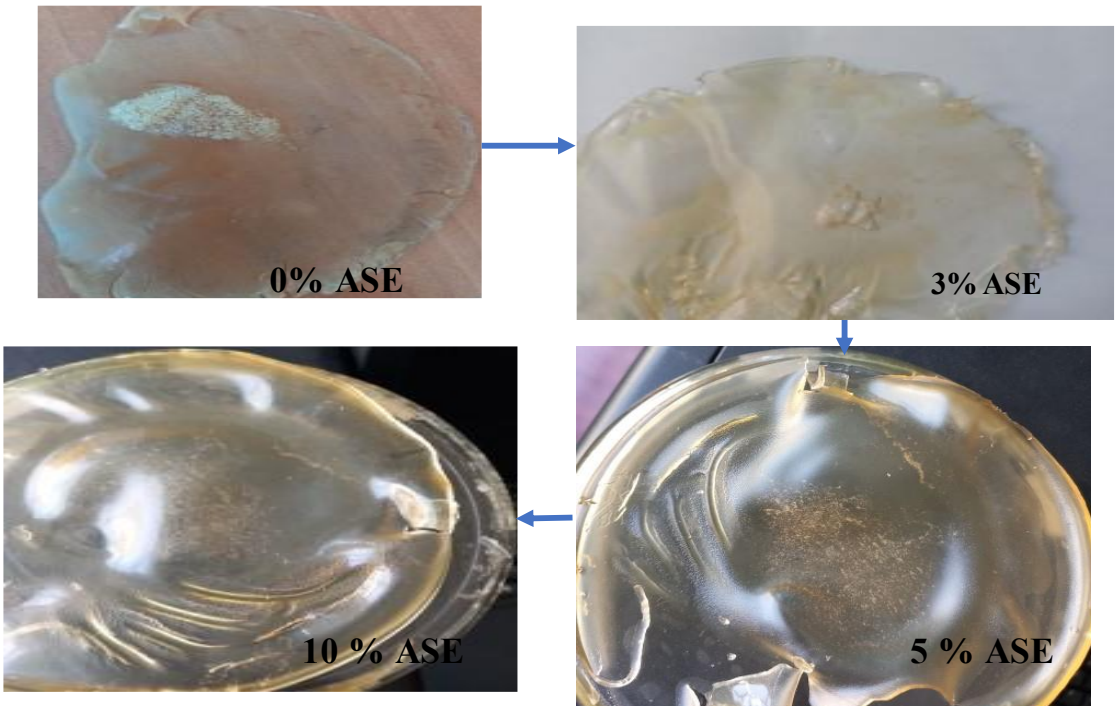


Figure 4. *9* Gelatin based film incorporating with ASE at different percentage (0%, 3%, 5% and 10% ASE)

