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Determination of Phytochemical in The Seed and Seed Coat of (*Gnetum gnemon L.*) Melinjo Fruit

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DECLARATION

I hereby declare that the work embodies in this report is the result of my own research except individual citations and summaries that I have explained their sources.

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Phytochemical Analysis of Seed and Seed Coat of Melinjo Fruit

(*Gnetum gnemon L.)*

ABSTRACT

Plants are the major sources for the production of natural products. Nowadays, demand of natural products is increasing since it can be as source of drug production, foods, cosmetic and agriculture industries. This is due to bioactive compound, naturally chemical compound generated from the plants, called phytochemical. Phytochemicals can be found in wide range of plant components as in the roots, stems, leaves and seeds that provide health benefits for human health. The Melinjo fruit (*Gnetum gnemon L*.) is perennial and an evergreen tree, native to Southeast Asia and has been planted around the world, primarily for its wonderful fruits. According to the recent research, it revealed that each part of the Melinjo fruit gives beneficial to humans due to the antioxidant, anti-inflammatory, anti-microbial and anti-diabetic properties. Furthermore, it is reported that phytochemicals such as saponins, tannins, flavonoids, alkaloids, terpenoids, quinones, and phenolic compounds have been found in Melinjo fruit (*Gnetum gnemon L*.). Thus, this study was performed to analyse the phytochemical content of Melinjo fruit seeds and seed coats using various extraction solvents in order to know whether it can be used for beneficial purposes.In this research, cold maceration extraction was applied to prepare the plant extarct and qualitative screening of phytochemicals in Melinjo fruit was carried out. The result present that both seed and seed coat of *Gnetum gnemon L.* contain phytochemicals. Nevertheless, it demonstrated that methanol was the most effective solvent, extracting the majority of phytochemical classes. Thus, *Gnetum gnemon L.* appears to be useful to human health. It should therefore be investigated to determine whether it may be safely used in the pharmaceutical and nutraceutical industries to improve people's health condition.

Keyword: Plant, Natural Product, Phytochemical, Melinjo fruit (*Gnetum gnemon L*.), Extraction

Analisis Fitokimia Benih dan Lapisan Benih Buah Melinjo

(*Gnetum gnemon L.*)

ABSTRAK

Tumbuhan merupakan sumber utama penghasilan produk semula jadi. Pada masa kini, permintaan terhadap produk semula jadi semakin meningkat kerana ia boleh menjadi sumber pengeluaran ubat, makanan, kosmetik dan industri pertanian. Ini disebabkan oleh sebatian bioaktif, sebatian kimia semulajadi yang dihasilkan daripada tumbuhan, dipanggil fitokimia. Fitokimia boleh didapati dalam pelbagai jenis komponen tumbuhan seperti pada akar, batang, daun dan biji benih yang memberi manfaat kesihatan untuk kesihatan manusia. Buah Melinjo (*Gnetum gnemon L.*) adalah pokok yang berbuah lebih dari satu musin, ianya berasal dari Asia Tenggara dan telah ditanam di seluruh dunia kerana buahnya yang enak dan boleh dijadikan sebagai sayur. Menurut penyelidikan baru-baru ini, ia telah mendedahkan bahawa setiap bahagian buah Melinjo memberi manfaat kepada manusia kerana sifat antioksidan, anti-radang, anti-mikrob dan anti-diabetes. Tambahan pula, dilaporkan bahawa fitokimia seperti saponin, tanin, flavonoid, alkaloid, terpenoid, kuinon, dan sebatian fenolik telah ditemui dalam buah Melinjo (*Gnetum gnemon L.*). Oleh itu, kajian ini dilakukan untuk menganalisis kandungan fitokimia benih buah Melinjo dan lapisan benih menggunakan pelbagai pelarut pengekstrakan untuk mengetahui sama ada ia boleh digunakan untuk tujuan yang berfaedah. Dalam penyelidikan ini, pengekstrakan maserasi sejuk telah digunakan untuk menyediakan ekstrak tumbuhan dan saringan kualitatif fitokimia dalam buah Melinjo telah dijalankan. Hasilnya menunjukkan bahawa kedua-dua benih dan lapisan benih *Gnetum gnemon L*. mengandungi fitokimia. Ia juga menunjukkan bahawa metanol adalah pelarut yang paling berkesan, mengekstrak sebahagian besar kelas fitokimia. Dengan ini, fitokimia yang berada di dalam *Gnetum gnemon L.* boleh dimanfaatkan kepada manusia. Namun begitu, ia perlu disiasat dengan lebih teliti untuk menentukan sama ada ia boleh digunakan dengan selamat dalam industri farmaseutikal dan nutraseutikal untuk peningkatan status kesihatan manusia.

Kata kunci: Tumbuhan, Produk semulajadi, Fitokimia, Buah Melinjo (*Gnetum gnemon L.*), Pengekstrakan

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LIST OF ABBREVIATIONS

CHAPTER 1

INTRODUCTION

1.1 Research Background

Plants are a valuable source of medicine and play a vital part in health care. The plant has excellent vitamins and minerals; it also generates many phytochemicals like phenolic acid, flavonoids, isoflavones and carotenoid. It is being used since ancient times. In those times, traditional medicine principles use local knowledge, skills, beliefs, and experiences to support local people's welfare. The plant used for traditional medicine includes various substances to treat both chronic and infectious diseases (Barua et al., 2015). Thus, it is unsurprising that 80% of the developing world's population, particularly in Asia, continues to rely on traditional medicine for health protection; the usage of traditional medicine demonstrates a long history of human interaction with the environment (Karunamoorthi et al., 2013). Industrialized economies rely on medicinal plants indirectly for their prescription medications as well. Around 18% of the top 150 prescription medications and 25% of modern medicine are plant-based (Kate, 2000). Thus, this demonstrates how plants are used to address medicinal conditions.

Phytochemical are non-nutritious plant-derived act as bioactive substances which adequate to prevent diseases. Besides protecting the plants from diseases like virus infection and resisting fungi, they also influence the plant's texture, aroma and colour. Phytochemical already present in foods that currently consumed regularly. It can be found in many foods, such as bananas, blackberries, tomatoes, legumes, whole grains, nuts, beans, mushrooms, herbs, and spices (Manthai, 2000). It essentially promotes health advantages and serves as a shield against diseases in the human body (Koche et al., 2018). Phytochemicals can be found in various part of the plant section, including the root, stem, leaves, flower, fruit and seeds (Alamgir, 2018). Yet, these phytochemicals' levels differ significantly from plant to plant depending upon the climate, variety, and growing conditions.

Recently, plant-derived bioactive substances or secondary metabolites are very significant due to their varied functions. The medicinal plant component is the most abundant source of biomaterials for traditional and modern medicine, food supplements, nutraceuticals, and chemical entities for synthetic pharmaceuticals (Ncube et al., 2008). According to Monika (2020) phytochemicals either alone or in combination, have enormous medicinal potential for healing a variety of ailments. This is because of their biological features, which include antibacterial action, antioxidant activity, hormone metabolism modulation, detoxification enzyme regulation, immune system stimulation, and anticancer activity. For example, antioxidant usually contains in carotenoids (carrot), anthocyanins (berries) and sulfides (garlic, onion). Consuming these mentioned fruits and vegetables helps protect cells against free radical damage and prevent them from becoming damaged by oxidation. Based on Scheck et al. (2006) research, extraction of the mature roots of *Scutellaria baicalensis* (Baikal skullcap), a traditional Chinese medicinal plant, inhibits the proliferative effects on various cancer lines due to the abundant flavonoid from the mature roots of the plant.

Gnetum gnemon L. or the other name is Melinjo, native to Southeast Asia, the western Pacific Ocean islands of Assam, Indonesia, Malaysia, the Philippines, and Fiji. It is often found in dry to humid tropical forest and lower montane forest, but it is also frequently cultivated as a houseplant, particularly in Southeast Asia (Manner, 2006). *The Gnetum gnemon L.* tree is a slender, shade-tolerant shrub that can reach a height of 15 to 20 cm. The branches grow in whorls from the solid and sturdy root system's base. Grey bark with conspicuously elevated rings where elder branches have broken off. Melinjo leaves vary in size and shape, but are typically 20cm long and 4cm wide. It is ovate-oblong in shape, elliptic in shape, and lanceolate in shape. Melinjo leaves are dark green, smooth, and glossy, opposite in arrangement, and sharp at both ends. Each fruit contains a single big ellipsoid or oval seed (Barua et al., 2015). Additionally, Melijo fruits three times a year in Indonesia and Malaysia; March-April, June-July, and September-October.

Moreover, according to Supriyadi et al. (2019) of *Gnetum gnemon L.* shows it has good advantages for human benefits due to its anti-diabetic and antioxidant activities that extracted from their seed protein using enzymatic hydrolysis. This is because the immature seed has a good source of protein, anti-diabetic peptide and antioxidant. As per researchers reported, the seeds have anti-microbial, antioxidant, anti-bacterial, anti-aging, tyrosinase inhibitory, and anti-inflammatory activities, (Ikeda et al., 2018; Barua et al., 2015). And furthermore, *Gnetum gnemon L.*, which is high in bioactive compounds such as flavonoids, tannins, saponins, and stilbenoids, contains trans-resveratrol, which has been demonstrated in trials to be beneficial in the treatment of diabetes and cardiovascular disease (Frankel, 1993)

Thus, an extraction method is needed to identify the particular bioactive component in the plants. Extraction is the practice of separating bioactive compounds of plants using standard methods and selective solvents. Plant extracts are complex mixtures of bioactive constituents in liquid, semisolid, or dried powder form, intended for oral or external use. The components are extracted in various ways, including infusions, decoctions, tinctures, powdered and fluid extract (Thakur, 2020). For small scale research setting, Soxhlet and Maceration extraction is commonly used. Meanwhile, several advanced extraction techniques have been produced in line with the current technology, especially for big scales research settings, such as ultrasound-assisted extraction (U.A.E.), microwave-assisted (M.A.E.) and supercritical fluid extraction (S.F.E.) (Gahlot et al., 2018).

