THESIS

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EXTRACTION OF BIOACTIVE COMPONENTS OF WALNUT PRESS-CAKE AND APPLICATION OF IT AS ANTIOXIDANTS

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I. INTRODUCTION

Walnuts (Juglans regia L.) recognized as one of the most important nuts worldwide, are presently cultivated commercially throughout southern Europe, northern Africa, eastern Asia, the United States, and western South America. Based on recent reports, walnuts seem to attain huge attention for their increasing health-promoting properties largely contributed by the abundance of bioactive compounds such as plant sterols, dietary fiber, and polyphenols (Fukuda et al., 2003).

In this research, we focused on walnut press-cakes polyphenol and their antioxidant activity. The cultivar, environmental factors, cultivation practices, and the oil recovery processes decide the composition as well as the quality of walnut press-cake, but it is still remarkably rich in oil (20–36 %), proteins (30–42 %) (Emre Bakkalbasi, 2015; Martínez et al., 2010; Santos et al., 2018), dietary fiber, phenolic substances, and minerals. Due to the polyunsaturated fatty acids of the oil, the press cake is sensitive to oxidation, especially when ground into a fine powder. This behaviour was attributed to the high vitamin E content of the cake which can reach 174 mg/kg (Emre Bakkalbasi, 2015) but other antioxidants such as phenolic compounds may contribute to oxidative stability since most of the phenolics initially present in the walnut kernels remain in the cake after cold pressing.

The experiments are to extract the polyphenol from walnut press-cake, and further analyze the antioxidant activity in combination with sunflower oil. Extraction efficiency can be improved by using innovative technologies, the research compares the application of ultrasound-assisted extraction (UAE) and water bath extraction (WBE) methods. Research by (Han et al., 2018) and (Tabaraki & Rastgoo, 2014) has shown that ultrasound assistance is the best method to intensify the recovery of phenolic compounds and the phenolic compounds extracted from walnut shells and husks are potentially good natural sources of antioxidants for the food and pharmaceutical industries. Mixed solvent of water and alcohol (specifically acetone-water) is used to extract polyphenols. To determine the antioxidant capacity of walnut-pressed extract, the total polyphenol and antioxidant capacity were measured by Total polyphenol content (TPC) and Ferric-reducing antioxidant power (FRAP) methods, and the remaining extract was dried for the next experiment. To test the effectiveness of this powder in food processing, the residue powder was mixed with sunflower oil, and the free fatty acid measurements were taken.

II. LITERATURE REVIEW

2.1 Characteristics of walnuts

2.1.1 General introduction of walnuts

Walnut is the edible seed of a drupe of any tree of the Genus Juglans, particularly the Persian or English walnut, they are rounded, single-seeded stone fruits of the tree commonly used for food after fully ripening between September and November, in which the removal of the husk at this stage reveals a browning wrinkly walnut shell. The shell surrounds the kernel, which is usually made up of two halves separated by a membranous partition, and this shell turns to be hard and brittle during ripening. Walnut seed kernels are covered in a brown seed coat that is rich in antioxidants, to protect the oil-rich embryo from atmospheric oxygen, thus preventing rancidity.

According to (FAOSTAT), in 2022, 3,8 million tons of whole walnut was harvested in the World, China is the leading world producer (they contribute 31% of the total), followed by the USA, Iran, Turkey, Ukraine, Romania, France, and India, but production in other countries such as Chile and Argentina have increased rapidly in recent years.

2.1.2 Nutrition fact and health benefit of walnuts

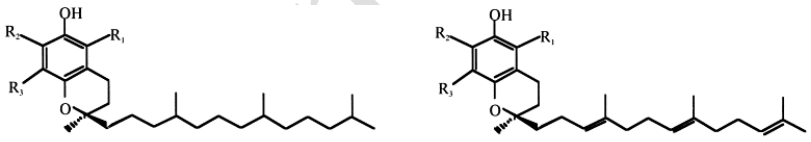
The walnut kernel is a rich source of proteins, fats, vitamins, minerals, and polyphenols. In detail, the health-promoting properties of walnut kernels are attributed to the polyunsaturated fatty acids (such as α linolenic acid), phenolics, phytosterols, tocols, B-vitamins, phospholipids, sphingolipids, carotenoids, proteins, dietary fiber, and non-sodium minerals sterols, pectic substances, phenolic acids, and related polyphenol, which makes the fruit indispensable for human nutrition. Some prominent compositions of walnut kernels and their health benefit are classified in subsections below.

Walnuts have abundant vitamin sources.

Vitamin E, a major lipid-soluble chain-breaking antioxidant found in membranes, acting as membrane stabilizer, has been shown to have significant health benefits in preventing a variety of diseases as an important factor in the defence of polyunsaturated fatty acids against the destruction of peroxidative. Vitamin E is a term used to refer to a group of minor but ubiquitous lipid-soluble compounds, encompassing four tocopherols (α-, β-, γ-, and δ-) and four tocotrienols (α-, β-, γ-, and δ) (Figure 1). Tocopherols and tocotrienols play two main roles as antioxidants, firstly as scavengers of lipid peroxyl radicals, and secondly

as singlet oxygen quenchers and chemical scavengers (Munné-Bosch & Alegre, 2002). In addition, Vitamin E with ability to donate phenolic hydrogen to lipid free radicals is the main contributor to the activity of tocols. In walnut, tocopherols are present in two main forms: alpha-tocopherol and gamma-tocopherol, γ-tocopherol has been shown to inhibit platelet aggregation and delay arterial thrombus formation in rats (Saldeen et al., 1999), while α-tocotrienols have a cholesterol-lowering effect (Qureshi et al., 1995), anticancer and tumor suppressive activities (Nesaretnam et al., 1998), antioxidant properties and anti-aggregation of blood platelets. Importantly, these effects of γ-tocopherol are more potent than α-tocotrienol.

A 100-gram of walnuts contains approximately 21 mg of gamma-tocopherol (vitamin E), and this amount provides 140% of the daily intake. As a strong fat-soluble antioxidant, vitamin E is required to protect the mucus and skin cell membranes against the damaging effects of free radicals and to maintain their unity. In addition, walnuts further contain important vitamin structures such as riboflavin, niacin, thiamine, pantothenic acid, vitamin B6, and folate/B9 (Sen.S.M, 2013). As walnut has a preventive effect on inflammation, they decrease the risk of high blood pressure; they also prevent arterial illnesses that can result in heart, and brain stroke, chest diseases, and colon and prostate cancer.



tocopherol

tocotrienol

tocopherol	R1	R2	R3
α-	CH_3	CH_3	CH_3
β-	CH_3	H	CH_3
γ-	H	CH_3	CH_3
δ-	H	H	CH_3

tocotrienol	R1	R2	R3
α-	CH_3	CH_3	CH_3
β-	CH_3	H	CH_3
γ-	H	CH_3	CH_3
δ-	H	H	CH_3

Figure 1. The chemical structure of Vitamin E isoform (Manolescu et al., 2008)

Walnuts are a protein, fat, and carbohydrate source

Walnut proteins can be categorized into four major categories: albumin, globulin, prolamin, and glutelin, study by (Sze-Tao & Sathe, 2000) accounted that glutelin is the major portion of protein in walnut. For instance, a number of these bioactive components have been reported to have free radical-scavenging capacities and have been shown to protect LDL-cholesterol from oxidative modification in vitro. Proteins (up to 24%) and lipids (up to

70%) account for more than 84% of the walnut kernel weight, however, walnut lipids are highly susceptible to oxidation leading to quality deterioration, shelf-life reduction, and consumer rejection. It has been reported that walnuts are an important source of essential polyunsaturated fatty acids (particularly linoleic acid), which may be able to alleviate several diseases and disorders. In the case of walnut residue, 65% of the protein was found (Gu et al., 2015), reported by (Arranz et al., 2008) showed that walnut residue contributed the most to walnut antioxidant activity, and walnut oil contributed less than 5% of walnut total antioxidant activity, which provided enough information for walnut residue active ingredient extraction. In addition to proteins and lipoids, walnut provides acceptable amounts of thiamin, riboflavin, vitamins, potassium phosphorus, etc (Sze-Tao & Sathe, 2000).

Walnuts are an excellent energy-dense, high-calorie source of food providing 630kcal per 100 grams, and 65% of walnuts are fat by weight. However, the study of (Sabaté et al., 2005) indicated that walnuts do not increase the risk of obesity when applying walnut-supplemented diets.

Walnuts in terms of heart health

Walnut is considered the number one heart-health-friendly hard-shell fruit, 10% of their energy is owned by Alfa-linoleic acid (ALA), a polyunsaturated n-3 fatty acid (n-3PUFA) found in vegetables are rich in phytomelatonin and contain more polyphenols than any other nut (Hayes et al., 2016). The bioavailability of ALA is almost complete as, like other unsaturated fatty acids, it is nearly 100% absorbed from the diet (Kris-Etherton et al., 2000). The high content of PUFA (linoleic and linolenic acid) in walnuts has been suggested to reduce the risk of heart disease by decreasing total and low-density lipoprotein (LDL) cholesterol and increasing high–density lipoprotein cholesterol (HDL-cholesterol), reducing inflammation and improving arterial function, and contributing nutrients that are essential to a healthful lifestyle (Papoutsi et al., 2008). In addition, recent studies indicated that ALA metabolism gives rise to vasodilatory and anti-inflammatory oxylipids, which might underlie the beneficial effect of walnut consumption on endothelial function, in addition, ALA is neuroprotective, although polyphenols from walnuts probably synergize for beneficial effects on brain function (Ros et al., 2018).

Researchers have proven that walnut consumption protects the body against heart disease, certain cancer types, diabetes type 2, and other health issues. As walnut helps blood flow more easily through the veins, it reduces the risk of heart attacks. For example, (Fukuda

et al., 2004) examined the in-vivo antioxidative effect of a polyphenol-rich walnut extract on oxidative stress in mice with type 2 diabetes and proved the effect of ellagitannins as an effective antioxidant, which is responsible for the suppressive effect on oxidative stress of diabetes.

Walnuts as an antioxidant source

Walnut consumption reduces cholesterol, oxidative stresses caused by free radicals, and the inflammations that damage health (Anderson et al., 2001; Morgan et al., 2002). Walnuts contain more than 20 mmol antioxidants per 100 grams, mostly in walnut pellicles (Blomhoff et al., 2006). In walnuts, antioxidant substances are 2-15 times as potent as vitamin E, which has powerful antioxidant properties that protect the body against disease-causing natural chemicals, and a handful of walnuts contain twice of antioxidants in comparison to other commonly consumed nuts (Vinson & Cai, 2012).

According to research by (Fukuda et al., 2003), most of the antioxidant capacity of walnut press-cake results from polyphenolic content, and prominant, ellagic acid, a major component of polyphenol in walnut press-cake as well as the whole walnut kernel has been proposed to exert antiatherogenic, anticarcinogenic, and antioxidative properties (Anderson et al., 2001).