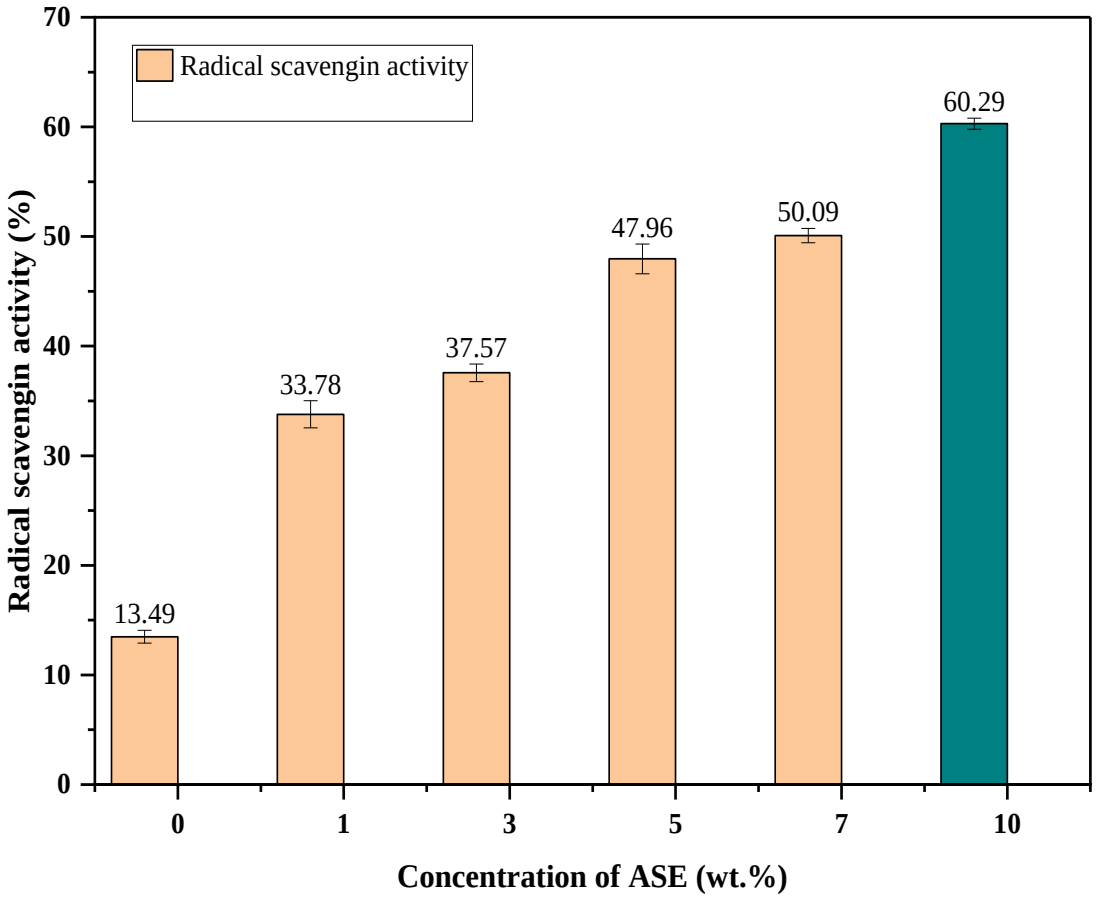


Figure 4. 10 The effect of concentrations of avocado seed extract on the radical scavenging activity of gelatin film at (ASE= 0 g, ASE =0.1/4 g gelatin, ASE =0.12g/ 4g gelatin, ASE= 0.2g/4g gelatin, ASE= 0.28g/ 4g gelatin, ASE= 0.4g/4g gelatin)

The results are in agreement with a study by Wu et al. (2013) who showed that the DPPH radical scavenging activity of silver carp skin gelatin film increased as the amount of green tea extract added to films increased. From the figure 4.10 with the increasing concentration of avocado seed extracts, the scavenging effect increased significantly ($p < 0.05$) from 13.49 % to 60.29 %. This result was similar to the result obtained in (Rahmi Nurdiani et al. 2022) which was done on fish gelatin film incorporating with mangrove leave extract, it is from 12.39% to 57.32%. Active packaging films' ability to combat free radicals is influenced by antioxidant chemicals' antioxidant activity (Hanani, Yee, & Khaizura, 2019). The antioxidant activity value obtained increased proportionately with the content of B. gymnorhiza and S. alba leaf extracts. Chin, Lyn, and Hanani

(2017) demonstrated the effects of antioxidant activity in active packaging with Aloe vera inclusion. The values were quite high, ranging from 65.78% to 74.76%. These numbers are far greater than those found in this study.

4.6 FTIR analysis of gelatin film incorporated with avocado seed extract

The infrared spectra of the control film and films incorporated from 3%, 5% and 10 % avocado seed extract (Persea *americana mill.*) are shown in Fig.4.11. The major peaks of the control film (Fig. 4.11) are present at 3280 cm^{-1} (amide-A, N–H stretching), 2933 cm^{-1} (amide-B, C–H stretching), 1628 cm^{-1} (amide-1, C=O stretching), 1544 cm^{-1} (amide-II, N–H bending), and 1241 cm^{-1} (amide III, C–N and N–H stretching). The addition of ASE to the gelatin film also displayed characteristic peaks in the spectral region, which was similar to that of the control film. As can be seen in Fig. 4.11, the spectrum of the films incorporated with different concentrations of ASE showed that some of the peaks shifted to lower or higher wavenumbers in comparison with the spectrum of the control film. The amide A peak shifted to lower wavenumbers, which could be attributed to free O–H and N–H groups forming hydrogen bonding with the carbonyl group in the films. A band was found at the wavenumber of 1000 cm^{-1} in the spectra of all films. It was possibly associated with a skeletal stretching vibration of C=C bonds. Therefore, the results illustrated that the addition of ASE might alter the intermolecular interactions in the gelatin films. The results obtained showed on infrared spectra of gelatin films incorporated with avocado seed *(Persea americana mill.)* extract.