1.2 Problem Statement

Ethnomedicine is the oldest form of illness and infection treatment. Every year, several studies are conducted on different plants that can cure human diseases and be used for traditional beauty applications. There is no research on Melinjo fruit (*Gnetum gnemon L.)* phytochemicals extraction in seed and seed coat. While previous work on the phytochemicals research of Melinjo fruit (*Gnetum gnemon L.*) was conducted in Indonesia, no such study has yet been conducted in Malaysia.

Not to mention, geographic distribution and plant growing condition is greatly influenced by the environment. Thereby, certain phytochemical level will be changing based on the climatic factors and agronomic conditions such as the development phase, pH of the soil, fertilizer, cultivation, harvest and storage operation (Borges, 2018).

1.3 Research question

Would Melinjo fruit (*Gnetum gnemon L.*) contains significant phytochemicals that have potential usage in nutraceutical and pharmaceutical industry?

1.4 Objectives

1. To determine the phytochemicals in the seed and seed coat of Melinjo fruit (*Gnetum gnemon L.*) using different extraction solvents.

2. To determine the most effective solvent to extract phytochemical between seed and seed coat of Melinjo fruit (*Gnetum gnemon L.*).

1.5 Hypothesis

H_0: The phytochemical in the seed and seed coat of Melinjo fruit (*Gnetum gnemon L.*) cannot be determined using different extract solvents.

H_1: The phytochemical in the seed and seed coat of Melinjo fruit (*Gnetum gnemon L.*) can be determined using different extract solvents.

1.6 Scope of study

This study focuses on analysing phytochemicals in Melinjo fruit (*Gnetum gnemon L.)* to identify whether it can be used safely for healthy purposes. In this study, cold maceration extraction was performed for the preparation of plant extract. The solvent used is chloroform, ethyl acetate, methanol and distilled water. Following that, qualitative screening was carried out.

1.7 Significance of study

To examine the possible potential of Melinjo fruit (*Gnetum gnemon L.*) and their biological activity whether it can be used for healthy purpose. Nowadays, Melinjo fruits has been consumed by people as health supplement products. It is believed to prevent or treat disease, such as anti-diabetic, anti-cancer and dementia. Thereby, the purpose of this study was to gain understanding of their properties and efficiency. In this study, the part of Melinjo fruit which contain the most phytochemical content was identified and analysed. This information was useful and can be used for future research.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural product

Natural products (NP) are chemical substances isolated from living organisms. Biosynthesis, extraction, identification, quantification, structural characterization, physical and chemical properties, and reactions are all-natural product chemistry. They are formed by the primary or secondary metabolic pathways (Bhat et al., 2005). NPs may be derived from microorganisms, plants, and animals' cells, tissues, and secretions (Neelam, 2014). It serves as an endless source of drug production, new pharmacophores and chemotypes, and scaffolds for amplification into effective drugs for various diseases and other helpful bioactive agents (Finar, 2006).

NPs have established a unique chemical diversity over millions of years, resulting in various biological activities and drug-like properties. For millennia, the natural product was used, in particular, as an active ingredient in herbal treatments, long before the rise of industrial chemistry pharmacology's (Sorokina and Steinbeck, 2020). Due to economic considerations, personal values, or the difficulties of consuming prescription drugs, the primary care alternative for most is conventional treatments such as Indian Ayurvedas, the traditional medicine of China, or African medication for many people around the world. NPs have now been one of the most valuable tools for discovering novel lead compounds and scaffolds in modern pharmacology (Newman, 2016; Mirza et al., 2015).

Anti-fungal and anti-biotic based on NPs derived from microorganisms are widely used. NPs or their variants are often used to treat tumours, cardiovascular disorders, diabetes, and other diseases (Dutta et al., 2019). For example, Srokina and Steinbeck (2020) show the data of NPs is responsible for more than half of all new medicines produced between 1981 and 2014. This increasing fascination with NPs, their applications, derivative products are also researched in the food industry (Ahmed et al., 2011), cosmetic and agriculture industries, resulting in the development of new natural pesticides (Mahesh et al., 2019; Sparks et al., 2019).

For instance, polyphenols are a typical natural substance used in cosmetics. Polyphenols are secondary metabolites of plants that comprise a wide variety of chemicals based on the number and location of hydroxyl groups attached to benzene rings (Vermerries and Nicholson, 2006). This group includes phenolic acids, flavonoids (e.g. flavonols, proanthocyanidins), tannins, coumarins, and stilbenes. The flavonoid group contains antioxidant, anti-inflammatory, and anti-bacterial characteristics, which improve skincare products (Zillich et al., 2015).

A clinical study about the efficiency of anti-wrinkle formulation involving flavonoids from ginkgo and a combination of tea and rooibos was carried out by Chuarienthong et al., (2010). According to the findings, the ginkgo formula is the safest anti-wrinkle remedy because it can improve skin moisturization and smoothness while reducing skin roughness and wrinkles.

2.1.1 Uses of natural product

Wound healing is an intricate procedure that restores the appearance and function of damaged tissues. Cytokine and multiple growth factors released at the wound site carefully control the process. Any changes obstructing the healing process can exacerbate tissue injury and lengthen the recovery process (Maklebust, 2005; Gopinath et al., 2004). A study from Rao et al., (2015) proposing the use of turmeric, Curcuma longa (C. longa), a family member of the ginger (Zingiberaceae), can act as a wound-healing agent. Anti-infectious, anti-inflammatory, anticarcinogenic, antioxidant and wound healing have been identified in biological functions of C. longa. In this study, since the curcumin's aqueous solubility, bioavailability and permeability are very poor, the researcher tested curcumin incorporated with other formulations (curcumin, glucosyl-THC) tetrahydro curcumin (THC)) through topical dressing on a wounded rat model.

The highest result of wound healing was Glucosyl-THC at a 2% concentration of curcuminoids, with 21 days of full recovery. Curcumin's bioavailability and pharmacological activities can be improved by glycosylation of the hydrophobic molecule. Rafaie et al., (2015) demonstrated that a hyaluronic acid-based nanocarrier gel core containing curcumin was effective at healing rats' burn wounds. Sharma et al., (2018) also discovered that mice treated with conjugated curcumin and hyaluronic acid (HA) healed more rapidly than animals treated with curcumin alone or without HA. Studies proved that incorporating chemicals like gelatin, oleic acid and hyaluronic acid, with curcumin which acquire excellent water absorption and liquid solution, improved curcumin bioavailability and promoted better wound healing.

Diabetes mellitus (DM) is a group of chronic and metabolic disorders due to persistently high blood pressure. The failure of the body to produce sufficient insulin or the body cannot respond efficiently to the insulin produced. Adjustment in various signal pathways such as JAK-STAT, AMPK, and PI3K is linked to the pathogenesis of DM diseases (Herman and Zimmet, 2012; Karter et al., 2013). According to Solayman et al. (2016), it is mentioned that different group types of polyphenols give other action mechanisms. In a study by Kurimoto et al. (2013), the leading anti-diabetic polyphenols that may enhance glucose absorption are black-soybean polyphenols that increase hyperglycemia and insulin sensitivity through the regulation of AMPK and anthocyanins and procyanidins. Meanwhile, Ong et al., (2011) had identified polyphenols in *Vernonia amygdalina* (Bitter Leaf) dominate anti-hyperglycemic effect, most likely by raising GLUT4 translocation and inhibiting hepatic G6Pase.

Research by Ortsäter et al., (2012) also talks on how Epigallocatechin gallate (EGCG), a green tea polyphenol, can enhance endothelial dysfunction and metabolic IR in skeletal muscle and liver. EGCG was also observed to reduce IR, increase glucose-induced insulin secretion, and delay b-cell death in the db/db mouse.

As an outcome, polyphenols may have the potential treatment for DM and obesity and cardiovascular disease, where IR and endothelial dysfunction have a reciprocal relationship (Keske et al., 2015).

2.1.2 Consumption of natural product in worldwide

Medicinal plants make almost 10% of all vascular plants, with an estimated 350,000 to nearly half a million types (Joppa et al., 2011). Plants have been utilized in medicine for centuries and continue to be used now (Grover, 2002). It is a fact that all societies have created a way of therapy based on flora indigenous to their environment (Fakim, 2006). Hundreds of higher plants are still farmed globally to extract necessary chemicals in medicine and pharmacy (Houghton, 1995). Plant therapeutic effects led to these benefits for pharmaceutical drugs derived from specific plants (Kinghorn et al., 2020).

In relation to the Korean Law of Acceleration of 'Natural Product Drug' in 2000, the term 'natural product drug' is defined as drugs researched and made using organic elements including new ingredients and efficacy (Ahn, 2017). The name "botanical drug" was coined by the United States to denote natural products manufactured from plant compounds such as algae and micro fungus.