Walnut oil

Walnut is also a good source of vegetable oil, which content of walnut kernels is 52–70% oil and can be applied for cooking and as an ingredient in the painting and cosmetics industry. Walnut oil is very susceptible to oxidation and rancidity and is consequently unsuitable for long storage, refining, deep frying, or any other cooking applications owing to its low smoking point, the oil is used directly (without refining) for edible purposes, mainly as a salad dressing and cooking (Poggetti et al., 2018). Furthermore, walnut oil is used as a component of dry skin creams, antiwrinkle and anti-aging products due to its high content of essential fatty acids particularly linoleic and linolenic acids, according to (Mao-Qiang et al., 1993), these acids are crucial agents responsible for the regulation of transepidermal water loss, which is one of the most important functions of the skin.

2.1.3 Walnut flour, a by-product of walnut extraction

Walnut flour is obtained by milling the press cake from walnut oil extraction, which contains polyunsaturated fatty acids and other bioactive compounds. The press-cake is rich

in the brown skin that covers the kernel which is important to protect fatty acids from oxidation, and the presence of phenolic compounds is responsible for this action (Burbano & Correa, 2021). The components of walnut flour are used in the formulation of various functional food products, including meat, dairy, and bakery products, which have positive effects on nutritional, functional, and sensory characteristics, for instance, a high capacity for water absorption, solubility, bulk density, and rapid viscosity. The use of press-cakes in bakery products has been extensively applied to improve their nutritional quality, modifying color, specific volume, and texture, and improving their antioxidant properties (Emre Bakkalbasi, 2015). However, there are relatively few studies on walnut powder's use in food, walnut residue from oil extraction is usually used as low-value feedstuff, fertilizer, and other materials. To a certain extent, walnut residue is uneconomically used, which has hindered the development of the industry.

2.1.4 Walnut consumption and dietary intake

There are many ways to consume walnuts, including unprocessed, roasted, salted, and flavored. Besides enhancing the flavor of yogurt, pizza, and cake, the walnut husk can also be sprinkled on salads and desserts, including walnut ice cream, and is widely applied in the confectionery industry. However, it is undeniable that walnuts are dense in energy and excessive consumption can easily lead to weight gain, in addition, walnuts also contain a large amount of fibers, which also cause bloating symptoms or stomach pain. It is recommended by many guidelines that walnuts be consumed regularly and in sufficient quantities to benefit health, and that 30 grams of walnuts per day be consumed to reduce chronic diseases and improve cardiovascular health (Brown et al., 2021).

2.2 Antioxidants

Antioxidants are compounds that either delay or inhibit the oxidative - a chemical reaction that can lead to polymerization and other chain reactions and produce free radicals by scavenging peroxidation initiating species, bind metal ion catalysts, decomposing primary products of oxidation to non-radical species and breaking chain reactions, decrease oxygen concentrations, intercept singlet oxygen reaction to prevent continued hydrogen abstraction from substrates (Ribeiro et al., 2019). Free radical is a general term used for reactive oxidant species (ROS), this group includes hydroxyl radicals OH-, Hydrogen peroxide (H_2O_2), peroxyl radical (ROO·), and reactive nitrogen species including nitric oxide (NO), one of the causes of degenerative diseases, damage DNA, cell membranes, and

other parts of cells (Pisoschi & Pop, 2015). The effect of antioxidants depends on several factors including antioxidant structure, oxidation condition, and the nature of the sample being oxidized. Antioxidants act at different levels in the oxidative sequence involving lipid molecules, in walnuts, this oxidation mechanism is mainly conducted by polyphenols and their subclass such as phenolic acids, flavonoids, and tannin which scavenge those free radicals. There are several clinical studies suggesting that the antioxidants in fruits, vegetables, tea, and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic diseases including heart disease and some cancers. Due to their unique properties of extending the shelf life of food products without adverse their sensory or nutritional qualities, antioxidants have become an essential part of preservation technology and contemporary health care. Antioxidants for use in food systems must be inexpensive, nontoxic, and effective at low concentrations; highly stable and capable of surviving processing; have no odor, taste, or color of their own; easy to incorporate and have good solubility in the product (Gulcin, 2020).

In walnuts, antioxidants are present in both nutritive and non-nutritive compounds, the nutrients antioxidants in walnuts such as protein, lipids, vitamins, and minerals have been well described in the previous section, and some outstanding non-nutritive compositions in terms of phytochemicals will be listed in the following sections. The high antioxidant capacity of the walnut kernel is appraised in two major fractions walnut oil and defatted walnut. While the contribution of oil to the total antioxidant capacity of walnuts is less than 5%, most of the antioxidant capacity in walnuts results from phenolic compounds in the defatted walnut fraction (Arranz et al., 2008).

2.3 Non-nutritive antioxidants in terms of phytochemicals- Polyphenols

Walnuts are abundant sources of natural antioxidants, particularly polyphenols, which include phytochemicals such as flavonoids, isoflavones, phenolic acid, sterols, lignins, and other low-molecular-weight compounds, these polyphenols can serve as an excellent alternative to synthetic counterparts

2.3.1 Polyphenol classification

The polyphenol family contains one of the most abundant and extensively studied molecules that naturally occur in the plant kingdom, and innumerable phenolic structures have already been characterized (Bravo, 2009). Polyphenols are strong antioxidants that complement and add to the functions of antioxidant vitamins and enzymes as a defense

against oxidative stress caused by excess reactive oxygen species (ROS), ultraviolet radiation, mechanical damage, or certain pathogens and predators (Tsao, 2010). Studies have also shown that different polyphenol subgroups may differ significantly in stability, bioavailability, as well as physiological functions related to human health, and all of them less or more exhibited antioxidant activity. This family includes an abundant diversity of substances that contain a polyphenol group, mainly flavonoids, phenolic compounds, tocopherols, phytosterols, tannins, and a variety of other polyphenolic plasma antioxidant capacity and/or decrease plasma oxidized compounds that possess hydroxy groups bound to an aromatic group (Li et al., 2021) as illustrated in Figure 2. Although polyphenols are chemically characterized as compounds with phenolic structural features, this group of natural products is highly diverse and contains several sub-groups of phenolic compounds, according to the mass spectrometry results, ellagic acid (EA) is the predominant polyphenol in walnuts (Vu et al., 2018).

Polyphenols are a structural class of mainly natural, but also synthetic or semi-synthetic, organic chemicals that are characterized by the presence of one or more aromatic rings bearing one or more hydroxyl moieties (Manach et al., 2004; Ververidis et al., 2007). Natural polyphenols generally occur in conjugated form with one or more sugar residues associated with a hydroxyl group, although direct linkage of the sugar unit to an aromatic carbon atom can also occur (Zhang & Tsao, 2016). In nature, polyphenols can occur in two main dependent and/or independent ways, one route involves the binding of two-carbon units, that is, activated acetate, to form polyketides, which undergo subsequent cyclization into polyphenols. Another mechanism is the shikimic acid pathway, by which most phenolic compounds are biosynthesized, via this route, the derived carbohydrate precursors of the glycolysis and pentose phosphate pathways are converted to the aromatic amino-acids phenylalanine, tyrosine, and tryptophan (Cutrim & Cortez, 2018). This is an extremely wide and complex group of plant substances.

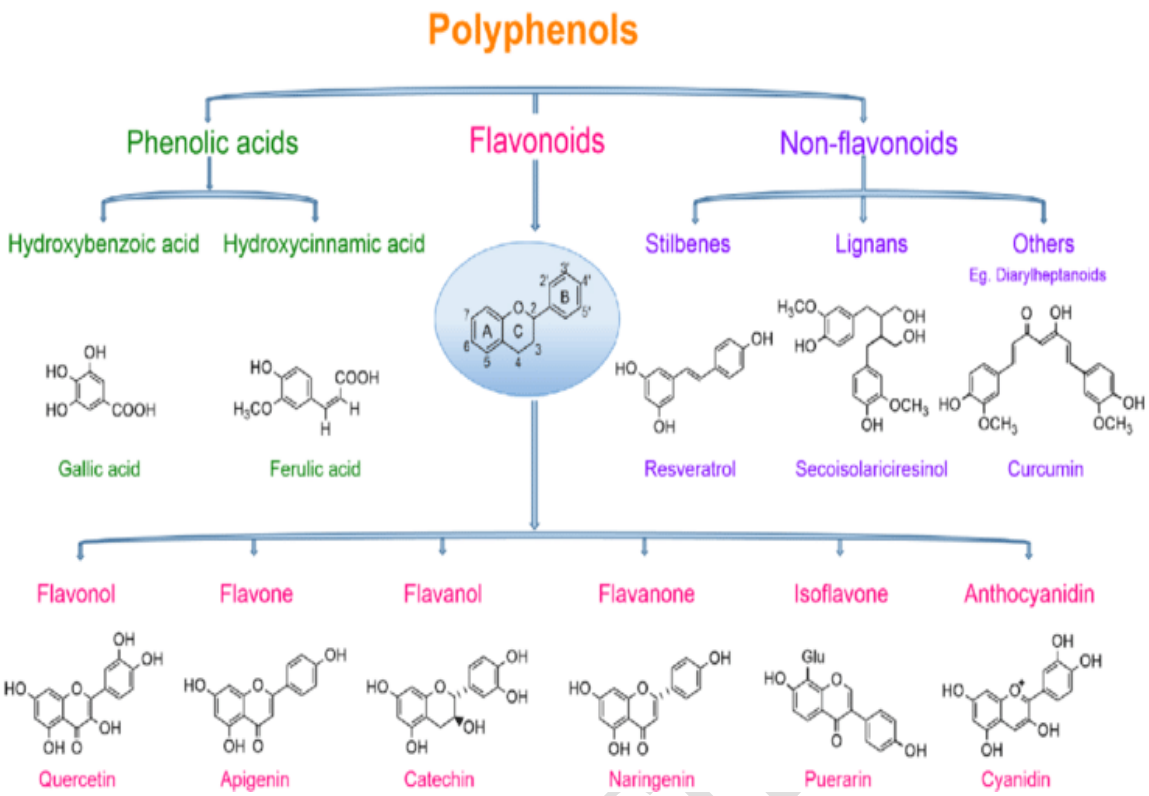


Figure 2. Classification of polyphenols with their basic chemical structure and typical representatives (Rambaran, 2020)

Polyphenols have several industrial applications, such as in the production of paints, paper, and cosmetics, as tanning agents, and as natural colorants and preservatives in food industry. The content of polyphenolics and their structure (the number and position of the hydroxyl groups in a molecule) significantly influence the pharmacological properties of medicinal plants (Zagoskina et al., 2023).