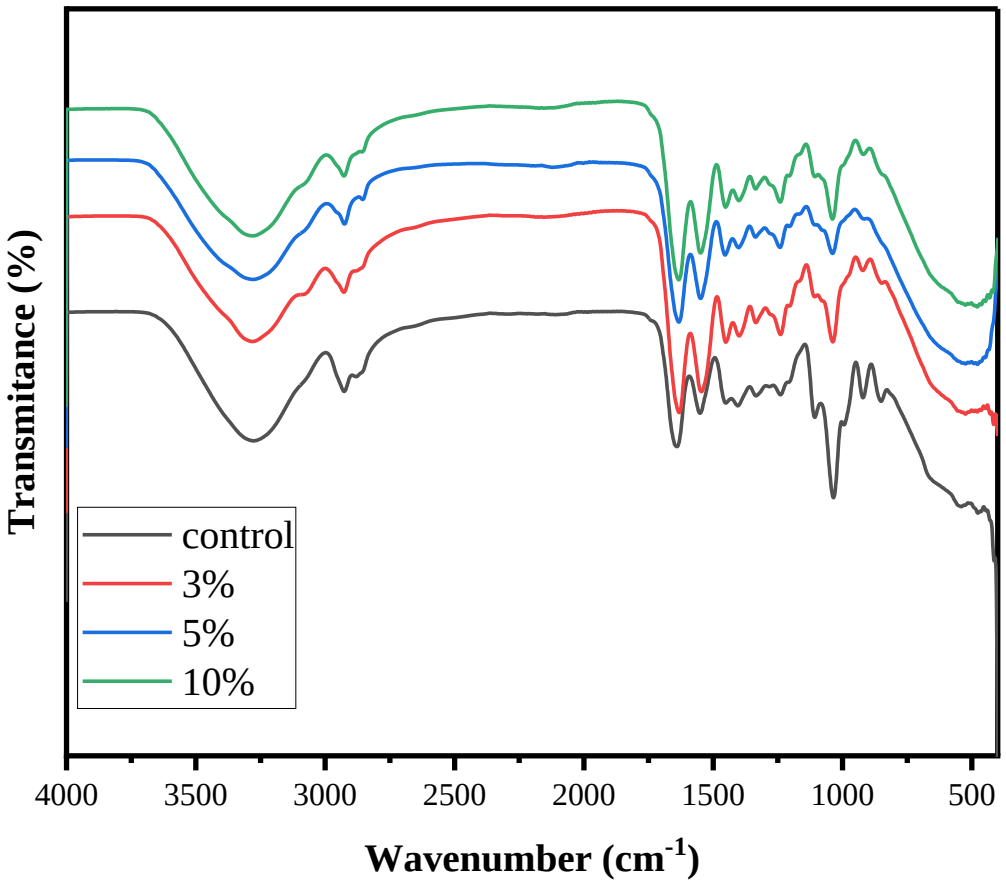


Figure 4. 11 FTIR spectra of films (---) Neat gelatin film (---) 3% ASE/4g gelatin films (---) 5% ASE /4g gelatin films (---) 10% ASE /4g gelatin films

4.7 Water solubility capacity of the gelatin film incorporated with avocado seed extract

Water solubility is a reflection of a film or coating's water resistance (de Alvarenga Pinto Cotrim et al. 2016). When designing food packaging, it is crucial to consider the film's water solubility (Chen, Y et al. 2018). The decrease in value is attributed to high protein-polyphenols interaction in which it formed stronger film network structure. Control films had the higher solubility rate due to the OH group presence in gelatin which affects the gelatin film by its hydrophilic nature. On the other hand, the solubility of film is a critical metric in food packaging (Zhao Y et al. 2021). The mass of film sample introduced into the oven for the whole weight percentage of extract were 21 mg and the mass remaining after 24 hours drying in the oven, the values were recorded.