While various merchants sell botanical products as food additional, the US Food and Drug Administration (FDA) has only authorized a few products for use as prescription medications (Liscinsky, 2012). Veregen®, green tea (*Camellia sinensis Kuntze*) is the first FDA-approved botanic medication and it is used to treat perennial warts and genital warts. (Blumenthal, 2007). The FDA then certified a New Drug Application (NDA) for FulyzaqTM, a blood-red latex derived from the South American croton tree (*Croton lechlerii Müll. Arg*) (Liscinsky, 2012).

In Europe, the term herbal medical product (HMP) refers to all medications containing one or more herbal product forms. Additionally, the Chinese invented traditional Chinese medicine (TCM) as a prescription for traditional medicines, owing to their affinity for eastern ideals. TCM has spread to 183 nations and areas globally in the last decade, accelerating globalization. (Xu, 2019). The main factor for TCM growth is that The State Food and Drug Administration (SFDA) has consistently deregulated the restrictions on TCM drug prices to encourage economic development in China (Xu, 2009).

In Korea, the "Natural Product Drug R&D Acceleration Law" was enacted, and the law requires institutes to provide a five-year plan for the research and development of natural products before they may be introduced. In 2013, multiple institutes raised millions of dollars for the botanical drug's commercialization. These findings indicate that botanical medications have achieved commercial success in the domestic market. Pharmaceutical companies were encouraged by these findings and refined their strategies for developing botanical drugs. Twenty-four businesses develop and manufacture botanical pharmaceuticals, with 23% being novel products. (Ahn, 2017).

2.2 Medicinal plant as source of natural product

Internationally, regardless of human development, cancer has been the leading cause of death (Zainal et al., 2016). According to Bray, (2018) study on world cancer data for 36 cancers in 185 countries in 2018, lung cancer is the most frequently diagnosed type of cancer (11.6 %), followed by female breast cancer (11.6 %), prostate cancer (7.1 %), and colorectal cancer (7.1 %). (7.1%) Lung cancer is the most prominent reason of death from cancer, followed by colorectal, liver, and stomach cancers. As a result, the use of medicinal plants to treat or minimize the activity of cancer is being reviewed.

Ellagic acid, abundant in fruit like nuts and berries, possesses anti-proliferative effects and induces apoptosis by caspase activation. This prevents tumour development caused by polycyclic aromatic hydrocarbons while hindering CYPlAl-dependent activate benzo[a]pyrene (Kaur et al., 2006).

Pomegranate fruit juice (PFJ) contains a high concentration of ellagic acid, which has been found to delay cancer development. After treatment with PFJ, the proliferation and migration of the ovarian cancer cell line were significantly inhibited. Ellagic acid and luteolin inhibit MMP-9 and MMP-2 expression in a dose-dependent manner. This lowered MMP-2 and MMP-9 expression successfully prevented tumour formation in ES-2 cell line produced nude mice cancer models (Liu et al., 2017). Ellagic acid therapy increased the capacity of human pancreatic adenocarcinoma cells to undergo apoptosis, induced mitochondrial depolarization, and decreased cell growth (Umesalma et al., 2010).

Icariin, a prenylated flavanol glycoside, was isolated from Herba Epimedii (*Berberidaceae*). Icariin causes an increase in the Bax/Bcl2 ratio, cytochrome c release, PARP cleavage, and caspase activation (Li et al., 2010; Wu et al., 2015). Icariin increased endoplasmic reticulum stress-related molecules and decreased anti-apoptotic Bcl-2 protein expression in human adenocarcinoma and esophageal cancer cells, according to Di et al., (2015). Meanwhile, Liu et al., (2015), demonstrated that Icariin induced an apoptotic cascade in human hepatocellular carcinoma cells through a Fas-mediated caspase-dependent pathway. This illustrates how Icariin reacts to a distinct type of tumour and inhibits its activity.

2.3 Phyto-chemistry

Plants are autotrophic organisms whose metabolism consists of two main processes; there is the primary metabolic process in all animals and plants, whereas a secondary metabolic process occurs in plants. Phytochemicals are different from primary metabolites. Primary metabolite includes carbohydrates, protein, vitamins, fats, and minerals needed daily plant maintenance. Meanwhile, phytochemistry is related to secondary metabolites. The word "phyto" comes from the Greek word. Phyto-chemistry could be defined as "plant chemistry" as bioactive chemicals in plants with beneficial effects above those provided from macronutrients and micronutrients (Hasler, 1999).

Phytochemistry is gaining popularity in research due to the compounds produced by plants (Egbuna et al., 2018). They consider secondary metabolites derived from structural compositions and biosynthetic processes, roles, and modes of action in biological systems. They act as a barrier against illness and injury and add to the plant's colour, aroma, and flavour. Phytochemicals are mostly plant-derived compounds that protect plant cells from pollution, stress, dehydration, ultraviolet radiation, and pathogenic attack (Gibson et al., 1998; K, 2000).

Some phytochemicals are unnecessary or needed to support life by the human body. (Heneman, 2008). Yet, phytochemicals have medicinal properties to help prevent or treat some common diseases (Hertog et al., 1993). Many of these advantages point to phytochemicals playing a part in disease prevention and treatment. As a result, several studies have been conducted to uncover phytochemicals' positive health effects and discover new plant natural products that are useful in the pharmaceutical industry, complementary medicine systems, nutraceutical industry, and dietary supplement industry (AlAli et al., 2021). Their role in the medical and pharmaceutical sectors for discovering new drugs are getting more attention from opportunists to make as a business.

After getting known, secondary metabolite's roles impact human health when their dietary consumption is essential. A wide array of dietary phytochemicals can be found in fruits, legumes, vegetables, nuts, whole grain, herbs, spices and seeds (Mathai, 2000). The common source is cabbage, carrots, onion, broccoli, tomatoes, grapes, raspberries, legumes, beans and soy food (Moorachian, 2000). The plant components, including root, stem, leaves, flowers, fruits and seeds, phytochemical substances can be discovered. Numerous phytochemicals, most notably pigment compounds, are highly concentrated in the outer layers of plant tissue. The degree varies according to the variety and the plant's processing, cooking, and growing conditions (Mamta et al., 2013).

Several phytochemical classifications are depending on their chemical structures and properties. One among them is flavonoids, tannins, saponins, quinones, terpenoids and alkaloids. (Vega and Oomah, 2013).

2.3.1 Flavonoids

Flavonoids are polyphenolic compounds in a wide range of fruits and vegetables. Flavonoids can be seen as free radicals or as glycosides. Yellow and white plant pigments contain these compounds. More than 4000 flavonoids have been discovered, with 500 included in their natural state. Polygonaceae, Rutaceae, Leguminosae, Umbelliferae, and Compositae are all rich in flavonoids (Kesarkar, 2009). They are widely distributed among vascular plants and can be found in almost every part of the plant (Harbone, 2013).

Flavonoids are polyphenolic, water-soluble compounds containing 15 carbon atoms. They consist of two rings of benzene connected by a three-carbon chain. A third middle ring is formed if the carbon in the chain is linked to carbon either by an oxygen bridge or directly in one of the benzene rings. (Panche et al., 2016). The six significant subtypes of flavonoids are chalcones, flavones, flavanones, anthoxanthines and anthocyanins. (Martens, 2010). Anthoxanthins are responsible for the yellow colour of individual petals, while anthocyanins are responsible for the red colour of buds and the purple-red colour of autumn leaves (Mierziak, 2014). Flavonoids are found in various plant-based foods and beverages, including vegetables, fruits, chocolate, tea, and wine, and are thus referred to as dietary flavonoids.

According to a study conducted by Kesarkar (2009), flavonoids have anti-inflammatory properties. This is because quercetin inhibits lipoxygenase and cyclooxygenase activity. They contribute to the release of arachidonic acid, a compound critical for the inflammatory response in general. Lipoxygenase is found in neutrophils and is responsible for producing of chemotactic compounds from arachidonic acids, which results in the release of cytokines. Thus, when quercetin is used as a phenolic molecule, it blocks the routes for both of them, thereby lowering the production of these inflammatory metabolites.

Other than that, flavonoids have antioxidant activity. Research by Panche et al., (2016), the most effective flavonoids for protecting the body against reactive oxygen species are flavones and catechins. Radicals and oxidative stress damage cells and tissues irreversibly when they cause or are caused by external damage during regular oxygen metabolism. (Grace, 1994). Nijveldt et al., (2001) mentioned the increased formation of reactive oxygen species through injury causes the endogenous scavenging compounds to be consumed and depleted. Flavonoids can function in conjunction with endogenous scavenging compounds. According to Hanasaki et al., (1994), research flavonoids can directly neutralize superoxide, while others can scavenge peroxynitrite, a highly reactive oxygen-derived radical. They encountered that flavonoids such as epicatechin and rutin are impactful radical scavengers, and that rutin's scavenging ability is attributable to its inhibitory effect on the enzyme xanthine oxidase (XO).

2.3.2 Alkaloids

Undoubtedly, alkaloids are a vast class of natural substances derived through secondary metabolism, each of which contains a nitrogen atom in the central location of the molecule (does not include nitrogen in an amide bond or peptide). Natural alkaloids are classified according to their chemical makeup and metabolic pathways. They are classified into three classes that are heterocyclic alkaloids, protoalkaloids (non-heterocyclic), and pseudoalkaloids. (Ranjitha and Sudha,2015). They're often present in plants, but they've been discovered in insects, animals' microorganisms and aquatic invertebrates (Bribi, 2018; Robert, 2013). Alkaloids are commonly developed to aid plant survival in the ecosystem since they are allelopathic compounds, which means they got the ability to act as a natural herbicide by protecting plants from pests (Jing et al., 2014).