2.3.2 Polyphenol in walnut

Among common foods, walnuts are one of the most important sources of polyphenols, with a reported content of up to 2500 mg/100 g, this amount is highest in comparison with other nuts (Vinson & Cai, 2012). Some studies have demonstrated that the antioxidant capacity was mainly attributed to the phenolic compounds (Cao et al., 1997; Prior et al., 2001). The phenolic compounds present in the walnuts are mainly hydrolyzable tannins and phenolic acids, the former accounting for 60–80 % depending on the nut variety, while the flavonols vary from 26 to 35% (Ojeda-Amador et al., 2018), their chemical characteristics and biological effects are well documented in the next sections. Besides that, a wide range of biological activities of polyphenols in walnuts are demonstrated by ellagitannins, mainly pedunculagin (~1600 mg/100 g) (Cerdá et al., 2005) and their

metabolites, suggesting a potential beneficial effect on human health (Sánchez-González et al., 2017). According to Sánchez, the presence of free ellagic acid in human plasma could be due to its release from the hydrolysis of ellagitannins, enabled by physiological pH and/or gut microbiota. However, the mechanisms of action supporting such benefits are still under debate. A recent meta-analysis reported a reduction of adiposity, LDL-cholesterol, and glucose following the consumption of ellagitannin-rich foods, particularly walnuts (García-Conesa et al., 2018).

Walnut polyphenols can lower blood lipids (total triglycerides and total cholesterol) and LDL, increase the concentration of HDL and inhibit pancreatic lipase activity (Haider et al., 2011), thereby reducing the risk of cardiovascular diseases. The major point of attraction to researchers and food manufacturers nowadays is the potent antioxidant properties of polyphenols and their preventive role in various oxidative stress-related.

2.4 Phenolic compounds

2.4.1 Phenolic acids

Phenolic compounds are the main classes of secondary metabolites, not only present in walnuts but also in fruits and vegetables in abundance. This term of an aromatic ring with at least one hydroxyl group, phenolic is highly diversified and comprises several subgroups, ranging from rather elementary substances, such as phenolic acids or less common stilbenes, to highly polymerized molecules, such as tannins, derived from the elementary compounds and defined according to the number of phenol rings and structural elements that bind these rings to another (Cheynier et al., 2015; Tsao, 2010). Two classes of phenolic acids, hydroxybenzoic acids, and hydroxycinnamic acids are found in plant materials (Shahidi & Naczk, 2003). Hydroxybenzoic acids include gallic, p-hydroxybenzoic, vanillic, syringic, and protocatechuic acids, among others; while hydroxycinnamic acids include coumaric-, caffeic-, ferullic acids (Figure 3).

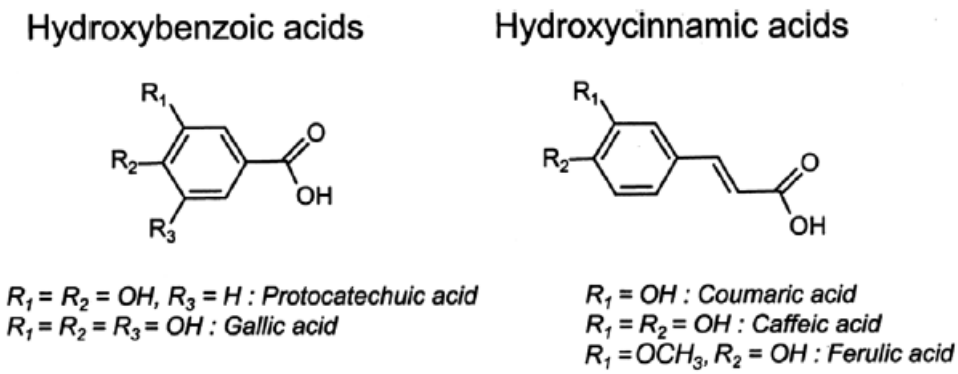


Figure 3. Chemical structure of hydroxybenzoic acids and hydroxycinnamic acids (Cutrim & Cortez, 2018)

Among the antioxidants, phenolic acids are known to be excellent natural antioxidants that have piqued the interests of food and pharmaceutical industries by expressing different antioxidant potentials, as protective substances against numerous kinds of stresses that can be triggered by environmental conditions, pathogens, and injuries. At a low concentration, phenolics act as antioxidants and protect food from oxidative rancidity (Karakaya, 2004). More information on the phenolic compounds of various nuts are clarified (Bodoira & Maestri, 2020), while the case of walnuts is more specifically described by (Jahanban-Esfahlan et al., 2019), particularly the antioxidant content of phenolic extracts obtained from different parts of the walnut tree and fruit. Synthetic phenolic antioxidants currently permitted for use in foods are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary-butylhydroquinone (TBHQ), in addition to octyl gallate (OG) and dodecyl gallate (DG) (Makahleh et al., 2015). These synthetic phenolic antioxidants are deliberately added to products to prevent or delay the onset of lipid oxidation during the processing and storage of fats, oils, and lipid-containing foods and have been used by the food industry for some 60 years.

The phenolic compounds and other substances in walnuts have been proven to improve human health and the evaluation of the beneficial effects generally lies in the total phenol content (TPC), total flavonoid content (TFC), and total condensation tannin content (TCTC) linked with antioxidant activity, in this project, total phenolic content of walnut press-cakes was determined. Flavonoids, phenolic acids, tannins, and naphthoquinones extracted from phenolic substances of walnuts are all phenolic compounds and plant polyphenols are derivatives of phenol, which are second only to cellulose, hemicellulose, and lignin in plants (Ni et al., 2022).

2.4.2 Flavanoids

Flavonoids are naturally occurring polyphenolic compounds, which represent the largest part of dietary polyphenols (up to 60%) (González-Vallinas et al., 2013). Flavonoids provide the most essential link between diet and the prevention of chronic disorders, they have the potential to protect from viral infections as well as several diseases, such as diabetes and cardiovascular, inflammatory, and neurological diseases (Androutsopoulos et al., 2010). Owning to their omnipresence and impressive biological function/activities they continue to be thoroughly investigated as potential drugs of food supplements. Majorly, flavonoids are electron donors and synthesized via condensation of a phenylpropanoid with three molecules of malonyl coenzyme A, which are relatively inactive (Hudson & Lewis, 1983), this reaction is catalyzed by the enzyme chalcone synthase which leads to the formation of chalcone which is subsequently cyclized under acidic conditions to form flavonoids (Shahidi & Naczk, 2003). Their common organization is 15 Carbon flavan structure (C6–C3–C6), which corresponds to two aromatic rings (rings A and B) combined with three carbons to produce an oxygenated heterocycle (ring C) (Figure 4).

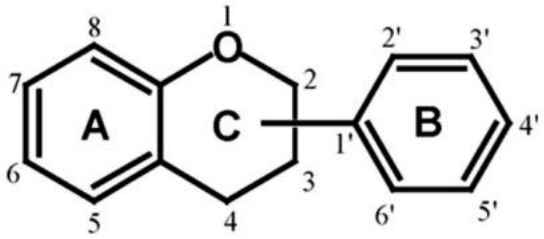


Figure 4. Basic backbone of flavonoids (Cutrim & Cortez, 2018)

There are various classes of flavonoids, namely flavones, flavanols, flavanones, flavanonols, isoflavones and flavans. The biochemical activities of flavonoids and their metabolites influence the phenoxyl radical stability and the antioxidant properties due to differences in the substitution pattern of the A and B rings on their chemical structures and the relative orientation of various moieties in the molecules (Figure 2). Their antioxidant behavior in lipid peroxidation systems has been investigated and compared to that proper of other flavonoids (Cholbi et al., 1991; Fraga et al., 1987; Mora et al., 1990; Peterson & Dwyer, 1998). Isoflavones, flavanones, methylated flavones, and flavonols as less polar flavonoids are extracted with organic compounds like chloroform, dichloromethane, diethyl ether, or ethyl acetate, while flavonoid glycosides and more polar aglycones are extracted using alcohols or alcohol-water mixtures. Flavonoids exert their antioxidative activity by

effectively scavenging various free radicals (such as superoxide anion and peroxynitrite), regulating oxidative stress-mediated enzyme activity by forming complexes with copper and iron (Veitch & Grayer, 2008; Vidisha et al., 2020). To explain the high activity of flavonoids, both preventive mechanisms and chain-breaking mechanisms are proposed (Musialik et al., 2009). Several in vitro studies have demonstrated that flavonoids inhibit lipid peroxidation and low-density lipoprotein (LDL) oxidation by scavenging peroxyl radicals (C. Rice-Evans et al., 2000; de Whalley et al., 1990).

2.4.3 Tannins

Tannins (commonly referred to as tannic acid) are water-soluble polyphenols that are present in many plant foods of varying molecular sizes and complexities. Tannins are highly hydroxylated molecules and can form insoluble complexes with carbohydrates and protein. Plant tannins can be subdivided into two major groups: (1) hydrolyzable and (2) nonhydrolyzable or condensed tannins. Hydrolyzable tannins contain a central core of polyhydric alcohol such as glucose, and hydroxyl groups, which are esterified either partially or wholly by gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) (Chung et al., 1998). Condensed tannins comprise a group of polyhydroxy-flavan-3-ol oligomers and polymers linked by carbon-carbon bonds between flavanol subunits (Schofield et al., 2001). The phenolic groups of tannins bind very tightly with the -NH groups of peptides and proteins, they prevent their hydrolysis and digestion in the stomach and therefore are known to be anti-nutritional in nature (Shahidi & Naczk, 2003). Subsequently, (Anderson et al., 2001) reported that ellagitannins were the most abundant phenolics in walnuts, characteristics of this substance is mentioned.

2.5 Tocopherols and other low-molecular-weight compounds

Walnuts are considered a good source of tocopherols and essential fatty acids, according to studies by (Halvorsen et al., 2002; Kornsteiner et al., 2006; Mishra et al., 2010), walnuts rank as one of the highest content of antioxidants among all the studied seeds and nuts. In general, the antioxidant activity of tocopherol is in the order $\delta < \gamma < \beta < \alpha$, recent literature results indicate that tocopherol may exert a prooxidant is temperature-dependent (Szewczyk et al., 2021)**.** In the case of walnut oil, it exhibits a high tocopherols content, mainly in the form of its gamma-tocopherol isomer, this form represents about 85% of the total tocopherols in the walnut oil and the other main isomers are δ and α-tocopherols, therefore, according to (Ojeda-Amador et al., 2018) partially defatted residual cakes contain

a much lower amount of tocopherols due to the apolar characteristics of these compounds, which remain in the oily phase during the extraction.