Table 4. *11* Water solubility potential of gelatin-based packaging film with and without ASE

Film samples	**Mass remaining(mg) after 24-hour, 105 ^{O}C**	**Film solubility (FS, wt.%)**
Control	1 ± 1.64	100 ± 4.80
3 % ASE	0.565 ± 1.14	56.5 ± 2.67
5 % ASE	0.449 ± 0.86	44.9 ± 1.84
10 % ASE	0.364 ± 1.16	36. 4 ± 1.47

According to our findings. Hydrophilic and hydrophobic components influence solubility. The control film had the highest solubility % of all the generated films (100%). That was expected, especially considering the hydrophilic properties of gelatin and alginate (Coy-Barrera et al.2020) . It was observed that the solubility of the films is inversely proportional to the ASE concentrations used. The solubility continuously decreased after adding the ASE, as shown in Table xx Furthermore, the lowest solubility was noted for 3% -ASE (36.4 %), followed by 5%-ASE (44.9%) and 10 %- ASE films (56.5 %). The result obtained in this study was in better agreement with (Moufida Chaari et al. 2022) which was done on gelatin containing beetroot peel extract film with the lowest solubility of 32.62%. The treatment with 3, 5 and 10 Wt.% ASE is the most appropriate concentration for obtaining the characteristics of water solubility. As a result, this film proved to be the most appropriate for food packing with regard to the water solubility of the film. Our results are in agreement with previous studies who reported that gelatin film incorporated with natural extracts showed a significantly lower solubility (Jamróz E et al.2019).

5. CONCLUSION AND RECCOMENDATION

5.1 Conclusion

The influence of process parameters on the extraction of avocado seeds in order to determine the better extraction conditions for obtaining extracts containing maximum number of bioactive substances (TPC) and having high antioxidant activity were examined under this investigation. An experimental approach was developed, which allows the optimal process parameters to be found by a reduced number of experiments. Four-step experimental procedure is developed for optimizing the extraction of bioactive substances from avocado seed and the better processing conditions were determined as follows: 100% ethanol, 70 ^{O}C, solid to-solvent ratio of 1:12.5, process duration of 4 hour. From analysis, the optimized conditions using an extraction temperature of 70 °C, an extraction time of 4 hour, a solvent ratio (Ethanol: water) of 100:0 v/v% and solid to solvent ratio of 1:12.5 could produce the optimum extraction yields, TPC and DPPH radical scavenging activity of 14.30 ± 0.20 %, 64.61 ± 0.72 mg GAE/g extract 71.65 %, respectively. Based on these results of total phenolic content (TPC), the antioxidant activity of the avocado seed extract was conducted. According to the result obtained the maximum radical scavenging activity of the seed extract were 71.6% or in terms of $Ic_{50,\ the}$ concentration at which the amount of DPPH radical reduced by 50 % was 1.97 mg AAE/ml. The IC_{50} values were the concentration needed to reduce the DPPH percentage by 50 %. Or at the concentration of 1.97 mg AAE/g dw it can inhibit the oxidation of the sample product by 50 %.

The ASE incorporation in the films interacted with the gelatin in the matrix. The Incorporation of the ASE into gelatin films decreased the water solubility of the film to 36.4 ± 1.74%, which was a great prospect for its application in food packaging. The antioxidant activities monitored by the DPPH method showed high antioxidant activities by the incorporation of ASE. Packaging films made from ASE and gelatin provide a new way to delay lipid oxidation and prolong the shelf life of foods. The highest radical scavenging activity of the gelatin film incorporated with avocado seed extract was 60.29 % which was lower than the actual antioxidant of avocado seed extract which was 71.65%. This may be the existence of competitive compounds in the interaction with polymer matrix could limit the activities of the extract. By using GC-MS analysis the available compounds which were responsible for the antioxidant activity are identified. Among the

compounds identified, the highest abundance compounds were 9 ,12-Octadecadienoic acid Z, Z methyl easter (44.99%) and n-hexadecanoic acid, methyl easter (22.95%) has a great potential for antioxidant activity. In conclusion, the avocado seed *(Persea americana mill.)* extract (served as an important material which can be successfully employed as a natural active material to produce gelatin films with good antioxidant properties with better hydrophobic property of the film. Based on the FTIR analysis, avocado seed extract showed relatively high antioxidant properties and offers adequate integrity (miscibility) with gelatin. Therefore, this study showed that gelatin film incorporated with avocado seed extract is a potential material for developing active packaging film, as it boasts superior antioxidant characteristics.