Not only have alkaloids been used as a natural pesticide for plants, but they have also been used extensively in medical properties, particularly in modern medicine. Tubocurarine, for example, is a component of toxic curare that is used in surgery as a muscle relaxant. (Kurek, 2019). Robert et al. (1998) state that quinine is an effective anti-malarial medicine that is progressively being superseded by more effective and less risky prescription pharmaceuticals. Quinidine, another alkaloid discovered in Cinchona species, is used to treat arrhythmias, or irregular heartbeats.

Besides, Berberine, a representative isoquinoline alkaloid, has anti-diabetic properties (Chang et al., 2015). According to studies from Leng, (2004) and Lee, (2006), Berberine has been shown to decrease hyperglycemia, increase insulin secretion, decrease body weight and fat levels, and aid in the reduction of glucose tolerance and insulin resistance by activating the AMPK pathway, elevating glucagon-like peptide-1 (GLp-1), suppressing reactive oxygen species (ROS), reversing mitochondrial dysfunction, and alleviating oxidative stress and restraints affected by endothelial microparticles (Chang et al., 2015).

2.3.3 Terpenoids

The most common and structurally diversified natural ingredients are terpenoids, also called isoprenoids. The suffix "ene" meant olefinic bounds and was originally used as the term "terpenes" for turpentine present in hydrocarbons. Terpenoids are categorized according to the number and chemical composition of carbons formed in isoprene units, followed by cyclical rearrangements and carbon skeleton rearrangements, with the isoprene law serving as an analytical function. (Zwenger and Basu, 2008). 2-methyl beta-1,3-diene (C5H8) is isoprene, the "building stone" for terpenoids. A single isoprene cell is characterized by hemiterpenoids, the most fundamental class of terpenoids.

Terpenoids are generated in the cytosol by the mevalonate (MVA) pathway or in the plasticidal 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Cheng et al., 2007). Even though there are exceptions and cross-talk between the two pathways, the MEP pathway produces hemi-, mono-, di-, and triterpenoids, while the MVA pathway produces sequin triterpenoids (Maffei, 2011). Baser et al., (2010) indicate that terpene products are often associated with essential oils consisting of steam, hydraulic or dry distillate fractions, or mechanical fragments responsible for the fragrances, smells, and odours of several plants. The different boiling range makes two groups, the mono- (140-180oC) and sesquiterpenoids (>200oC). Usually, terpenoids are found in the herbal plant, citrusy fruits in orange or yellow colour (Yamamoto et al., 2013).

One of the many uses of terpenoids is as anti-microbial activity. According to Raut and Karuppayil (2014), vaporized thyme oil is effective against *Staphylococcus aureus* and a small number of other multi-drug resistant bacteria found in the respiratory system. This may be explained through research Dorman et al., (2000) demonstrated that terpenoids have a large and diverse spectrum of anti-bacterial activity against Gram-positive and Gram-negative bacteria. Because they are lipophilic, they can easily cross the cell wall and membrane. Bacterial cell death is caused by the breakdown of membrane integrity and ability, leakage of cell material, denaturation of cytoplasmic proteins, and deactivation of cellular enzymes.

Besides, terpenoids have anti-cancer activity. Research from Ludwiczuk et al. (2017) shows that the mechanism of action depends on the induction of apoptosis or necrosis to inhibit tumour cell proliferation. Terpenoids have also been shown to have anti-cancer properties when used with chemotherapy agents. β-Caryophyllene enhances the anti-cancer efficacy of paclitaxel by facilitating its passage across cancer cell membranes (Lesgards et al., 2014). It is also mentioned that Eugenol blocks cell growth of 50 percent% of the lines in melanoma cells within 24 hours and induces apoptosis of healthy cells at a concentration of 0.5 M in cervical cancer cells without toxicity.

2.3.4 Tannins

Tannins are water-soluble phenol molecules produced and collected as secondary metabolic products by higher plants. Tannins are polyphenols with molecular weights ranging from 500 to 3000 Da. Its molecular weight will increase 20,000 da in complexes with saccharides, alkaloids, and proteins exhibiting phenol-like reactions. Tannic acid's chemical composition is determined by the plant species that produce it (Krzyzowska et al., 2017). Tannins are classified into two major groups: hydrolyzable tannins and condensed tannins. Ellagitannins are hydrolysable tannins; gallotannines are complex tannins. On the other hand, Procyanidins are condensed tannins with a condensed carbon chain (Khanbabaee and Van, 2001). Hydrolyzable tannins are several phenolic acids linked via an ester bond to a central glucose residue.

In contrast with condensed tannins, their structure lacks sugar and is related to flavonoids (Lochab et al., 2014). Condensed tannins have greater anti-bacterial, anti-viral, and anti-fungal effects than hydrolyzed tannins and are more resistant to microbial degradation.

Tannin is abundant in practically every part of the plant, including the leaves, berries, bark, stems, leaves, plant galls, and seed. In the tree growth regions, for example, in the secondary phloem and xylem and the cortex-epidemic layer, tannins are detected in the stem tissue.

Studies conducted by Beretta et al., (2009) hydrolyzable tannins have cardiac defensive effects by stabilization, degradation of the aortic wall by pericardian tissues and inhibition of enzymes (Karthikeyan, 2007). Specifically, hydrolyzable tannins exert anti-ischemic and vasorelaxant activity mostly through the interaction of multiple factors, including activation of the cyclooxygenase pathway, inhibition of TNF-alpha, activation of endothelial nitric oxide synthase, and scavenging free radicals and reactive oxygen species.

Furthermore, condensed tannins have long-standing anti-hypertensive and vasorelaxant characteristics that have added to the effects of the endothelium, including nitric oxide, and an essential impact in cardiac defence against cardiac defence isoproterenol-induced myocardial infarction (Magos et al., 2008).

Tannins also has wound healing properties (Halkes et al., 2001). Recent research has discovered that tannin can form complexes with various biomolecules, suggesting that toxic chemicals in burnt tissue may help precipitate (Chokotho and Hasselt, 2005). Tannin also allows cure wounds, slow scar progression, and remove reactive oxygen compounds in skin tissue formation. Tannins have the following benefits that are pain relief, secondary infection prevention, plasma depletion, and prolific epithelialization (Hupkens, 1995).

2.3.5 Quinones

Quinones are secondary metabolites of an aromatic (hexacyclic saturated) di-one or di-ketone system that are mostly isolated from plants. They're procured by the oxidation of hydroquinone's. For instance, quinones occur naturally in a broad variety of forms, including naphthoquinones, anthraquinones, benzoquinones and polyquinones

(Eyong and Kenneth, 2013). There are wide-ranging Quinones present in fungus, bacteria, and various plant species. However, they are found in only a few species. Sea urchins, aphids, lac beetles, and some scale insects are all species that obtain quinone from the plants they ingest (Alamgir et al., 2018)

Studies from Kobayashi et al., (2011) shown, after undergoing mammalian pols and lipopolysaccharide (LPS) that been induced in mouse macrophage cells, the effect of naphthoquinones on activation of nuclear factor (NF)-κB is useful and could be a strong anti-inflammatory chemopreventive agent and possess a great anti-microbial activity (Alibi et al, 2021). It is also reported by Gafner et al., (1996) that quinones have great anti-bacterial and anti-fungal activities from naphthoquinones derived from *Newbouldia laevis* roots against *Escherichia coli, Bacillus subtilis, Cladosporium cucumerinum and Candida albincans.*

2.3.6 Phenolic Compounds

Phenolic substances are natural metabolites that develop biogenetically through the phenylpropanoid pathway. Their structure is composed of an aromatic ring with more hydroxyl substituents (Tsao, 2010). Phenolic chemicals regulate several metabolic functions in plants, including structure and development, pigmentation, and disease resistance (Naumovski, 2015). The three most abundant phenolic chemical classes in the human diet are phenolic acids, flavonoids, and tannins (Balasundram et al., 2006; King et al., 1999).

Phenolic acids have two small groups that are hydroxycinnamic and hydroxybenzoic acids. Phenolic acid is a significant part of fruits and vegetables (Balasundram et al.,2006). They act as acids due to their carboxylic groups. These chemicals contribute to the colour stability, fragrance character, and antioxidant capabilities of the product (Fleuriet and Macheix, 2003). Ellagic and gallic acids are two of the most abundant hydroxybenzoic acids in nut and berry products (Maas et al., 1991). Caffeic, ferulic, p-coumaric, and sinapic acids are the top prevalent hydroxycinnamic acids and aromatics found in nature. Typically, chlorogenic acid is plentiful in grains, coffee beans, and sunflower seeds. It is created when caffeic acid and quinic acid combine (King et al., 1999). Chlorogenic acid is a crucial substrate for the enzymatic browning of fruits like pears and apples and is an essential member of this group in terms of food material (Bravo, 1998).

Research from Kwon et al., (2008) and Aryaeian et al., (2017) stated phenolic compound help reduce hyperglycemia and increase the synthesis of acute insulin and sensitivity to insulin. Enzymatic pancreatic α-amylase and α-glucosidase are also involved in glucose metabolism and the intestinal absorption of hydrolyzed carbohydrates. Kwon et al., (2008) indicate that at 99.6%, catechin was the most efficient α-glucosidase inhibitor, followed by caffeic acid, resveratrol, and rosmarinic acid. Not only that, the phenolic compound extracted from food such as berries, wheat, apple, mung beans and maize are a few examples that are effective inhibitors of these enzymes (Randhir and Shetty, 2007).