Polyunsaturated fatty acids were the main group of fatty acids found in walnuts, ranging from 73.07 to 80.98% (Li et al., 2007). The major fatty acids found in walnut oil are oleic (18:1 n-9), linoleic (18:2 n-6), and linolenic (18:3 n-3) acids, those ratios are important to the economic and nutritional value of the nut. Linoleic acid is the primary fatty acid, followed by linolenic, palmitic, oleic, and stearic acid. Walnut oil has a high linoleic acid content, which makes it unsuitable for cooking since it can char, but walnuts are a wonderful ingredient for breads, muffins, cakes, and biscuits (Zwarts, G.P. Savage, D.L. McNeil, 1999)

(Anderson et al., 2001) demonstrated that walnut extract containing gallic acid, ellagic acid, and flavonoids suppresses the oxidation of human LDL in vitro, thus protecting lymphoid cells against the cytotoxic effects of oxidized LDL (Lee et al., 2004). Following by (Kris-Etherton et al., 2000), argued that ellagic acid and flavonoids in walnuts have potential serum cholesterol-modulating effects, and one group of flavonoids has cardioprotective effects .

III. EXPERIMENTAL

3.1 Walnut extraction in general

The updated outlines for the extraction techniques employed in determining the phenolic compounds in plant materials from conventional to unconventional methods are presented in this section. Conventional extraction methods are mostly designated by utilizing a larger volume of extraction solvents and manual procedures that are mostly dependent on the investigator and labor-intensive; thus, the techniques are not ideally consistent (Alara, Abdurahman, & Ukaegbu, 2018; Alara, Abdurahman, Ukaegbu, et al., 2018). Since conventional extraction methods suffer some drawbacks, it is important to overcome these challenges, this brought about the use of advanced extraction methods, which have been generated with the purpose of filling the missing gaps of conventional methods (Shams et al., 2015), the commonly used extraction methods summarized in Table 1 (Singleton et al., 1999).

Table 1. Various extraction methods (Alara et al., 2021)

Various extraction methods	
Conventional methods	**Advanced methods**
Infusion	Pressurized liquid extraction
Decoction	Supercritical fluid extraction
Maceration	Microwave-assisted extraction
Percolation	**Ultrasound-assisted extraction**
Reflux extraction	Enzyme-assisted extraction
Soxhlet extraction	Pulsed electric field extraction.
Water bath(indirect heating)	

Each technique has its advantages and disadvantages, but the main goal of the chosen method is the achievement of complete extraction of the compounds of interest and avoidance of their chemical modification, and two experimental design methods were conducted for the screening and optimization of the process factors. The present study is a preliminary investigation of the total polyphenol compound extracted by different extraction methods, based on this, we conclude the effectiveness of the extraction method.

3.1.1 Conventional extraction- Waterbath extraction (WBE)

As we know, the biggest advantage of heating in a water bath is that the temperature can be precisely controlled and the reactants are heated evenly, in this process, the plant material is slowly heated to achieve maximum extraction, while the rising in temperature also helps release it's inner components into the solvent environment.

Conventional extraction and concentration of polyphenols using a water bath is typically conducted at temperatures ranging from 20 to 50°C, temperatures above 70°C cause rapid polyphenol degradation. An increase in temperature increases the efficiency of the extraction since heat renders the cell walls permeable, increasing the solubility and diffusion coefficients of the compounds to be extracted and decreasing the viscosity of the solvent, thus facilitating its passage through the solid substrate mass. However, the use of temperatures higher than 50°C decreases the total polyphenols and proanthocyanins yield, which is probably due to their degradation.

3.1.2 Advanced extraction- Ultrasound-assisted extraction (UAE)

Due to problems associated with high processing temperatures and long processing times in conventional extraction procedures, there is an essential need to promote the development and application of alternative extraction techniques for phenolic compounds. Possible alternatives ultrasound-assisted extraction, microwave-assisted extraction, ultrasound-microwave-assisted extraction, supercritical fluid extraction, and subcritical water extraction have recently gained a high interest (Solana et al., 2015) due to their simplicity, shorter extraction times and reduced organic solvent consumption.

Ultrasonic-assisted extraction (UAE) has been revealed as one of the most valuable methodologies for bioactive compound extraction from plant matrices. Ultrasound-assisted extraction consists of the application of ultrasonic waves that cause expansion and compression cycles in which the expansion creates liquid bubbles while the compression leads to bubbles collapse, causing cavitation. Cavitation causes cell rupture near the solid surface, which improves solvent penetration and can also break down cell walls. Using UAEs increases mass transfer, cell disruption, penetration, and capillary effects, without causing significant changes in the structural and functional properties of most target compounds, these changes are related to the frequency and power of the ultrasonic vibration, besides mechanical and thermal effects of ultrasound waves (Esclapez et al., 2011). Using sonication improves extraction efficiency and rate, reduces temperature and solvent usage,

and facilitates compound solubilization, is a relatively cost-effective procedure for extracting phenolic compounds from plant materials (Luque de Castro, 2007). Ultrasonic probes and baths are the two most common systems used for laborious extraction. Ultrasounds assistance has been shown to intensify the recovery of phenolic compounds from walnut shell (Han et al., 2018) or walnut husk (Tabaraki & Rastgoo, 2014) but has not increased the phenolic content of extracts obtained from pistachios (Fregapane et al., 2020).

3.1.3 Solvent use for extraction

The yield and rate of polyphenolic extraction are related to the solvent characteristics. It has been observed that methanol is more efficient in the extraction of lower molecular weight polyphenols while aqueous acetone is a suitable solvent for the extraction of higher molecular weight flavanols (Metivier et al., 1980; Prior et al., 2001). However, many phenolic compounds are subject to degradation or undergo undesirable oxidation therefore significantly decreasing the phenolic yield in the extract.

The extraction of substances under certain conditions, also known as extraction according to the same principle of "similar compatibility"(‘Introduction on Extraction and Analysis of Polyphenols’, 2020). In the first step, the proper extraction procedure has to be considered, the decision on the extraction method to be employed is influenced by the chemical nature of the substance, sample particle size, and also by the presence of interfering substances. Besides solvent polarity, solvent pH, solubility is also influenced by both extraction time and temperature. A higher temperature simultaneously increases solubility and mass transfer velocities as well as decreases viscosity and surface tension of solvents contributing to a higher extraction rate (Brunner, 2005, 2013). For the elimination of unwanted compounds such as waxes, fats, terpenes, and chlorophylls, additional steps may be introduced (Beatriz Díaz-Reinoso et al., 2006). Lastly, mathematical models for optimizing the overall extraction have been highlighted for controlling the process.

Polyphenolic compounds can be extracted from fresh, frozen, or dried walnut press-cake samples. Before extraction, pretreatment techniques like drying, crushing, and grinding may be required depending on the samples. After extraction, isolation, and purification are done to obtain the active compounds. The selection of the drying procedure impacts the total phenolic content, for particular, the freeze-drying retains higher phenolic content levels in plant samples than air-drying.

Solid-solven extraction method is used in the current study which has been widely accepted due to its ease of use, efficiency, and wide-ranging applicability. There are a number of solvents which are commonly used in the process, such as alcohols (methanol, ethanol), acetone, diethyl ether, and ethyl acetate, often mixed with water in different proportions. However, there are some disadvantages associated with the use of these solvents, including a potential hazardous effect on human health and residues of the solvents remaining in the final products. In order to solve those problems, additional purification steps must be performed, which adds time and expense to the process. Additionally, by using pure organic solvents, very polar phenolic acids (benzoic, cinnamic acids) cannot be extracted completely. In such cases, mixtures of alcohol-water, particularly acetone–water are suggested and conducted in this study.

3.2 Extraction of polyphenol from walnut press-cake

3.2.1 Sample preparation

The walnut kernels were purchased from local market in Budapest, Hungary.

The partially defatted cake was collected the day after pressing and was stored at -18°C after having spent about 48 hours at room temperature. When extraction campaigns were planned, 100,0 grams were grounded with a juice grinder, sieved below 600 μm (Retsch, Germany), and divided into smaller batches stored at – 18°C until use (Figure 5). A sample of the freshly pressed cake was stored at room temperature in a food-grade kraft paper bag after gentle deagglomeration to mimic the storage conditions of flour in mills. 10,0 grams of walnut press-cake was extracted with 300,0 mL solvent and gently shaked by using a 500,0 ml Erlenmeyer flask.

Figure 5. Ground walnut press-cake

3.2.2 Chemicals and reagents

The use of an alcoholic solution provides satisfactory extraction. As polyphenols are associated with other biomolecules like proteins, polysaccharides, terpenes, chlorophyll, lipids, and inorganic compounds, methanol, acetone, or water alone are inefficient solvents for extraction of total phenols from powdered plants. In this study, aqueous mixture of acetone and distilled water is used to optimize the polyphenol yields.

3.2.3 Preparation of polyphenol extracts from walnut press-cake

3.2.3.1 Waterbath extraction

In this study, an Erlenmeyer flask (500,0 mL) was performed on a Tecator shaking water bath shaker (Figure 6) with fixed agitation and at 60°C temperature: the extraction was conducted with 70% acetone solvent (which means 700,0 ml acetone: 300,0 ml water) and respectively provide by 1:30 solid-solvent ratio (in detailed 10,0-gram sample into 300,0 mL solvent). The sample was collected after 60 minutes. After extraction, the obtained extracts were filtered through a 0.45µm cellulose filter, and reconstituted filtrates were properly diluted up to the required concentration for further analysis (Figure 7).

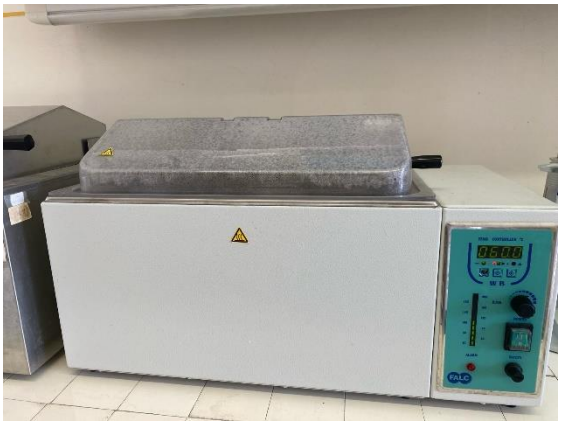


Figure 6. Tecator shaking water bath shaker

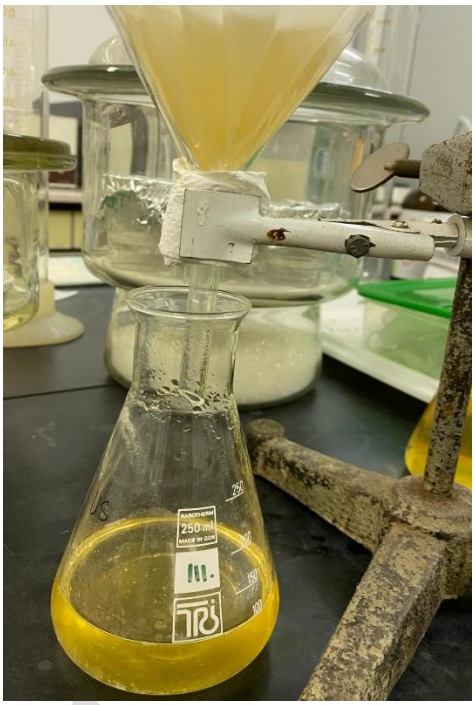


Figure 7. Waterbath extraction's extract

3.2.3.2 Ultrasound- asissted extraction

Ultrasound-assisted extractions in this study were performed by using an ultrasonic bath. The amount of 10,0- gram walnut press-cake flour was mixed with 300,0 mL appropriate solvent (70% acetone) in a modified solid: solvent ratio (1:30) into a 500,0 ml Erlenmeyer flask and this extract was then conducted in an Ultrasonic Bath (Vevor 3L)(Figure 8). Samples were treated for 60 minutes with power ultrasound, high intensity, and low frequency at 35 °C temperature. The extraction was conducted under fixed sonication. Sonication was performed at 40 kHz. After extraction, the bottles were cooled to room temperature using a cool water bath and then the extracts were filtered through a 0.45µm cellulose filter paper, and reconstituted filtrates were properly diluted up to the required concentration for further analysis (Figure 9).