5.2 Recommendation

Given the proven potential to extract antioxidants from avocado seed (*Persea americana mill*.) extract, which is a waste product, and use this to make bioactive packaging film, additional work is still required to investigate the other characteristics of the film. Therefore, it is strongly advised that future studies that would supplement the current work and attempt to investigate the process pay close attention to the following points.

- To increase the amount and performance of bioactive compounds, future studies should focus solely on working in an inert atmosphere and away from light, which would greatly prevent the oxidative damage of polyphenolic compounds. They should also take care to dry them away from oxidation facilitating factors like heat, the presence of oxygen, and metal ions
- Studies should concentrate on the evaluations of the economic benefits and in vivo activities of these extracts before their commercial exploitation because quantitative analysis of phytocompounds (antioxidants) in ethanolic/water extracts by spectrophotometer analysis suggested that total phenolic content and antioxidant activity were present at the highest concentrations in ASE.

- Various testing systems have shown that extracts and fractions from avocado seeds have antioxidant properties; therefore, in future work on avocado byproducts, especially in avocado seeds, HPLC (Higher Performance Liquid Chromatograph) should be used for

both quantitative and qualitative analyses in order to more accurately ascertain which component fractions are responsible for the antioxidant activity of the investigated extracts.

- During the extraction of avocado seed extract for the development of bioactive packaging film, there are several observable complications. Foam generation during the film-forming solution (gelation) process was one of the issues that remained unresolved. It is recommended that the researchers implement mechanisms to address this issue if they plan to conduct the same type of research in the future.

- Making the film less water-soluble than it was done in this work could be the subject of another future investigation. Because decreasing the film's water solubility minimizes oxidation and the transfer of free water to the product, In the manufacturing of bioactive films, it is crucial for extending the shelf life of the product inside.