Next is phenolic acid as an anti-microbial agent. The study from Cueva et al., (2010) revealed they have anti-microbial properties and can also be used as food preservatives. The chemical structure of phenolic acids, incredibly saturated chain length, location, and some substitutions in the central benzene ring has been used to determine their anti-microbial ability. Phenolic acids have a weaker anti-bacterial activity than their methyl and butyl esters. By extending the length of the alkyl chain, the movement of phenolic acids is dramatically improved. Merkl et al., (2010) show that when compared to their phenolic acid monomers, oligomers have more activity. Anti-microbial activity of hydroxybenzoic and hydroxycinnamic acids varies depending on the number of hydroxyl (OH) and methoxy (OCH3) functional groups in the compound (Sanchez et al., 2011). Almajano et al., (2007) say pH has a crucial role as phenolic acids' anti-bacterial ability of phenolic acid is inversely proportional to changes in ph. The anti-bacterial activity of hydroxycinnamic acids is greatly reduced as the double bonds are decreased.

2.3.7 Saponins

Saponins, or glycosides, are a complex group of compounds with a composition that Includes one of more steroid or triterpenoid aglycone sugar chains. That are commonly distributed in the plant kingdom (Mazza et al., 2007). Physicochemical and biological properties reflect their structural diversity and can be implemented in various conventional and industrial applications. Saponin is derived from the Latin word sapo (soap). It refers to the capability of saponins to produce continuous froth when shaken with water, even in dilute solutions (Love and Simons, 2020).

Saponins widely found in agriculturally important plants, especially legumes. Many of these legumes are common in human diets. The saponins have exceptionally high in soy beans (Glycine max) and chickpeas (*Cicer arietinum*) (Rupasinghe et al., 2003). Species of plants, genetic source and plant component being studied, the environmental and agronomic factors related to plant growth and treatments post-harvest, such as storage and care, all affect plant material saponin quality. (Mazza et al., 2007).

According to Malinow et al., (1977) studies, saponin gives hypocholesterolemia effect, reducing the plasma cholesterol level. Besides Sidhu et al., (2004) evaluated the impact of soy saponins on cholesterol absorption in rats and discovered that at a concentration of 2.0 g/L, soy saponin decreased cholesterol absorption by approximately 50%. As a result, because soy saponins lack carboxyl groups, they have a low affinity for cholesterol and require more significant to block cholesterol absorption. This could be supported by the research of Rideout et al., (2010) on daily consumption of saponins gives effect in lowering liver and blood cholesterol concentration in animal studies chosen.

Apart from reducing cholesterol, saponin also has antioxidant properties. Saponin is a natural antioxidant because it binds to cholesterol and slows cholesterol oxidation in the colon. According to Reshef et al., (1976) and Ruiz et al., (1996), DMPP (2,3dihydro2,5dihydroxy6methyl4Hpyran4one) is abundant in legumes such as soybean, peas, and chickpeas. Additionally, the presence of DDMP saponins contributes to the protection of proteins against free radical attack, preventing lipid peroxidation or DNA degradation.

2.4 Botanical Description of Melinjo (*Gnetum gnemon L.*)

2.4.1 Taxonomy

Gnetum gnemon L. is a *Gnetum* genus found in Southeast Asia and the western Pacific Ocean islands, ranging from Mizoram and Assam in India to Indonesia and Malaysia, the Philippines, and Fiji. Gnetum, joint fir, two leaves, Melinjo, Belinjo, Bago, and tulip are some of the common names for this plant. (*Gnetum gnemon L.*) is from the Gnetaceae family. The order for *Gnetum gnemon L.* is Ephedrales. The Ephedra is a genus of desert shrubs that grow short, straggling, or ascending. The leaves are opposite or whorled along the nodes of green branchlets, and are reduced to sizes about one centimetre long. Gnetidae is the subclass and Gnetopsida is the class for *Gnetum gnemon L*. Plante is the kingdom of (*Gnetum. Gnemon L*). and Tracheophyta under phylum sector.

2.4.2 Botany

The tree is small to medium in size, growing to a height of 15 to 20 centimeters and bearing evergreen leaves. Branches typically develop in whorls from the base and are firmly entrenched with a solid tap root system. The trunk of *Gnetum gnemon L*. does not grow buttresses; instead, standard swollen rings across the girth mark the location of old branches. The stem is very well, with a cylindrical bole and a diameter of up to 40 cm.

The leaves are petiolate, ovate-oblong or elliptic in shape, 10 to 20 cm long and 4 to 7 cm wide, reticulately veined, glabrous and glossy, dark green in color, apex acute to sub acuminate, margins whole and base acute

As gymnosperms species, *Gnetum gnemon L*. had aggregated cones or strobili at the terminal stem axis in female plants and staminate strobili at the terminal 3-5 cm long axis in male plants and only a few ovules on a spike mature into seeds. Fruits are ellipsoid, typically in clusters, that grow 1-3.5 cm long and half as wide when fully ripe, turning yellow to orange-red then purple (World Agroforestry Center, 2021). The fruits and leaves are common in Indonesian cuisine (Konno et al, 2013).

Figure 2.1: Melinjo fruits (seed and seed coat)

2.4.3 Geographical Distribution

Based on World Agroforestry Center, *Gnetum gnemon L.* is an old-world species that are indigenous to the area defined by Takhtajan, which extends from Northeast India to Fiji as the location where the angiosperm was either born or cradled. According to the study of Barua et al., (2015) two types of shrub from *Gnetum gnemon L*. variety were discovered in Northeast India, which is *Gnetum gnemon* var. *griffithii* (Parl.) Markgr. And *Gnetum gnemon* var. *brunonianum* (Griff.) Markgr. were found. Besides, *Gnetum gnemon L*. is very famous in Indonesia, such as Java, Kalimantan and Sulawesi Island and called as Melinjo. The tree can be found in dry to humid tropical rainforests to lower montane forests at elevations of below 1700 metres (Manner, 2006). Melinjo also could be cultivated in orchards or home gardens.

Gnetum gnemon L. is a shrubby Phenerophyte that grows into small to medium-sized trees and is an integral part of the low-elevation rain forest ecosystem. The plant thrives in clear and partly shaded tropical and subtropical climates, with well-draining, mildly acidic soil to neutral to slightly alkaline (Manner, 2006).

2.4.4 Reproduction and Propagation

Melinjo is relatively easy to propagate. Cutting, air-layering, grafting, seed, or budding are all options for propagation (Cadiz and Florido, 2001). Some seeds can be grown from seedlings naturally found growing beneath bearing trees; they can then be harvested, grown in a nursery, and planted appropriately. Seedlings emerging naturally under bearing trees can be harvested and grown in a nursery until they are mature enough to be planted for a small number of trees. Large ripe fruits that have fallen from the tree are harvested to produce a more significant number of trees. The rind is cut, and the seed is dried and placed in the shade until an adequate amount is collected.

The seed is pre-germinated in a box filled with alternating layers of seed and sand. The germination bed is placed in a shady area. Phosphorus supplementation can support seedling growth and it can also improve by inoculation with the mycorrhizae fungi *Scleroderma sinnamariense*. Usually, seeds take 3 of regular watering, but if without pre-germination treatment, it should take 6 to 12 months. Then, the seedlings will be ready to be transferred to the nursery, where they will be grown for six months or more, initially in the shade, before being transplanted early in the rainy season (Manner and Elevitch, 2006).

For the alternative approach, air layering has the advantages of selecting the best mother trees, allowing the young plant to produce fruit within 2-3 years of planting, and allowing the collection of only female seed-producing plants. The position of cincturing is critical for air layer effectiveness; the top of the ring of bark to be extracted should be at the edge of a swollen node and rooting will take up to two months. After separation, the layers must be nurtured for some time before being planted in the field. By that means, they must be pruned to match top and roots and grown in the shade in pots (Cadiz and Florido, 2001).

2.4.5 Uses

Melinjo (*Gnetum gnemon L.*) plant widely planted in Southeast Asia, especially in Indonesia, is already becoming an ordinary vegetable for daily consumption. The most popular is called "*emping*", a type of crackers that slightly have a bitter taste. It is made from crushing the endosperms generated by shelling heated seeds, drying, and deep-frying them. Other than "*emping*", Melinjo leaves and fruits also be consumed as soup named "*sayur asem*" (Kato, Tokunaga and Sakan, 2009).

Studies from Mukhlisah et al. (2020) show that Melinjo leaves help preserve the quality of duck eggs. The preservation of the duck eggs is done by soaking fresh eggs into a vegetable tanner that contain tannin and, in this case, is Melinjo plant. Among the chemical compounds contained in Melinjo, especially in its seeds and leaves, are saponins, flavonoids and tannins. (Dewi et al., 2012). The proximate analysis of Melinjo leaves revealed a tannin concentration of 4.55%, indicating that it is one of the plant tanners. The studies present the relation between the level of extraction Melinjo leaf effecting on duck egg cavity.