Figure 8. Ultrasonic Bath (Vevor 3L)

Figure 9. Ultrasound assisted extraction's extract

3.2.4 Analysis of extracted polyphenol-Total Polyphenol Content (TPC) and antioxidant activity of the extract (FRAP)

3.2.4.1 Determination of total polyphenol content (TPC)

The contents of total polyphenol of the extracts were determined by Folin Ciocalteu's reagent assay according to a modified version of the procedure described by (Singleton et al., 1999). The procedure was conducted by preparation of reagents, 10,0 mL Folin reagent diluted with 100,0 mL of distilled water; 7,42 grams amount of Sodium carbonate solution was taken in a 100,0 mL volumetric flask and a small amount of distilled water was added in it, and shaken to dissolve Na_2CO_3, this volume was made up to 100,0 mL by adding more distilled water; Methanol: distilled water also diluted by 80:20 ratio, or mixed 40,0 mL methanol into 10,0 mL distilled water. Consequently, 0,1-gram gallic acid was also diluted by 0,9 mL methanol: distilled water following the prepared ratio previously.

Seven tubes of calibration solution are measured according to Table 2, the results are measured by gallic acid and then placed again in the water bath for 5 minutes to produce high color development, while the vibration gently shaking of the holder helps to neutralize the solution absorbance of the resulting blue color supernatant was read at 720nm using

Hitachi U-2900 spectrophotometer. For sample's absorbance measurement, 1,0 mL walnut press-cake extract was diluted in 9,0 mL acetone (70%), 1,25-1,25 mL Folin Ciocalteu's reagent was added to 7 centrifuge tubes containing 0,5; 1,0; 1,5; 2,0; 2,5 mL of extract. Similar to the standard calibration, 0,25-0,25 mL MeOH: DW was added into each tube. After one minute, the contents were mixed thoroughly with 1,0-1,0 mL of sodium carbonate solution and placed in water bath for 5 minutes at 50°C, ultimately absorbance value was measured by using a Hitachi U-2900 spectrophotometer. All measurements were repeated three times, and the content of total polyphenol in each extract was determined using a standard curve prepared for gallic acid and expressed as milligram gallic acid equivalents (mg/L GAE) (Figure 10).

Table 2. Calibration of FRAP reagents

	Folin	***MeOH:DV***	***Gallic acid***	***Na_2CO_3 after 1 min***	***µM***	***µg/mL***
1	1250 µL	250 µL	-	1000 µL	0	0
2	1250 µL	250 µL	-	1000 µL	0	0
3	**1250 µL**	**200 µL**	**50 µL**	**1000 µL**	**6**	**1,02**
4	**1250 µL**	**150 µL**	**100 µL**	**1000 µL**	**12**	**2,64**
5	**1250 µL**	**100 µL**	**150 µL**	**1000 µL**	**18**	**3,06**
6	**1250 µL**	**50 µL**	**200 µL**	**1000 µL**	**24**	**4,08**
7	**1250 µL**	**-**	**250 µL**	**1000 µL**	**30**	**5,1**

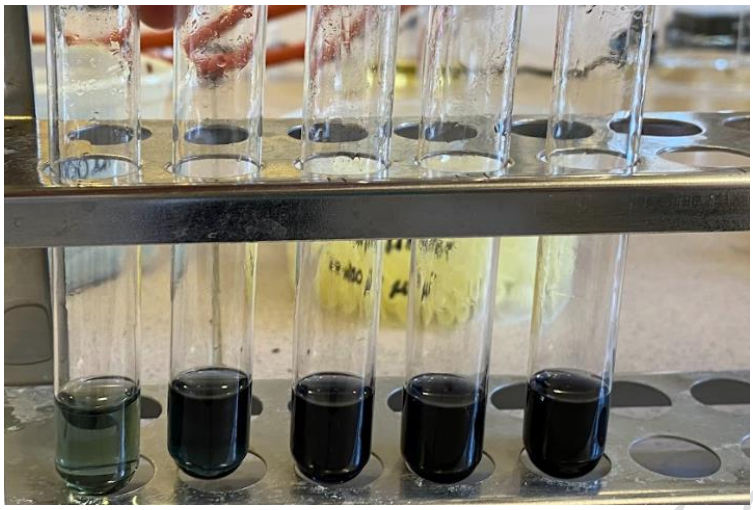


Figure 10. Preparation of Folin reagent by using gallic acid

3.2.4.2 Ferric-reducing antioxidant power (FRAP)

At Low pH, when a ferric tripyridyl triazine (Fe^{III}-TPTZ) complex is reduced to the ferrous (Fe^{II}) form, an intense blue color with an absorption maximum at 593nm develops. The reaction is nonspecific, and half reaction which has a less positive redox potential, under reaction conditions, than the Fe^{III}/ Fe^{II}- TpTZ half-reaction will drive Fe^{II} reduction. Test conditions favour reduction of the complex and, thereby, color development, provides that antioxidant is present. The FRAP assay results are presented with particular reference to the following: reaction kinetics and do-response relationships with solutions of ascorbic acid, uric acid, bilirubin, Trolox (water-soluble analog of vitamin E), alfa-tocopherol and albumin, with mixtures of these antioxidants and with plasma (Benzie & Strain, 1996).

Preparation of reagent

Reagents included 25,0 mL of 300,0 mmol/L acetate buffer, pH=3,6 (3,1 gram Na-acetate*3H_2O and 16,0 mL acetic acid per litter of buffer solution); TPTZ (Tripyridyl-s-Triazine, which mixed by 62,46 mg TPTZ/ 20,0 mL distilled water in 67 μL HCl and FeCl_3 *6H_2O (0,10 g FeCl_3 *6H_2O into 20,0 mL DW). Working FRAP reagent was prepared as required by mixing 25,0 ml acetate buffer, 2.50 ml TPTZ solution and 2.50 ml FeCl3*6H2O solution, this is sealed with aluminum foil to avoid sunlight (figure). Besides that, the ascorbic solution was also prepared by mixing 17,163 mg ascorbic acid solution with 10,0 mL of distilled water.

The standard curve was prepared according to Table 2 to read the absorbance value, with 7 tube content FRAP reagents, distilled water, and ascorbic acid, then placed into a water-bath

to incubate for 5 minutes, after incubating, the absorbance value was read by using Hitachi U-2900 spectrophotometer (Figure 11-12)

Table 3. Calibration of FRAP reagents

	FRAP reagent (µL)	**DW (µL)**	**AS (1 mM / L) (µL)**	**Ascorbic acid concentration**	
				(µM / L)	(µg / mL)
Blank	1500	50	-	0	0
Blank	1500	50	-	0	0
1	1500	40	10	6.451	1.056
2	1500	30	20	12.903	2.272
3	1500	20	30	19.354	3.416
4	1500	10	40	25.806	4.544
5	1500	-	50	32.258	5.688

A 30,0 mL diluted sample (extract: acetone 70% 1:9) was mixed well with 1,5 mL FRAP reagent and 20,0 µL distilled water, it again took 5 minutes incubate time in the water bath at 50°C. A total of 20,0 mL of sample was then added to each well of the spectrophotometer. Each sample was run in triplicate at 593 nm. The initial blank reading for each well with just FRAP reagent was then subtracted from the final reading of FRAP reagent with sample to determine the FRAP value for each sample.

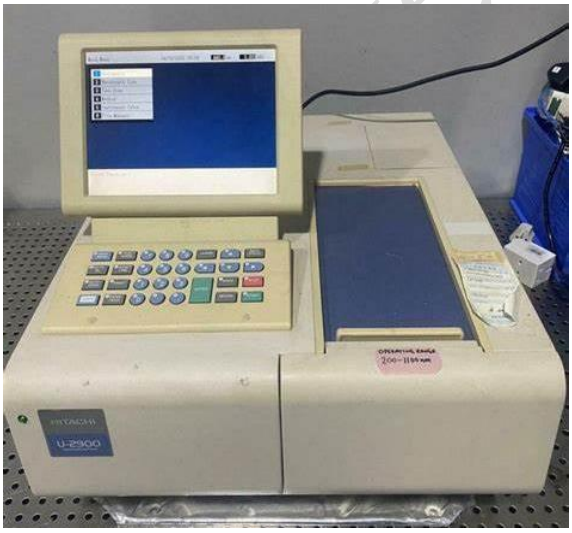


Figure 11. Hitachi U-2900 spectrophotometer

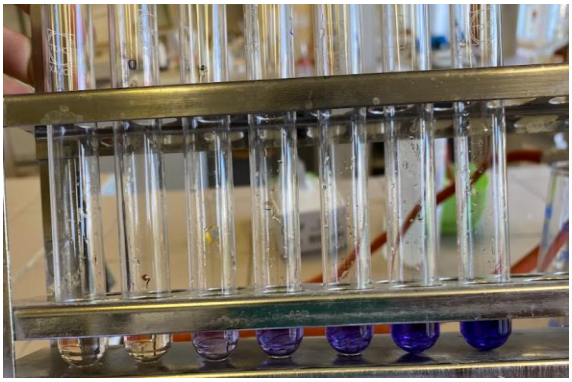


Figure 12. Preparation standard reagents with ascorbic acid

3.2.4.3 Colour analysis.

The color of the samples was measured with a Konica Minolta CR 400 digital color meter, which measures the color of the samples according to the C.I.E.Lab system. Experimentally, with the involvement of target persons, it was determined in what proportion the light rays of different colors (wavelengths) can be produced from a mixture of the three basic colors: R (red) 700 nm; G (green) 546.1 sq m; B (blue) light with a wavelength of 435.8 nm. However, not all colors can be produced with this model without using negative values, which is why the CIE XYZ system based on RGB metrics and transformed from it was created, which is still the basis of color measurement today. The instrument was calibrated using the standard white tile (L* 97,59, a* 0,24, and b* 1.87)