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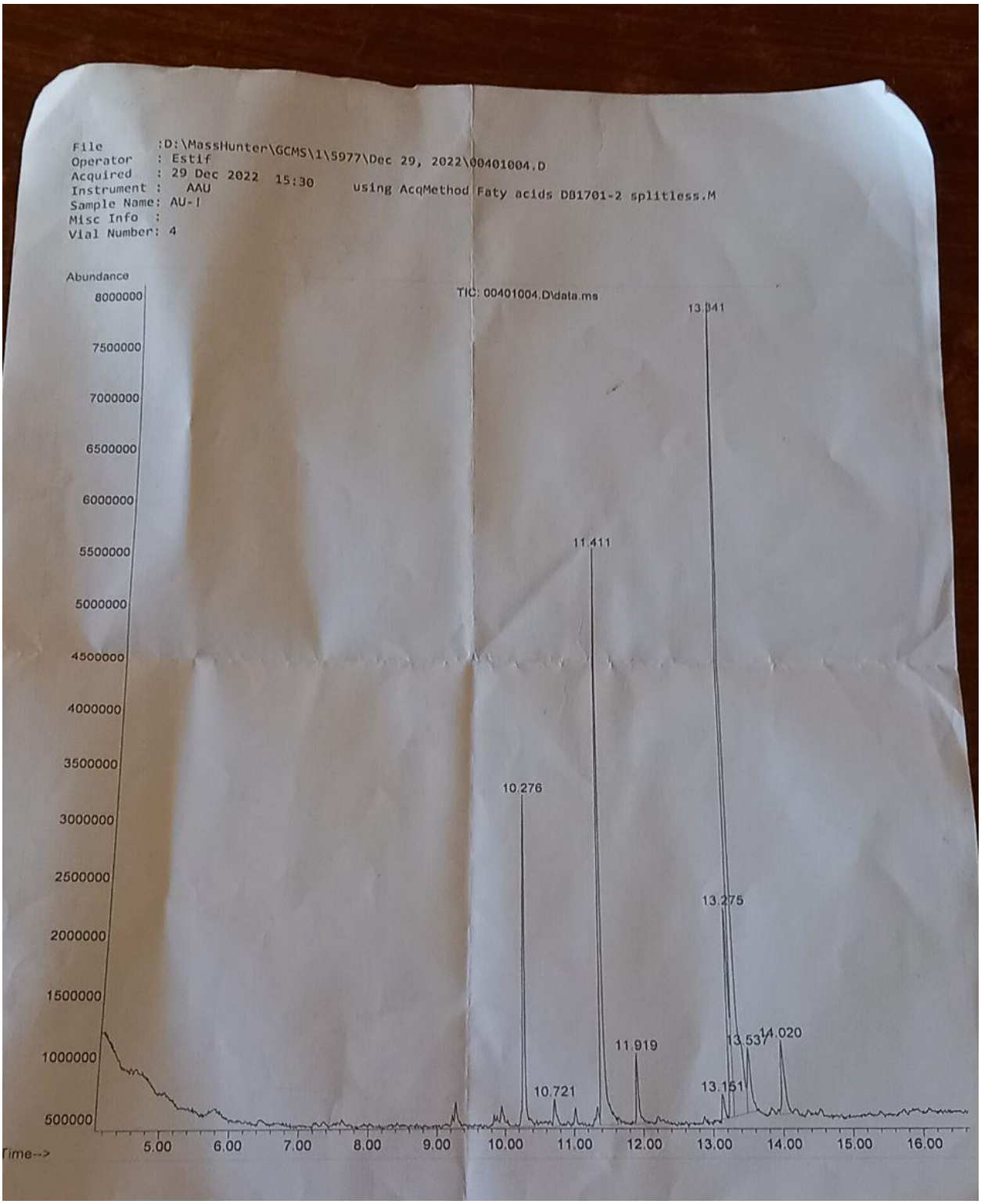
Appendixes