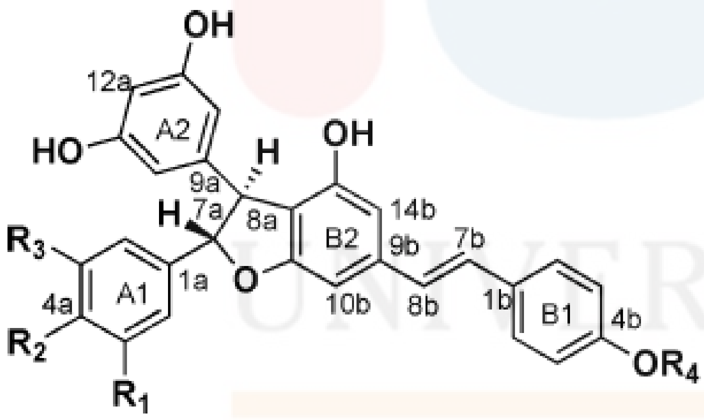
The higher the level of extract Melinjo leaf, the lower the depth of air cavity hence the higher the value of the duck eggs. This is because the tanning substance (tannin) in it blocks the pores of the eggshell, preventing CO2 gas from entering and maintaining the thickness of the egg white. The growth in pH can be slowed by preventing the evaporation of water and carbon dioxide (CO2) gas, and the thickness of egg whites can be preserved (Setiawan and Widianti, 2018).

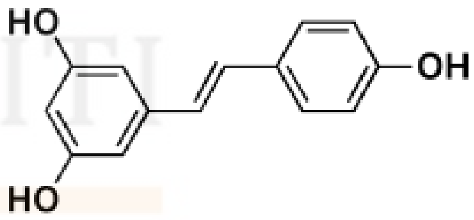
The research of Kustiningsih et al. (2020) uncovered that compiling the activated compound from Melinjo shells with Tio2 nanoparticles can help improve the effectiveness of adsorbents as degradation compounds. This in line with research by Rashid et al., (2018) and Alfat, (2009), observed CO gas concentrations were reduced by 91.50% and NO2 by 95.40% in exhaust gas emissions when activated coconut shell carbon media with TiO2 are used, while coconut shell started carbon media without TiO2 may minimize carbon dioxide by 83.10%and NO2 by 93.60%. After going under the parameter analysis to measure the quality of activated carbon such as Iodine number, surface area (BET), XRD, SEM-EDX, the evidence portrays good results on each of the parameters but not to forget, that the quantity of TiO2 nanoparticles and activated carbon used in the composite characterization affects the performance. Since resveratrol is a polyphenol compound in Melinjo plants, resveratrol has antioxidant and anti-bacterial functions (Hisada et al., 2005).

2.5 Chemical Constituents and Biological Properties of Melinjo (*Gnetum Gnemon L.*)

Gnetum gnemon L., popularly known as Melinjo in Indonesia, is a member of the Gnetaceae family. It has a high concentration of stilbenes such as resveratrol (Kato et al., 2009). Resveratrol is a synthetic polyphenol found in red wine and other foods. It is believed to have anti-aging qualities (Baur et al., 2006).

Based on an experiment conducted by Kato et al., (2009) using 50% EtOH extracting from dried Melinjo seeds, isolation of stilbene oligomers such as gnetin L (1), gnetin C (2), gnemonoside A (3), gnemonoside C (4), gnemonoside D (5) and a stilbenes monomer such as resveratrol (6), were discovered.





1: R_1 = OCH_3, R_2 = R_4 = H, R_3 = OH
2: R_1 = R_3 = R_4 = H, R_2 = OH
3: R_1 = R_3 = H, R_2 = OGlc, R_4 = Glc
4: R_1 = R_3 = R_4 = H, R_2 = OGlc
5: R_1 = R_3 =H, R_2 = OH, R_4 = Glc

6

Figure 2.2: Shows chemical structure of the stilbene derivatives from *Gnetum gnemon L.*

The EtOH extract of Melinjo seed was divided using DPPH radical scavenging. The DPPH radical scavenging activity of ascorbic acid and DI-alpha-tocopherol with all these stilbenes have the same outcomes (Baur et al., 2006). Gnetin L (1) and C (2) have a modest inhibitory action on pancreatic lipase, while porcine pancreatic α-amylase and anti-microbial activity against food microorganisms had a relatively poor inhibitory effect of the Gnetin C. (Hiroko et al., 2020). According to research from Brasnyó et al., (2011), extracted seed can remove active oxygen as an antioxidant, prevent absorption of fat, regulate blood sugar levels, enhance intestinal bacterial flora by inhibiting the growth of harmful enterobacteria, and extend the shelf life of food.

Research from Iliya et al., (2002) discusses anti-microbial activity in Gnetum gnemon L. It is proven Melinjo extract has a great deal of potential as a food preservative against such pathogenic bacteria. These could be supported by studies from Bloomfield, (1991) and Yasni et al., (2009) by the result from extracting Melinjo seeds and peel with well-diffusion procedure for *Staphylococcus aureus* and *Pseudomonas aeruginosa*, Melinjo extract possesses the same inhibition capacity in comparison to antibiotics (streptomycin 10ppm and penicillin G).

2.6 Extraction Methods

The term extraction has been used in pharmaceuticals to describe the process of separating medicinally active components from inactive or inert parts of plant or animal tissues by using selective solvents in standard extraction procedures (Ingle et al., 2017). Based on Bakar et al., (2013) research, the definition of extraction is by extracting an active, desirable property or a substance from the tissue of a plant for a specific purpose. It may be utilized in different activities such as additives, cosmetics, processing aids, or even food and functional properties for foodstuffs. Various industries can use an extract based on its chemical classification, although various industries mean different technologies used. Hence various solubility, stability and shape, other regulations, claim and problems arise.

It is crucial to identify an excellent raw material source for the extraction process. To ensure gain the high-quality extraction compound, it is best to understand the descriptive areas, the part with the most concentrated bioactive activity and how it be collected, and how the pre-preparation is set up since it may cause variability in the quality. All these plants' products are comparatively in liquids, semisolids, or powders intended solely for oral or external use. The extraction procedure aims to achieve the maximum biological activity from the extracts while maximizing the number of target compounds (Chang and Yang, 2002). The extraction techniques influence the extract yield and physical activity and affect their extraction solvent.

Based on Zhang et al., (2018), besides extraction techniques that may affect extraction solvent, temperature and duration also play a vital role in this procedure. Not only that, the selection of the solvent is the utmost critical for solvent extraction. For temperature, solubility and diffusion are increased at high temperatures. On the other hand, a too-hot temperature may allow solvents to evaporate, resulting in undesirable impurity extracts and the decomposition of thermolabile components (destruction due to change in response to heat). Many solvents extract active compounds from plant material, including methanol, ethanol, acetone, and water (Diem et al., 2014). Since plant material contains various bioactive compounds whose solubility properties vary in different solvents, the most appropriate extraction solvent is determined by the kind of plant material and the compounds to be isolated (Koffi et al., 2010). In particular, the smaller the particle size, the better the extraction result. The small particle size would improve the extraction efficiency due to increased solvent penetration and solute diffusion. However, too fine a particle size can result in excessive solute accumulation in the solid and difficulties subsequent filtration.

Years have passed, there are many extraction methods have arisen. The traditional extraction procedure is maceration, Soxhlet, percolation, reflux extraction. Typically, using organic solvents, requiring large volumes of solvents and long extraction times. Meanwhile, for modern extraction procedures is ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), as well as microwave-assisted extraction (MAE). They are effectively applied in natural product extraction as well, and they offer several advantages, such as lower organic solvent consumption, a shorter extraction time and more excellent selectivity.

2.6.1 Traditional Extraction Methods

The most common technique in the traditional extraction method is maceration. It is suitable for Small Manufacturing Enterprise (SME) level or small research settings. Maceration involves the soaking of plant materials, either coarse or powdered, into a stoppered container with a solvent and permitted for at minimum three days with daily agitation at room temperature. (Handa et al., 2008). After three days, the mixture is strained, the wet solid layer is pressed, and the mixed liquids are clarified by filtration or decantation. In this conventional process, the convection and conduction of heat is transferred and solvents indicate the compound shape generated from the samples. The process aimed to break and split the plant's cell wall and release soluble bioactive substances. (Azwanida, 2015). It is a simple extraction method but could lead to a long extraction time and low extraction efficiency. Also, proper management is needed to take care the ample of solvent used in this method.

Next is percolation. These are the most frequent extraction methods used to extract active ingredients when creating tinctures and fluid extracts (Pandey and Tripathi, 2014). The techniques used in percolation is similar to maceration but they used percolator. The percolator is a cone-narrow shape with two ends open. Plant materials are moistened with the solvent and then placed in a percolation chamber and let sit for approximately four hours. Afterwards, the plant material is rinsed multiple times with solvent until the active ingredient has been extracted. It is possible to use the solvent before reaching saturation point (Saxena et al., 2013). Compared to maceration, percolation is more efficient due to its continuous process in which the saturated solvent is always being replaced with the new solvent.

Hot continuous extraction, or the other name is Soxhlet extraction also one of the traditional extraction methods. In this extraction method, a porous or "thimble" bag made from hard filter paper, like a chamber shape of Soxhlet apparatus, a finely ground sample is inserted. The solvents are heated in the bottom bottle, vaporized in the thimble, dissolved into the condenser and dripped back into the flask. The liquid fluid is drained into the bottom flask agitated when it reaches the syphon handle. The advantages of this process are that a large amount of sample can be extracted with less solvent consumption and time taken (Zhang et al., 2018). It is only used for a small production operation but is much more cost-efficient and feasible when modified for a continuous extraction procedure on a large and medium scale.