3.3 Analyis of the antioxidant activity of extracted polyphenol by testing into oil

3.3.1 Antioxidant in terms of oxidative stability review

One of the major changes that occurs during processing, distribution, and final preparation of food is oxidation. Oxidation is initiated by homolytic breakage of a hydrogen bonded to carbon α to the double bond, resulting in lipid-free radicals. Free radicals are important chain-carrying intermediates in lipid autoxidation, which lead to the formation of hydroperoxides that further degrade to secondary oxidation products such as ketones and aldehydes, among others. These radicals react with atmospheric oxygen to form peroxides as the primary product of lipid oxidation (Coppin & Pike, 2001). The lipids involved in the oxidation process contain unsaturated fatty acids, which is hydrolysis and/ or oxidation products of triglycerides (TG) in vegetable oil, represented by oleic, linoleic, linolenic, and another long chain PUFAs, however, other unsaturated lipids present in fats and oils such as cholesterol and other sterols do become oxidized during this process. Free fatty acids (FFAs) are thought to be the preferred substrate for lipid oxidation. The greater the percentage of FFA in a sample, the greater the number of TAG hydrolyzed into FFAs, and the greater the potential for FFAs to produce primary oxidation products (i.e., oxylipins) and secondary volatiles (Shen et al., 2021). In edible oils, FFA are undesirable, as high FFA content results in increased losses during refining, oxidation of lipids initiates other changes in the food system that affect its nutritional quality, wholesomeness, safety, color, flavor, and texture. It is important to comply with the FFA index limits during oil processing since these compounds can create reactive oxygen species causing cell death and necrosis (Rockenfeller et al., 2010), as well as reducing immune cell function (Eftimiadi et al., 1987).

Oxidative stability is an important indicator to determine oil quality and shelf because low-molecular weight off-flavor compounds are produced during oxidation. Oxidation of edible oils is influenced by an energy input such as light or heat, composition of fatty acids, types of oxygen, and minor compounds such as metals, pigments, phospholipids, free fatty acids, mono- and diacylglycerols, thermally oxidized compounds, and antioxidants. Many efforts have been made to improve the oxidative stabilities of oils by systematic studies on the effects of these factors (Choe & Min, 2006).

The oxidation of edible fats and oils can be controlled by application of antioxidants, using processing techniques that minimize the loss of tocopherols and other natural antioxidants, inactivation of prooxidant metals and enzymes, minimizing exposure to oxygen, heat and light, hydrogenation of PUFA and the use of an inert gas or vacuum packaging to expel oxygen before long-term storage (Miraliakbari & Shahidi, 2008), in addition to reaching the expected free fatty acid value. Research has also focused on reducing this compound, we investigated the FFA composition before and after mixing with walnut press-cake extracted powder containing antioxidants to determine the stabilization of this combination without frying conditions by measuring the free fatty acids value, to conclude the effectiveness of this application in increasing the antioxidant properties of cooking oils, particularly in terms of limiting lipid oxidation.

3.3.2 Drying process

The extract obtained from the extraction process contains large amounts of solvents (water-acetone), the removal of these solvents from the extract is necessary, the purity of the product obtained is not only easier for further experiments, whereas the application of this substance as a powder is also preferred in food processing to limit unwanted reactions with solvents while optimizing production efficiency. An evaporation process is applied to separate the acetone from the extract during the first step of removing the solvent from the extract. The boiling temperature of acetone is much lower than that of water (56.08°C), this process helps remove acetone. However, further drying is required to remove any remaining water, which has led to the investigation of two drying methods, each providing a different result.

The solvent of the sample was evaporated by testing two different rotary evaporators, the first equipment lacked of vacuum pump, which helped to create the vacuum inside the flash by reducing the air pressure inside the flask, thus reducing the boiling point of solvents,

to increase the rate of evaporation and separation process. Simultaneously, this device uses oil bath instead of a water bath to heat the flask; in principle, an oil bath can operate without disturbing the composition of the sample if there is intervention from the vacuum pump. There is an excessive increase in the temperature in the oil tank that cannot be adjusted, thereby reducing the amount of bioactive compounds of the extract. The purification was then carried out in a completed Büchi Rotavapor R200 rotary evaporator (Figure 13), the accordance temperature required for evaporation was also adjusted to 60°C, obtaining the desired result.

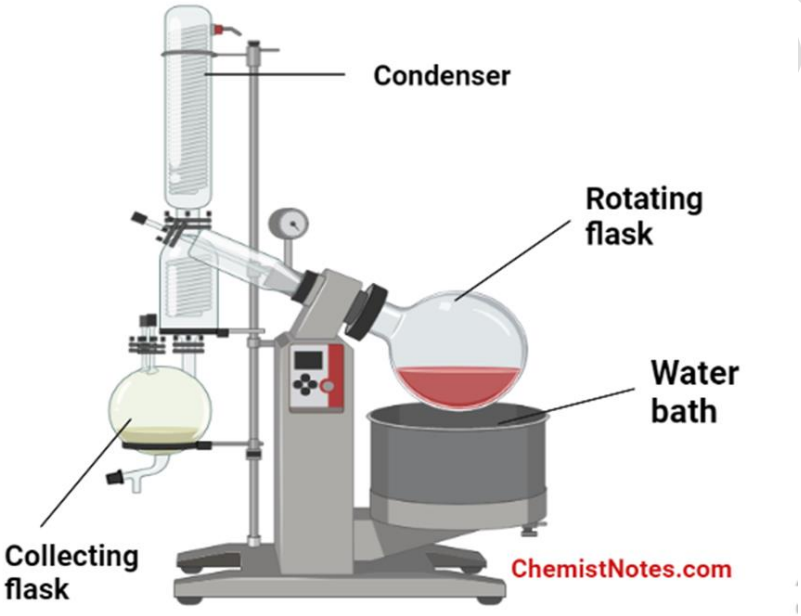


Figure 13. Rotary evaporator (Source:ChemistNotes.com)

A drying cabinet at 105°C was used for the second step of dehydrating the walnut polyphenol extract for 4 hours, however, this method is not suitable in the case of walnut polyphenol extract due to its deterioration under heat treatment, resulting in a caramel-colored powder as shown (Figures 14-15). Therefore, lyophilization was employed - which is widely applied as a dehydrated process through three main phases: The samples first mixed gently, and dispensed approximately 20,0 mL into glass Petri dishes, after storage in the dark at room temperature for 25 hours, the samples were placed into the freezer overnight until the ice starts to nucleate and ice growth follows and the shelf reaches the triple point (-40°C). Following the first phase, the sample pressure was lowered, and slightly heated to allow the frozen water in the material to sublimate (sublimation), this phase raised the shelf temperature to -20°C; In the final phase, the remaining ionically bound water molecules are completely removed by a process called adsorption, by raising the shelf temperature to a

higher level (25°C). After 5 days of lyophilization, the amount of powder as shown below is obtained that is much lighter than the original sample (Figures 16-17), a further examination is then conducted using this powder. Lyophilization is typically used to increase stability, extend shelf life as well as transport optimization.

Figure 14. WBE dried powder using drying cabinet

Figure 15. UAE dried powder using drying cabinet

Figure 16. Waterbath extraction dried powder

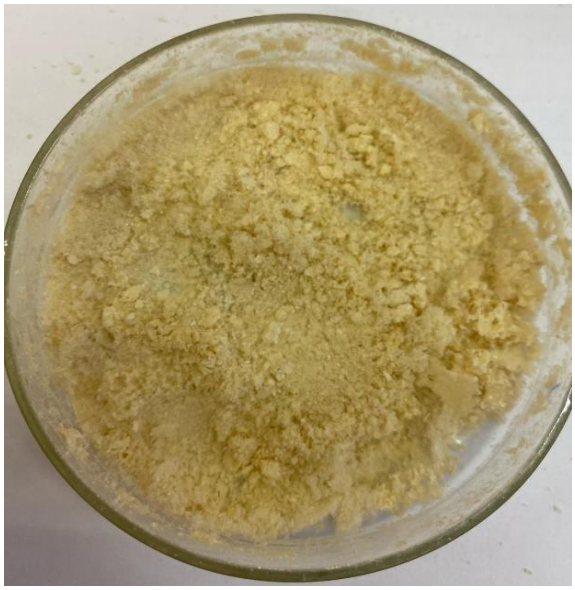


Figure 17. Ultrasound assited extraction dried powder

3.3.3 Antioxidants of walnut extract powder in application with sunflower oil

Refined sunflower oil Vénusz brand purchased from local market, Budapest, Hungary.

A different proportion of extract powder was mixed with refined sunflower oil, which was at 1000 and 2000 parts per million. In order to ensure optimal mixing, the mixture was placed in a water bath at 50°C for 60 minutes for the powder to dissolve, then stored overnight for the oil to blend with the antioxidants, sunflower oil was used as a control sample. The samples are then ready for free fatty acid determination.

3.3.3.1 Free-fatty acid (FFA) determination

Free fatty acid (FFA) value determined by AOCS Official Method Ca 5a-40 (AOCS, 1998) with using about 10.0-12.0 grams oil sample weighed with an allowance of ± 0.05g as error into 250,0 ml glass stoppered Erlenmeyer flasks and the weights were recorded

Chemicals: Ethanol 96%, Phenolphthalein indicator 1%, Potassium hydroxide solution 0,1N

Procedure

Add a few drops of phenolphthalein indicator into 50,0 ml ethanol, then neutralize by 0,1N Potassium hydroxide solution solution until the appearance of the first permanent pink color, then pour into Erlenmeyer flask which contains the well-mixed oil sample, and slightly boil the flask to dissolve the sample into this solution, then cool at room temperature. Burettes were filled with potassium hydroxide solution 0,1N. A few drop of phenolphthalein indicator was added to flask and titration with the potassium hydroxide done until sample change to light pink color (Figure 18).