Appendix A. Experimental result of total phenolic content versus literature

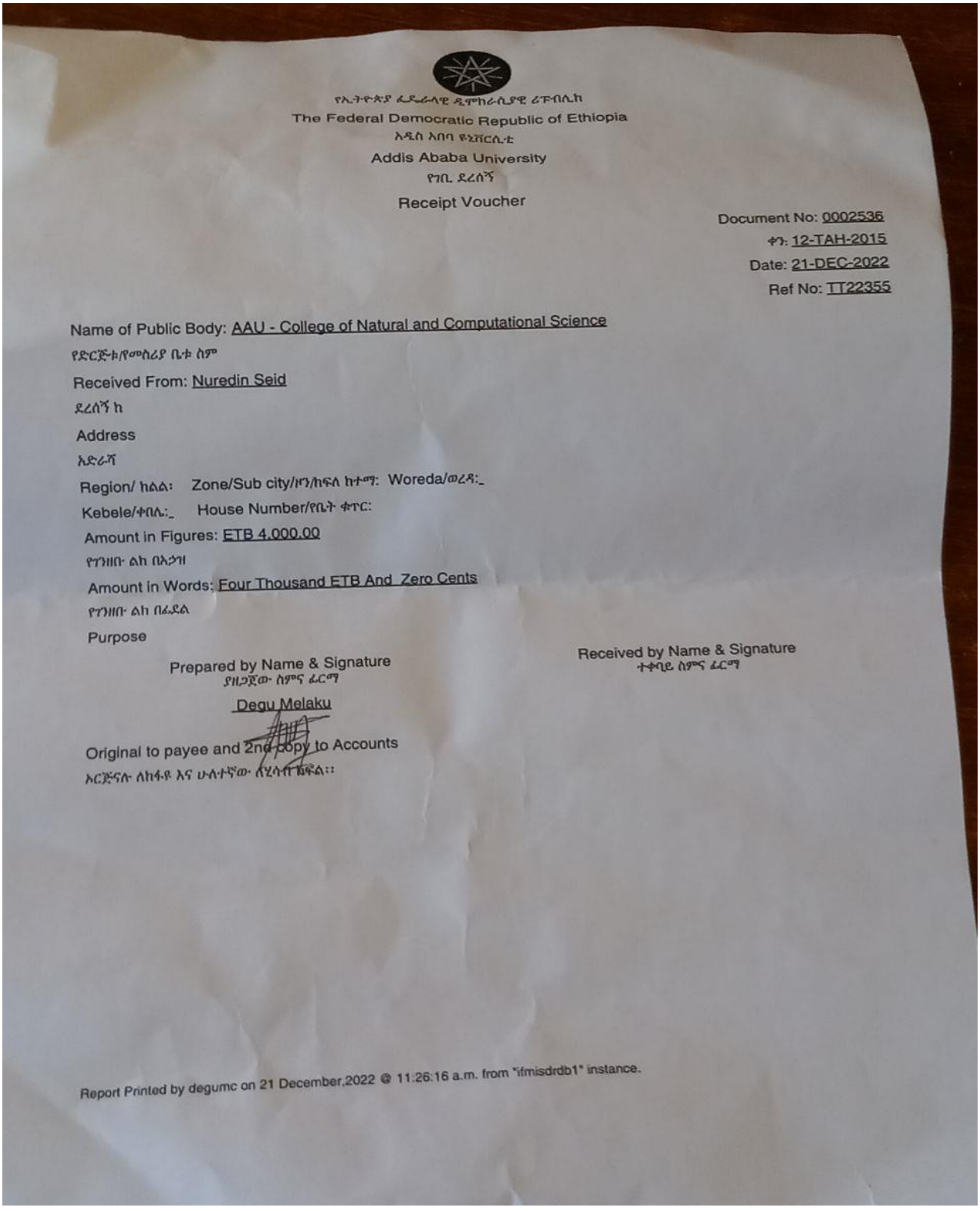
Biomass	Method	TPC	Reference
Avocado seed	Soxhlet with ethanolic extraction	8820 mg GAE/100G	Y.Y. Soong et al. 2004
Avocado seed	Accelerated solvent extraction (ASE) with ethyl acetate and 70% acetone	1699-6912 mg GAE/100g	J.G. Rodríguez-Carpena et al. 2011
Avocado seed	Agitation extraction	4500-7040mg GAE/100g	F.S. Gómez et al. 2014, A.F. Vinha et al.2013, G. Oboh et al.2013
Avocado Peel	UAE	6350mg GAE/100g	É.R. Daiuto et al.2014
Avocado peel	Accelerated solvent extraction	3300-17200 mg GAE/100g	J.G. Rodríguez-Carpena et al. 2011
Avocado seed and avocado Peel	Soxhlet extraction with ethanol/water (80:20%)	227.9 mg GAE/g of extract (Peel) 72.5 mg GAE/g of extract (Seed)	Melgar B, et al.2017

Avocado seed and avocado Peel	UAE (ethanol/water 80:20)	63.5-120.3 mg GAE/g(seeds) 57.3-59.2 mg GAE/g (Peels)	Tremocoldi MA et al. 2018

Appendix B. GC-MS chromatogram for avocado seed extract



Appendix C. Receipt for GC- MS analysis



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Addis Ababa University
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Receipt Voucher

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ቁጥ: 12-TAH-2015
Date: 21-DEC-2022
Ref No: TT22355

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Appendix D: GC-MS out put

TIC: 00401004.D\data.ms
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Peak #	Ret Time	Type	Width	Area	Start Time	End Time
1	10.276	VV	0.031	55039411	10.17	10.426
2	10.721	VV	0.043	6617275	10.684	10.851
3	11.411	VV	0.035	112899433	11.368	11.628
4	11.919	PV	0.039	14703591	11.805	12.093
5	13.151	BV	0.039	4845988	12.965	13.222
6	13.275	VV	0.035	39262717	13.222	13.302
7	13.341	VV	0.042	221055685	13.302	13.496
8	13.537	VV	0.052	18436527	13.496	13.639
9	14.02	VV	0.048	19399839	13.953	14.144