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Open Access Journal of Agricultural Research
ISSN: 2474-8846

Determination of Selected Phytochemicals and Nutritional Compositions in Melinjo (*Gnetum gnemon* L.) Fruit using Different Extraction Solvents

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Research Article

Volume 7 Issue 2

Received Date: July 11, 2022

Published Date: August 02, 2022

DOI: 10.23880/oajar-16000290

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 has 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 extract 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 to extract 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.

Keywords: Plant; Natural Product; Phytochemical; Melinjo fruit (*Gnetum gnemon L.*)

Abbreviations: UAE: Ultrasound Assisted Extraction; MAE: Microwave Assisted; SFE: Supercritical Fluid Extraction.

Introduction

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 [1]. 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 [2]. 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 [3]. Thus, this demonstrates how plants are used to address medicinal conditions.

Phytochemicals 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 [4]. It essentially promotes health advantages and serves as a shield against diseases in the human body [5]. Phytochemicals can be found in various part of the plant section, including the root, stem, leaves, flower, fruit and seeds [6]. 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 [7]. 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. [8] 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 [9]. The *Gnetum gnemon L.* tree is a slender, shade-tolerant shrub that can reach a height of 18 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 [1]. Additionally, Melijo fruits three times a year in Indonesia and Malaysia; March-April, June-July, and September-October.

Moreover, according to Supriyadi, et al. [10] of *Gnetum gnemon L.* shows it has good advantages for human benefits due to its antidiabetic and antioxidant activities that extracted from their seed protein using enzymatic hydrolysis. This is because the immature seed has a good source of protein, antidiabetic peptide and antioxidant. As per researchers reported, the seeds have antimicrobial, antioxidant, antibacterial, anti-aging, tyrosinase inhibitory, and anti-inflammatory activities, [1,11]. And furthermore, *Gnetum gnemon L.*, which is high in bioactive compounds such as flavonoids, tannins, saponoids, and stilbenoids, contains trans-resveratrol, which has been demonstrated in trials to be beneficial in the treatment of diabetes and cardiovascular disease [12].

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 [13]. 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.) [14].

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 and study of dietary compositions. 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 [15].

Hence, the objectives of this study were to: (i) determine the selected phytochemicals in the seed and seed coat of Melinjo fruit (*Gnetum gnemon L.*) using different extraction solvents; and to (ii) determine the most effective solvent to extract phytochemical between seed and seed coat of Melinjo fruit (*Gnetum gnemon L.*).

Materials and Methods

Plant Materials

Seed and seed coat samples of Melinjo fruit (*Gnetum gnemon L.*) were collected in Peninsular Malaysia. 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 [16].

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) [17].

Qualitative Screening of Phytochemical and Nutritional Composition

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

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 color if aurones and chalcones are present. In cases where no color 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 [18].

Test for Alkaloids (Wagner's 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. 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 are present.

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 [19].

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 are present [20].

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 color was observed if quinones are present [21].

Test for Phenols: A 0.5 g of the extract was treated with 3% ferric chloride ($FeCl_3$). Formation of deep blue color was observed if phenols are present [17].

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 are present [21].

Test for Carbohydrates (Molisch's 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 are present [22].

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 color were formed if protein is present [22].

Results and Discussion

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 1 and Table 2 show the result of the analysis for detected phytochemicals in seeds

and seeds coat of *Gnetum gnemon L.* respectively in this study.

In both seed and seed coat (Tables 1 and 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 (Tables 1 and 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 [23]. Besides, the role of quinones in *Gnetum gnemon L.* as anti-inflammatory agents where it can help to relieve pain [24] and demonstrated a high level of anti-microbial activity. Therefore, it has the potential for the treatment of bacterial infections [25]. 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 (Tables 1 and 2). The presence of alkaloids in *Gnetum gnemon L.* indicates the potency of anti-diabetic activity [10,26] where it has the opportunity to create a low-cost nutritious functional food.

Phytochemical test	Methanol	Chloro form	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 1: Phytochemical screening of seed of Gnetum gnemon L. by using various solvents.

Phytochemical test	Methanol	Chloro form	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 2: Phytochemical screening of seed coat of Gnetum gnemon L. by using various solvents.

In the seed of *Gnetum gnemon L.*, all extract of different solvents displays the positive result for the presence of terpenoids (Table 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 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 [27] and it exert anticancer 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 [28].

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 2). Yet, only methanol solvent in seed of *Gnetum gnemon L.* yielded a positive response (Table 1). It was detected by formation of orange colour. This can be corroborated in the study by Saraswaty, et al. [29] 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 [30] and anti-inflammatory action 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 [31]. 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 1). Nonetheless, tannins were detected in the seed coat (Table 2) of *Gnetum gnemon L.* using methanol, aqueous, and ethyl acetate solvent. Next, *Gnetum gnemon L.* seed and seed coat were only detected through methanol and aqueous extraction for phenols (Tables 1 and 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 [32]. 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 [33].

Therefore, the presence of tannins showed cardioprotective properties Karthikeyan, 2007 and anti-ischemic activity. It has the potential of preventing the development of certain coronary syndromes by hindering the atherogenic process and balancing blood pressure [34]. 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 [35,36]. Thereby, these valuable capabilities for public health could be utilised in the pharmaceutical and nutraceuticals industry.

But, 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 [37]. 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 [38].

Conclusion

The result of this study 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. There are several recommendations 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.

- **Conflict of Interests:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

- **Acknowledgement:** The authors would like to thank Universiti Malaysia Kelantan for providing the research facilities and Malaysia Ministry of Higher Education for Fundamental Research Grant Scheme (FRGS) (Ref: FRGS/1/2018/WAB01/UMK/02/1).

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