Figure 18. Free-fatty acids titration of ultrasound assisted extract powder mixed with sunflower oil

IV. RESULTS AND DISCUSSION

4.1 Total polyphenol content (TPC)

The method is based on the oxidation reaction between polyphenolic compounds with the Folin-Ciocalteu reagent. The concentration of garlic acid in samples was determined by using an equation that was obtained from a standard gallic acid curve (Table 4). The equation is given by attached graph (Figure 19):

Table 4. Concentration and absorbance of gallic acid

Concentration(ug/mL)	absorbance
0	0
1,02	0,083
2,64	0,206
3,06	0,319
4,08	0,48
5,1	0,493

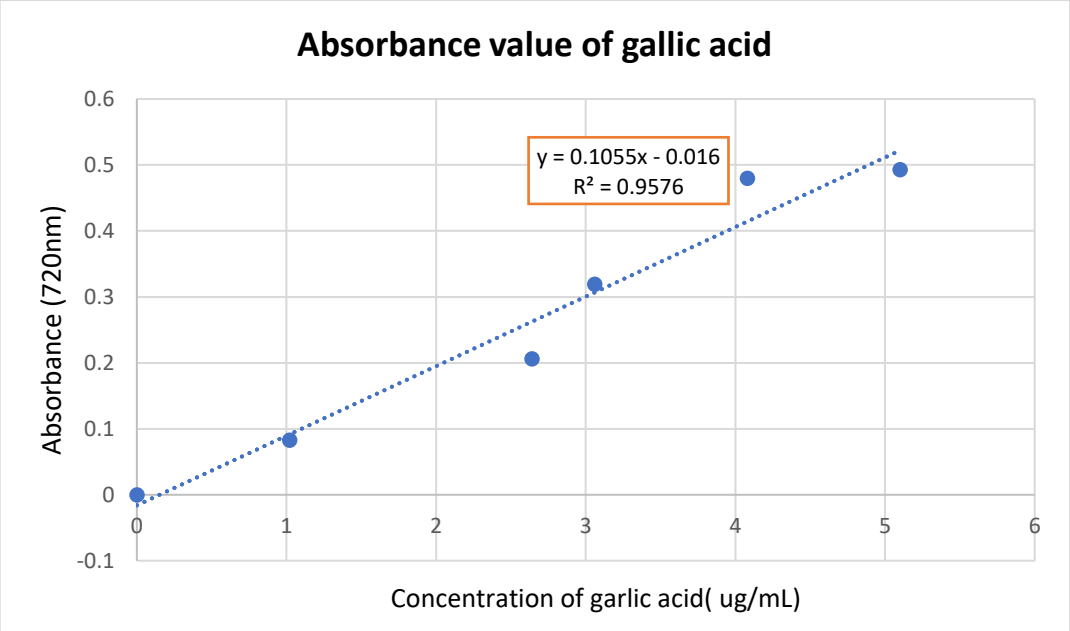


Figure 19. The absorbance value of gallic acid

We calculated the average absorbance value, and found different results between ultrasound-assisted extraction and water bath extraction, with ultrasound-assisted extraction nearly twice as good as water bath extraction (0,513 and 0,263)

Based on these given data and applying the formula, the total phenolic content calculated.

Equation of calibration line: y= 0,1055x – 0,016

TPC – Total Polyphenol Content (μg GAE/ g dry matter)

$$\mathbf{TPC} = \frac{\mathbf{A}}{\mathbf{tg\alpha}} * \frac{\mathbf{V_{all}}}{\mathbf{V_{sample}}} * \mathbf{D}$$

- A – Absorbance
- tgα – Slope of the calibration line
- V_{all} – Final volume
- V_{sample} – Volume of the sample
- D – Dilution

$$\mathrm{TPC_{water\ bath}} = \frac{A}{tg\alpha} * \frac{V_{all}}{V_{sample}} * D = \frac{0{,}263*2500*(30*10)}{0{,}1055*50} = 37393{,}36\ (\mu g\ GAE/g\ \ d.m)$$

$$\mathrm{TPC_{ultrasound}} = \frac{A}{tg\alpha} * \frac{V_{all}}{V_{sample}} * D = \frac{0{,}513*2500*(30*10)}{0{,}1055*50} = 72890{,}99\ (\mu g\ GAE/g\ \ d.m)$$

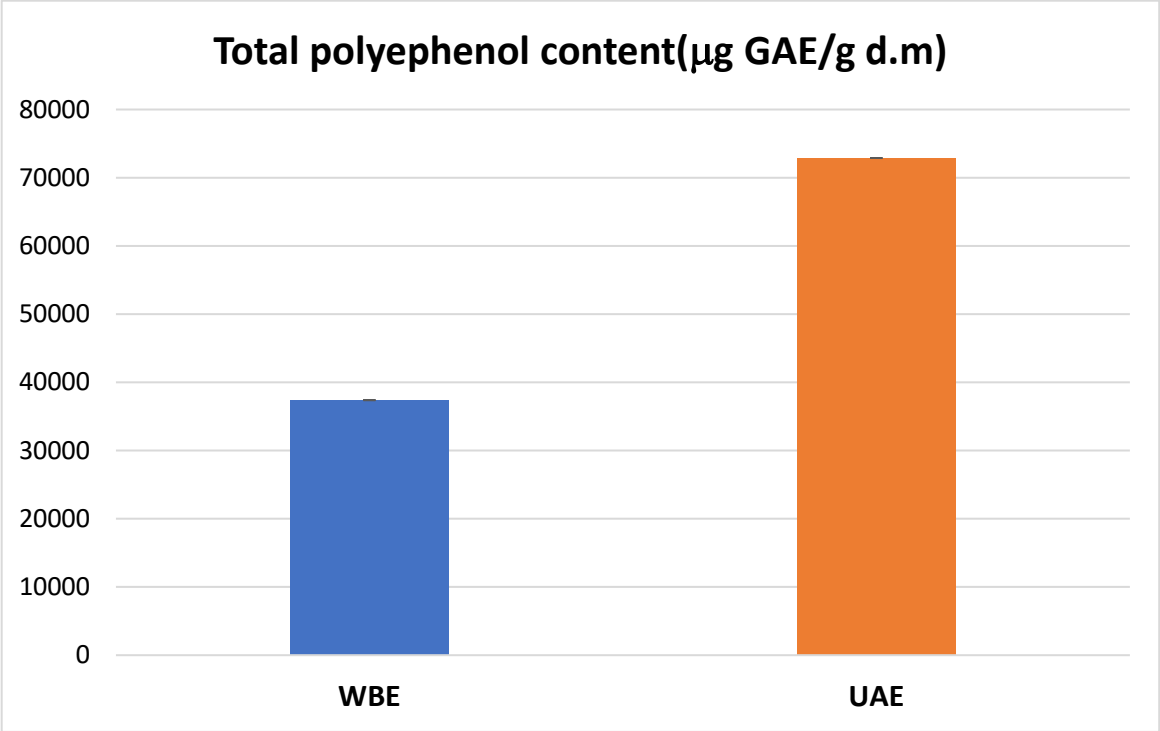


Figure 20. Total polyphenol contents (µg GAE/100g), WBE=water bath extraction, UAE-ulttrasound-assisted extraction

The significant difference in TPC content between the two extraction methods are shown. The differences can be explained due to the fact that most of the compound groups that are extracted into the solution medium in water bath extraction do not have more

phenolic character than ultrasound-assisted extraction, on the other hand, the mechanical characteristic of ultrasound waves helped to break the sample's cell walls, improve the solvent penetration and extraction efficiencies, this has been clarified in several articles and experimental results are also in line with these hypotheses. In addition, extraction conditions, represented by temperatures, and time linked to polyphenol properties may cause the differences.

The obtained results allowed optimizing the conditions for the extraction of antioxidant polyphenols from walnut press cake by using a technique considered as environmentally and financially friendly. Ultrasound assistance improves considerably both kinetics and yields of extraction of phenolics showing an efficient way to produce antioxidant-rich extracts at reduced time and energy. The effect of ultrasound is more significant while the operating conditions are generally less favorable for the extraction, the presence of acetone in the solvent improved also greatly the extraction process.

4.2 Antioxidant capacity (FRAP assay)

Construction of calibration curve

The calibration curve was constructed by using ascorbic acid standard. After preparation of different concentration of ascorbic acid standard, the absorbance of the solutions was measured at 592 nm. The resulting absorbance is shown in Table 5.

Table 5. Concentration and absorbance value of ascorbic acid

Concentration(ug/mL)	Absorbance
0	0
0	0
1,056	0,428
2,272	0,593
3,416	1,04
4,544	1,345
5,688	1,789

The equation of the calibration curve was obtained from the resulting absorbance versus concentration of the graph and the equation was: Y=0.3036X +0.0059, where $r^2 = 0{,}9907$ (Figure 20)

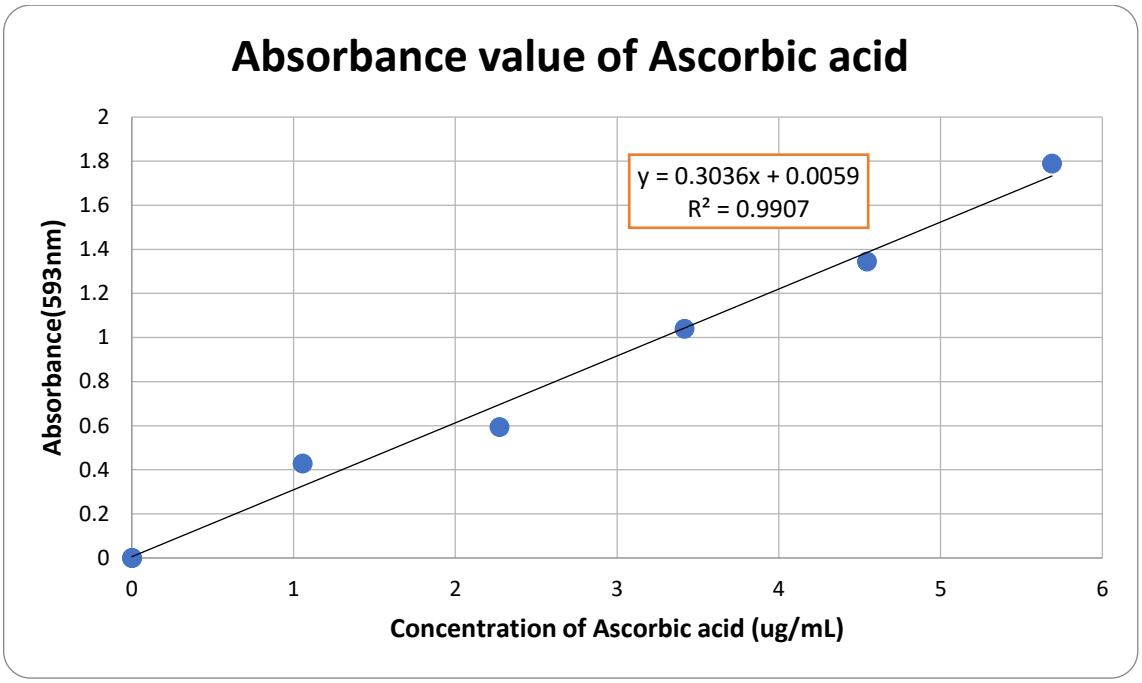


Figure 21. Absorbance value of ascorbic acid

The iron (III) reducing activity determination was expressed in µg ascorbic acid equivalents/g of dry matter weight as given by the formula:

$$\boldsymbol{FRAP = \frac{A}{tg\alpha} * \frac{V_{all}}{V_{sample}} * D}$$

- FRAP – The Ferric Reducing Ability of Plasma- antioxidant capacity(μg AAE/g dry matter)
- A – Absorbance
- tgα – Slope of the calibration line
- V_{all} – Final volume
- V_{sample} – Volume of the sample
- D – Dilution

$$FRAP_{WBE} = \frac{A}{tg\alpha} * \frac{V_{all}}{V_{sample}} * D = \frac{0{,}416 * 1550 * (30 * 10)}{0{,}3036 * 30} = 21255{,}49\ (\mu g\ AAE/g)$$

$$FRAP_{UAE} = \frac{A}{tg\alpha} * \frac{V_{all}}{V_{sample}} * D = \frac{0{,}440 * 1550 * (30 * 10)}{0{,}3036 * 30} = 22480{,}78\ (\mu g\ AAE/g)$$