Reflux extraction is a fluid-solid extraction process that happens for a period without losing solvent at a constant temperature, with recurring solvent evaporation and condensation. The system is commonly used in the herbal industry because it is practical, simple to use, and inexpensive (Wang et al., 2013). It is said that reflux extraction is has more advantages than maceration and percolation because they require less solvent used and extraction time. This can be proven research by Zhang, (2013) on comparison of the extraction method in traditional Chinese medicine of active ingredients. The reflux process is effective in decoction, and the maximum yield was achieved with 60% ethanol as an extraction solvent, baicalin and puerarin.

2.6.2 Modern Extraction Methods

A new technique of removing the bioactive compounds from microwave by-products that use microwave radiation is also known as a modern extraction method (Paré et al., 1994). Microwave radiation is a powerful electromagnetic wave that propagates at the speed of light in a vacuum. The interaction of microwaves with dipoles of polar (water and organic component) and polarizable materials (sample and solvent) generates microwave to heat. It is caused by the conduction of ions and the rotation of dipoles (Schmink, 2011; Sun et al., 2016). The dipolar cycle disrupts hydrogen bonding in the electromagnetic region, improving dissolved ion migration and causing the solvent's matrix penetration (Kaufmann and Christen, 2002). The radiation is generated by the moderate pressure of ionic currents, causing friction and releasing heat by the effect of the Joule. The energy of the microwave is used to convert the solid matrix into a solvent form, enabling quick and high-efficiency extraction. (Simone et al., 2020). However, poor heating happens in non-polar solvent due to energy only transmits by dielectric absorption. The technology is considered selective as it favours high polarity, high dielectric molecules and solvents (Belwal et al., 2018). The benefit of using MAE is it could provide synergic activity to speed up the extraction process and increase the yield because of the heat and mass transfers at MAE in the same direction (Simone et al., 2020).

Supercritical fluid extraction (SFE) is a sample preparation technique that minimizes the usage of organic solvents while enhancing sample throughput, whereas SFE is used as the extraction solvent. It is also known as dense-gas compression because it utilizes gases (CO2) and compresses them into a dense liquid. The material exhibits standard physical properties for both gases and liquids at the critical stage. Temperature, pressure, and sample volume all have a role in determining when a drug approaches its critical stage. Supercritical carbon dioxide (S-CO2) was frequently employed in SFE because CO2 is an excellent solvent for non-polar analytes and is inexpensive and nontoxic. Although S-CO2 has a low solubility for polar molecules, adding a tiny amount of ethanol or methanol can be changed to extract polar chemicals. Since S-CO2 vaporizes at average temperature, it produces analytes in concentrated form. SC-solvents' strength can be altered rapidly by modifying the temperature, heating, or adding modifiers, reducing extraction time. (Patil et al., 2014). But, the main disadvantages of this process are that the startup cost is prohibitive (Naudé et al., 1998).

Last but not least, ultrasound-assisted extraction (UAE) is under modern extraction method also to be known as sonification extraction. This is a highly advanced technology capable of extracting large amounts of bioactive compounds in the shortest amount of time. According to Handa et al., (2008), the range of ultrasound use in UAE is from 20 kHz to 2000 kHz. This is in line with Azmir et al., (2013) whereby it states that the cavitation phenomenon occurs due to the propagation of ultrasonic waves, causing the implosion of bubbles, which causes macroturbulence, high-velocity interparticle collisions, and perturbations inside the sample's microporous particles. Thereby, improving the extraction efficiency as the solute diffuses very fast from the solid stage to the solvent stage.

UAE too can be used for different solvents, such as acid, water and non-aqueous solvents. Furthermore, solvent selection is critical in ultrasound-assisted extraction, owing to its significant impact on cavitation efficacy and acoustic energy transference to reactants. Other strong values of choosing UAE in extraction method are its only low energy consumption and solvent usage.UAE also reduce the extraction time and it is a low-cost technology since the procedure is uncomplicated. For that reason, ultrasound-assisted extraction can be utilized in small and large phytochemical extraction scales (Azwanida, 2015)

CHAPTER 3

METHODOLGY

3.1 Plant Material

Seed and seed coat samples of Melinjo fruit (*Gnetum gnemon L.*) were collected. The samples were cleaned with tap water and rinsed with distilled water to remove dust and other inert materials. The cleaned samples were placed in the oven for 24 hours. The shaded dried samples were powdered using electrical blender (Durai et al., 2016).

3.2 Crude Extract Preparation

Dry powdered seed and seed coat materials were subjected to organic solvent extraction. Four organic solvents including aqueous, methanol, ethyl acetate and chloroform were use as solvent. 30 g of the seed and seed coat samples were taken into a separate conical flask and 200 ml of aqueous were added. The conical flask was kept on mechanical shaker for 100 r.p.m for 24 hours. The extract was filtered through Whatman filter paper. The filtrate will be collected and then concentrated using rotary evaporator to obtain the crude extract. Then, it was left in fume hood for 24 hours. The concentrated extract was stored in sterile containers in the refrigerator till further analysis. The extraction was carried out in duplicate. The procedures were repeated for other solvent extraction (methanol, aqueous, ethyl acetate, chloroform) (Sethupandian et al., 2016).

3.3 Qualitative Screening of Phytochemical

The condensed *Gnetum gnemon L.* crude extract of seed and seed coat were used for preliminary qualitative screening of phytochemical such as flavonoids, alkaloids, terpenoids, tannins, quinones, phenols, saponins, carbohydrates, and proteins.

3.3.1 Test for Flavonoids (Shinoda Test)

A 0.5 g of the extract was added with 1 ml of absolute ethanol (C_2H_5OH) and 3 drops of concentrated hydrochloric acid (HCl). Formation of red colour if aurones and chalcones are present. In cases where no colour will be observed, pieces of metallic magnesium will be added. Formation of orange, red or magenta colouration were observed if flavones and flavanols are present (Rondón et al., 2018).

3.3.2 Test for Alkaloids (Wagner Test)

Wagner's reagent was prepared. A 12.5 g of potassium iodide (KI) and 2.5g of iodine (I_2) were dissolved and the volume were made up to 250ml with distilled water (Kalpana et al, 2014). A few drops of Wagner's reagent were added to 1ml of extract along the side of test tube. Red brown colour precipitation or turbidity was observed if alkaloids were present (Kalpana et al., 2014).

3.3.3 Test for Terpenoids (Salkowski Test)

A 0.5 g of the extract was added with 2 ml of chloroform. Then, 3 ml of concentrated sulphuric acid (H_2SO_4) was added carefully to form a layer. A reddish-brown colouration of the interface indicated as presence of terpenoids (Kabubii et al., 2015).

3.3.4 Test for Tannins (Ferric Chloride Test)

A 0.5 g of the extract was boiled in 10 ml of distilled water in a test tube. The mixture was filtered. A few drops of 0.1% ferric chloride ($FeCl_3$) were added to the filtrate. Formation of blue-black precipitate indicates hydrolysable tannins are present. Formation of green precipitate if condensed tannins were present (Martha and Abdulmumin, 2016).

3.3.5 Test for Quinones

A 0.5 g of the extract was added with few drops of concentrated sulphuric acid (H_2SO_4). Formation of red colour was observed if quinones were present (Kalpana et al., 2014).

3.3.6 Test for Phenols

A 0.5 g of the extract was treated with 3% ferric chloride ($FeCl_3$). Formation of deep blue colour was observed if phenols were present (Sethupandian et al., 2016).

3.3.7 Test for Saponins (Foam Test)

A 0.5 g of the extract was added with 5 ml of boiling water in a test tube. The solution was allowed to cool and shaken well to mix thoroughly. Appearance of foam was observed if saponins were present (Kalpana et al., 2014).

3.3.8 Test for Carbohydrates (Molisch Test)

The extract was dissolved in 5 ml distilled water and filtered. The filtrates were used to test the presence of carbohydrates. 1 ml of the filtrate solution was treated with 2 drops of Molisch's reagent (α-naphthol dissolved in ethanol) in a test tube. 2 ml of concentrated sulphuric acid (H_2SO_4) was added on the side of the test tube. Formation of violet ring at the junction was observed if carbohydrates were present (Nagy, 2014).

3.3.9 Test for Proteins (Biuret Test)

1-2 spatula of the extract was added with 1 ml of water in a test tube. The mixture was stirred to mix well. 1 ml of sodium hydroxide (NaOH) was added and stirred. 2 drops of copper sulphate solution ($CuSO_4$) were added. Violet or pink colour were formed if protein was present (Nagy, 2014).

CHAPTER 4

RESULT AND DISCUSSIONS

Qualitative phytochemical study was performed through colour test, precipitation formation, and so forth. The change in particular colour and the production of precipitation revealed the presence of bioactive chemicals in *Gnetum gnemon L.* seeds and seed coats. Table 4.1 and Table 4.2 show the result of the analysis for detected phytochemicals in seeds and seeds coat of *Gnetum gnemon L.* respectively in this study.

Table 4.1: Phytochemical screening of seed of *Gnetum gnemon L*. by using various solvents

Phytochemical Test	Methanol	Chloroform	Ethyl Acetate	Aqueous
Flavonoid Shinoda test	+	-	-	-
Alkaloids Wagner test	+	-	+	+
Terpenoids Salkowski test	+	+	+	+
Tannins Ferric Chloride test	+	-	-	+
Phenols	+	-	-	+
Saponins Foam test	+	+	+	+
Carbohydrate Molisch test	-	-	-	-
Protein Biuret test	-	-	-	-
Quinones	+	+	+	+

Note: (+) indicate presence of phytochemicals, (-) indicate absence of phytochemicals.