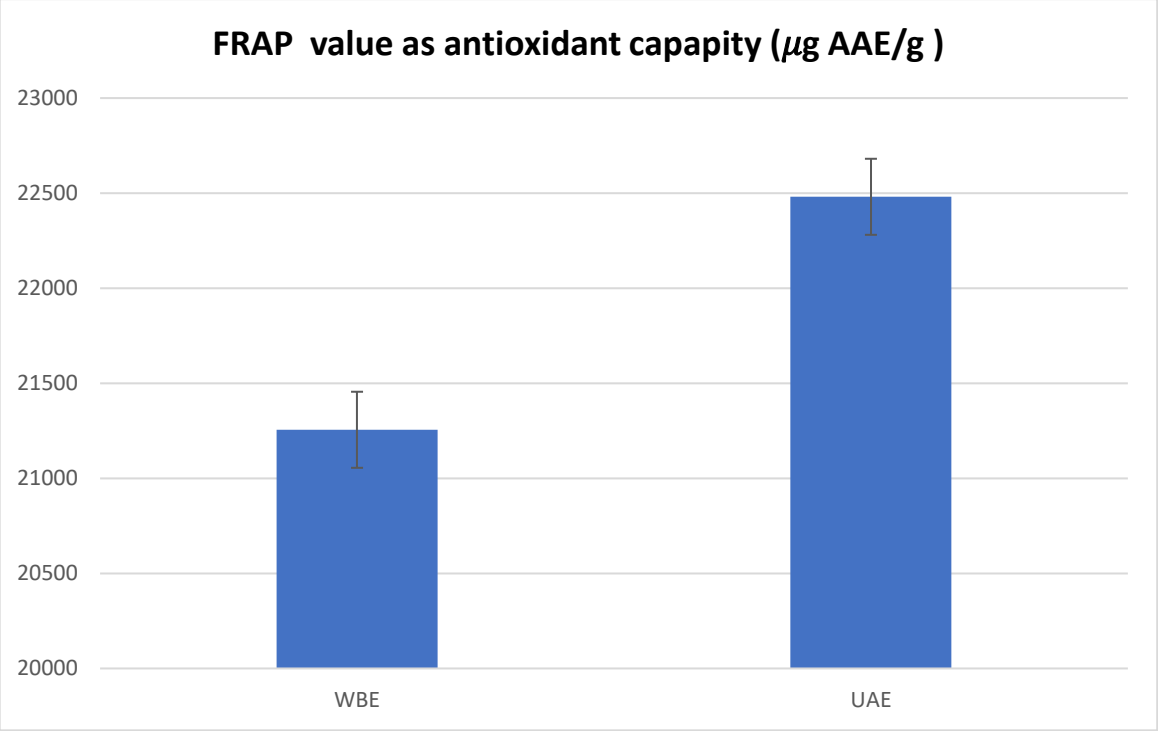


Figure 22. FRAP value as antioxidant capapity (μg AAE/g) , WBE=water bath extraction, UAE- ulttrasound-assisted extraction

As a result (Figure 22), there are differences in the yield of antioxidant capacity between the two extraction methods, the water bath extraction showed a lower antioxidant quantity whereas increased to higher concentrations through ultrasound-assisted extraction, in accordance with TPC values.

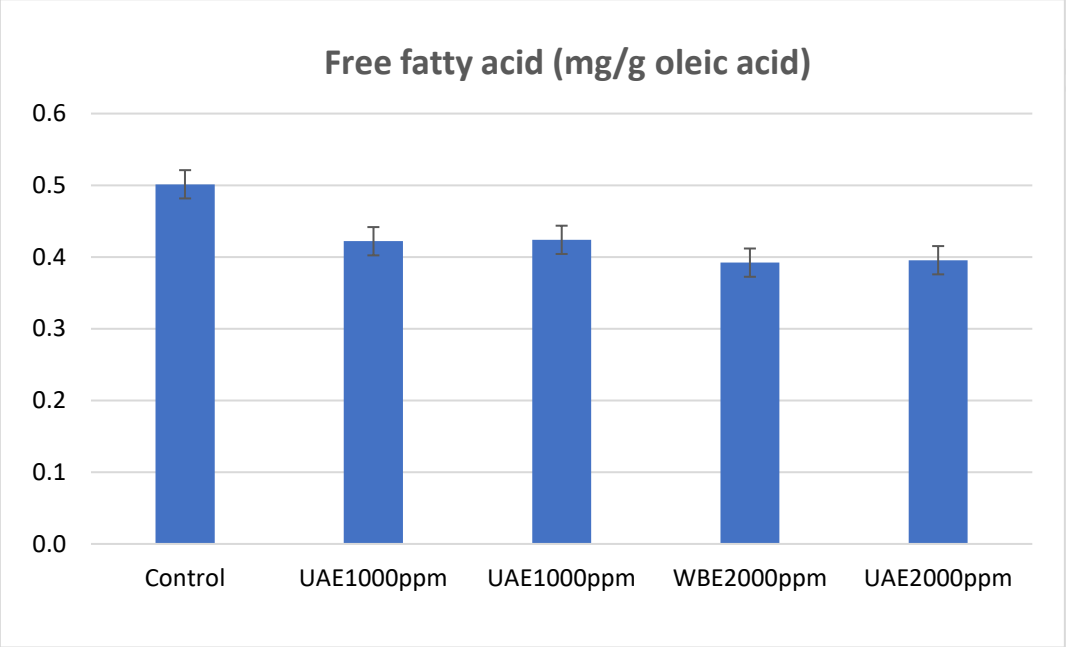
4.3 Color analysis

Sample	Color		
	L*	**a***	**b***
WBE extract	28,99 ± 1,03	0,87 ± 0,14	5,79 ± 1,04
UAE extract	25,33 ± 0,78	0,79 ± 0,08	5,35 ± 0,50
WBE dried powder	97,25 ± 0,30	0,22 ± 0,05	1,76 ± 0,27
UAEdried powder	98,51 ± 0,05	0,84 ± 0,01	(-2,16) ± 0,09

According to the results, the L* value of the samples increased, the powder dryed from WBE and UAE extract showed a significant change, i.e the powders became lighter. The red/green color factor (a*) gave positive values (0,22–0,87) for the samples, indicating that

the samples were reddish in color. The red color intensity decreased significantly in the WBE samples. The yellow/blue color (b*) had a positive value (1,76-5,79) for WBE-s and UAE extract, indicating yellow color intensity, and it decreased significantly during the drying process. The b* negative value in the UAE-dried powder indicated blue. The color difference (ΔEab) between the control and the samples was over 6.0, which shows a significant difference, meaning that the extent of the change can be easily detected with the naked eye.

4.4 Free fatty acids



The FFA values of the samples at levels of 1000 and 2000 ppm were kept lower than that of the control, indicating that the application of antioxidants contained in walnut press-cake powders prevented auto-oxidation, the most common oxidative reaction in fat and oil caused by the reaction between oxygen and unsaturated fatty acid. Simultaneously the usage of antioxidants from ultrasound-assisted extraction materials showed greater efficiency, mainly at higher concentrations of natural antioxidants (2000ppm), which resulting in the lowest level of free fatty acids. Therefore, walnut press cake can be recommended as a potent source of antioxidants for the stabilization of food systems, especially unsaturated vegetable oils.

V. CONCLUSION

The walnut kernel contains numerous nutrition compounds, making it widely consumed as a whole nut or walnut oil. However, walnut residues are frequently abandoned as a by-product of walnut oil extraction, thrown away as waste, used as animal feed, composted, or even polluting the environment. Recent studies on walnut residues have demonstrated their health benefits through various aspects, involving the analysis of bioactive components and their activity, providing a theoretical basis for further processing, my study aims to contribute to this research process by examining the walnut residue polyphenol content and its application as an antioxidant in food processing through two main experimental series.

In my first series of tests, I determined some nutritional properties of walnut press-cake by testing polyphenol content in particular and antioxidant activity in general by applying different extraction methods, involving both conventional and advanced extraction, intended for comparison of their performance. Simultaneously, prevalent laboratory analytical methods such as total phenolic content (TPC) and Ferric-reducing antioxidant power (FRAP), color analysis were employed. Based on the results, it can be affirmed that walnut press-cake extract has significant polyphenol content and antioxidant capacity, indicating this material's potential in food processing. On the other hand, the effectiveness of the extraction methods is shown, the application of the ultrasound-assisted extraction method indicated a higher quantity of antioxidants mainly due to its mechanism, this method not only helps improve the extraction efficiency but also the cost-effective procedure for extracting phenolic compounds from plant materials.

Subsequently, different evaporation and drying methods were applied, I tested two different rotary evaporators, the first equipment used oil-bath instead of a water bath, and due to a lack of an accompanying vacuum pump, the extract quickly evaporated, since it could not adjust the temperature below 70°C to prevent the bioactive components from being deteriorated by thermal effects. Those limitations have been overcome with the application of another rotary evaporator that uses a water bath to raise the rotating flask’s temperature and can be adjusted to reach the expected heat treatment. Subsequently, the evaporated extract still contained water, this solvent was also separated by applying two different drying methods, however drying by the dry cabinet at 105°C has caramelized the sample, ultimately, the freeze-drying is then applied and effectively achieves the desired result.

In the second experimental series, I mapped the basic attributions of the walnut press-cake extract dried powder as an antioxidant in application with sunflower oil whether it can modify the oil's physical characteristics, and the changes in the practical function of sunflower oil were examined. The samples were mixed with sunflower oil in different proportions, and then the free fatty acids value in terms of oleic acids content was determined. Result indicated that by mixing walnut press-cake powder with sunflower oil, free fatty acids are reduced, and the effectiveness of the process is proportional to antioxidant levels.

In this research, I gained a deeper understanding of the properties, and mechanisms of antioxidants, specifically polyphenols in walnut press-cake. I successfully analyzed the content and application of polyphenols in walnut press-cake. I have developed a suitable extraction method through the first experiment series, and besides the conducted methods, the comparison with other extraction methods, and different extraction conditions, such as temperature, time, solvent, and solvent ratio, have also drawn my attention. The most appropriate drying method was selected after approached different drying methods. The use of antioxidants to reduce unsaturated free fatty acids in cooking oil was also successfully evaluated. As a result of the experimental results, I believe it is necessary to further investigate the activities of walnut presscake bioactive compounds; reusing this material in food production is an interesting concept and deserves a lot of consideration.

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