Table 4.2: Phytochemical screening of seed coat of *Gnetum gnemon L*. by using various solvents

Phytochemical Test	Methanol	Chloroform	Ethyl Acetate	Aqueous
Flavonoid Shinoda test	+	+	+	+
Alkaloids Wagner test	+	-	+	+
Terpenoids Salkowski test	+	-	+	+
Tannins Ferric Chloride test	+	-	+	+
Phenols	+	-	-	+
Saponins Foam test	+	+	+	+
Carbohydrate Molisch test	-	-	-	-
Protein Biuret test	-	-	-	-
Quinones	+	+	+	+

Note: (+) indicate presence of phytochemicals, (-) indicate absence of phytochemicals.

In both seed and seed coat (Table 4.1 and Table 4.2) of *Gnetum gnemon L.*, all the extracts of different solvents (methanol, aqueous, ethyl acetate, chloroform) showed the positive results for the presence of saponins, which was detected by the appearance of foam on top of the test tube. Next, all extract expressed good result for the appearance of quinones in seed and seed coat of *Gnetum gnemon L.* which was observed with the formation of red colour (Table 4.1 and Table 4.2). This finding is in line with previous research stated that saponin in *Gnetum gnemon L.* possesses the ability to reduce cholesterol level in human body (Malinow et al., 1977). Besides, the role of quinones in *Gnetum gnemon L.* as anti-inflammatory agents where it can help to relieve pain (Kobayashi et al., 2011) and demonstrated a high level of anti-microbial activity. Therefore, it has the potential for the treatment of bacterial infections (Alibi et al., 2021). Apart from that, only the methanol, ethyl acetate and aqueous extract showed the presence of alkaloids in Wagner test for seed coat and seeds, which was detected by red brown precipitation (Table 4.1 and Table 4.2). The presence of alkaloids in *Gnetum gnemon L.* indicates the potency of anti-diabetic activity (Chang et al., 2015; Supriyadi et al., 2019) where it has the opportunity to create a low-cost nutritious functional food.

In the seed of *Gnetum gnemon L.*, all extracts of different solvents display the positive result for the presence of terpenoids (Table 4.1). These terpenoids were detected by the formation of reddish brown at the interface in Salkowski test. However, three of the four solvents tested were able to produce a favourable outcome in the seed coat, except for chloroform extracts (Table 4.2). Thereby, presence of terpenoids in four various extracts of seed and seed coat has verified that both seed and seed coat of *Gnetum gnemon L.* possess anti-microbial action (Raut and Karuppayi, 2014) and it exert anti-cancer activity by inducing cell death (apoptosis) in cancer cells without damaging the normal cells. Terpenoids also have the ability to stimulate immunological response along with protecting skin cells from ultraviolet radiation all of which these functions can be applied in the nutraceutical industry (Ludwiczuk et al., 2017; Lesgards et al., 2014).

Next, Shinoda test was carried out to determine the presence of flavonoid in seed coat of *Gnetum gnemon L.*, whereby all the tests revealed positive results (Table 4.2). Yet, only methanol solvent in seed of *Gnetum gnemon L.* yielded a positive response (Table 4.1). It was detected by formation of orange colour. This can be corroborated in the study by Saraswaty et al., (2017) which concluded that *Gnetum gnemon L.* seed coat contained the highest antioxidant activity. With the presence of flavonoids in seed coat and seeds, this discovery is consistent with the fact that *Gnetum gnemon L*. exhibits antioxidant activity which has the ability to mitigate oxidative stress (Panche et al., 2016) and anti-inflammatory action (Kesarkar et al., 2009) that help to regulate mediators involved in inflammation in human body. Furthermore, antioxidant effect in *Gnetum gnemon L.* contributes to the protection of cells against free radicals (Santoso et al., 2010) As a result, it has the potential to be used as a medicine in the nutraceuticals and pharmaceutical industries.

The following test is the Ferric Chloride test. It is revealed that only methanol and aqueous extracts in the seed of *Gnetum gnemon L.* contained tannins (Table 4.1). Nonetheless, tannins were detected in the seed coat of *Gnetum gnemon L.* using methanol, aqueous, and ethyl acetate solvent (Table 4.2). Next, *Gnetum gnemon L.* seed and seed coat were only detected through methanol and aqueous extraction for phenols (Table 4.1 and Table 4.2). Green precipitation was found in the presence of tannins, while deep blue was observed in the presence of phenols. According to the previous studies, methanol was the most effective solvent for extracting phenol compound. The best solvent to extract condensed tannins was aqueous and ethyl acetate to extract hydrolysable tannins (Rhazi et al., 2019). This demonstrated that the influence of the solvent's nature on phenolic compound extractions. Besides, based on prior findings hot water extraction procedure can generate higher yields. (Corral et al., 2020; Duraisamy et al., 2020).

Therefore, the presence of tannins showed cardioprotective properties (Karthikeyan., 2007) and anti-ischemic activity (Beretta et al., 2009). It has the potential of preventing the development of certain coronary syndromes by hindering the atherogenic process and balancing blood pressure (Hort et al., 2012). The presence of phenols in *Gnetum gnemon L.* confers an anti-diabetic action, aiding in the improvement of acute insulin secretion and sensitivity to insulin (Aryaeian et al., 2017; Seo et al., 2008). Thereby, these valuable capabilities for public health could be utilised in the pharmaceutical and nutraceuticals industry.

However, the Molisch and Biuret tests revealed an absence of carbohydrate and protein for both seed and seed coat of *Gnetum gnemon L.* For the Molisch test, a violet ring formation was unable to be observed at the test tube's junction, whereas for the Biuret test, a pink or violet colour was not formed. The negative results may indicate the minimal amount of carbohydrate and protein. Additionally, it is suspected due to the samples used are crude extracts, the presence of carbohydrate and protein in *Gnetum gnemon L.* may be affected by the impurities in the extract.

The occurrence of phytochemical classifications clearly demonstrates high efficacy of seed and seed coat extracts with different solvents. The present study shown that efficacy rises in the following order, chloroform <ethyl acetate < aqueous < methanol extracts of seed and seed coat of *Gnetum gnemon L.* This arrangement is according to polarity of a solvent, solubility of powdered seed and seed coat of *Gnetum gnemon L.* together with these phytochemicals are poorly soluble in it (Gupta and Dhawan, 2017).

Hence, this is in line with previous research that showed, methanolic extract of seeds of *Gnetum gnemon L.* provided the most significant level of saponins, quinones, alkaloids, flavonoids, tannins, terpenoids, and phenol because methanol was a universal solvent with the polar property (Abdul Mun'im et al, 2017)

CHAPTER 5

CONCLUSION

In conclusion, the result of this research showed that both of the seed and seed coats of Melinjo fruit (*Gnetum gnemon L.)* contain phytochemicals. It uncovers that seed coat extracts contain more classes of phytochemicals compare to seed. These results suggested that methanol is the best solvent for phytochemical compound extractions from the seed and seed coat of *Gnetum gnemon L.* The other key finding in this study is that the selection of particular solvent is vital for extracting bioactive compounds effectively. Furthermore, the temperature, solvent and sample ratio, as well as the polarity of the solvent, will give an impact on the extraction procedure

. There are several recommendations are suggested for future research such as, applying advanced extraction techniques. For example, microwave-assisted extraction (MAE). MAE is well-known for its ability to extract bioactive compound from material more rapidly than conventional extraction process. Besides, thin layer chromatography technique also can be applied since it provides a fast separation of compound, thereby reveal the quantity and nature of the mixture's components in *Gnetum gnemon L.* However, the phytochemical that found in *Gnetum gnemon L.* should be further isolated, purified, analysed and described in future research to gain a thorough understanding of this plant potential properties against disease. Additionally, owing to the lack of previous research on phytochemicals in the seed coat of *Gnetum gnemon L.*, the positive results for some classes of phytochemicals in the seed coat demonstrated in this work should be interpreted cautiously. A safety test for pharmaceutical and nutraceutical application should be conducted in order to generate novel medicines that fulfil safety standard that will be used to improves people health.

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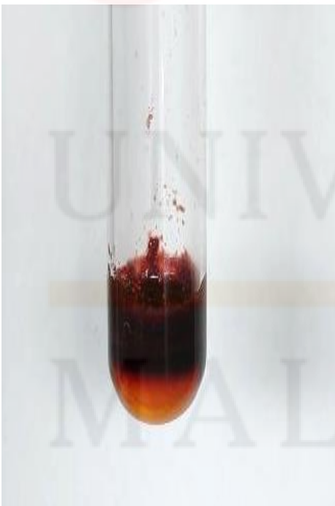
Table A.1: Phytochemical screening of *Gnetum gnemon L.*

Details	Descriptions
	Frothing in foam test indicates the presence of saponins
	Formation of red colour indicates the presence of quinones

Formation of red brown precipitation in Wagner test indicates the presence of alkaloids

Formation of orange colour in Shinoda test indicates the presence of flavonoids

	Formation of blue-black precipitation indicates the presence of tannins
	Formation of deep blue colour indicates the presence of phenols

Formation of reddish-brown colouration of interface in Salkowski test indicates the presence of terpenoids